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THE ROLE OF METABOLIC REWIRING IN ENDOCRINE RESISTANCE

BY

EYLEM KULKOYLUOGLU COTUL

DISSERTATION

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Doctoral Committee:

Professor William G. Helferich, Chair
Assistant Professor Zeynep Madak Erdogan, Director of Research
Professor Elvira de Mejia
Associate Professor Hong Chen

ABSTRACT

Breast cancer is the most common cancer type in women population affecting a large number of women worldwide. Due to its highly heterogenous molecular characteristics, there is not a default treatment strategy for breast cancer. Almost two third of breast cancer cases are estrogen receptor positive ER (+). Majority of breast cancer specific deaths in women with ER α (+) tumor occur due to metastases that are resistant to endocrine therapy. There is a critical need for novel therapeutic approaches to prevent or delay recurrence of ER α (+) tumors. The overall objective of this dissertation project is to define the interaction among endocrine resistance, nuclear export system, and cellular metabolic pathways, and to evaluate the metabolic impact of combinational ER α and XPO1 targeting by using combination of endocrine therapy agents and a XPO1-specific small molecule inhibitor (Selinexor-SEL) in different endocrine resistant ER (+) breast cancer cell lines. The long term goal is to develop effective treatment strategies to overcome endocrine resistance in ER (+) breast cancer.

In the first part of this project, we focused on the characterization of XPO1 protein in tamoxifen resistance. In this part, we used endocrine-sensitive and -resistant breast cancer cell lines (*in vitro*) and tumor xenograft models (*in vivo*) to test our hypothesis. Our results showed that expression profile of XPO1 protein has a pivotal role in the progression of tamoxifen resistance in ER (+) breast cancer cell models. We found out that XPO1 protein modulates tamoxifen resistance due to its function to determine subcellular localization of important kinases.

In the second part of the project, we investigated how nuclear export system contribute to tamoxifen resistance development by testing with a novel combinational targeting strategy using 4-OHT and SEL in Luminal B type breast cancer cell lines, and to investigate the metabolic outcomes of this novel therapy. To test our hypotheses, we used actual patient tumor samples and endocrine-sensitive and -resistant breast cancer cell lines (*in vitro*). Our findings indicated that XPO1 expression is significantly higher in Luminal B subtype compared to other molecular subtypes. We demonstrated that combined targeting of XPO1 and ER α rewires metabolic pathways (e.g. Akt pathway), and shuts down both glycolytic and mitochondrial pathways that would eventually lead to autophagy.

Lastly, we assessed the impact of other endocrine therapy options alone or in combination with Selinexor on metabolism in different endocrine resistant breast cancer cell lines and possible

metastatic organ sites. We used 3-D cell culture models and endocrine-sensitive and -resistant breast cancer cell lines (*in vitro*). Using a combination of transcriptomics, kinase arrays, metabolomics and metabolic flux experiments, we identified glutamine metabolism pathways to be rewired during endocrine resistance. In limited media conditions mimicking nutrient deprived tumor microenvironment, endocrine resistant cells were more dependent on mitochondria for energy production. Their glucose and fatty acid dependency decreased in the presence of SEL and cells were more dependent on glutamine. The effect of glutamine was dependent on conversion of the glutamine to glutamate and mitochondrial complex 1 activity. In order to examine metabolites that might result in the observed phenotype we performed GC/MS whole metabolite profiling and identified amino acid metabolism pathways to be upregulated when cells were treated with SEL.

In conclusion, our study indicated that remodeling metabolic pathways to regenerate new vulnerabilities in endocrine resistant breast tumors is novel, and given the need for better strategies for improving therapy response of relapsed ER α (+) tumors, our findings show great promise for uncovering the role ER α -XPO1 crosstalk plays in reducing cancer recurrences.

Keywords: Breast cancer, cancer metabolism, endocrine resistance, nuclear transport system

*This work is dedicated to my parents **Melek Kulkoyluoglu** and **Levent Kulkoyluoglu**, my dear husband **Ugur Cotul** and all my family and friends. Nothing in my life was possible without their unconditional love and support during these four years.*

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CHAPTER 1: INTRODUCTION

1.1. Overview

ER α is known to be present in 75% of breast cancers and is considered the most common drug target and prognostic marker defining breast cancer subtypes [1]. Effective and well-tolerated endocrine therapies were developed to avoid negative effects of radiation and chemotherapy, including tamoxifen, which is a selective estrogen receptor modulator (SERM) used to treat pre- and post-menopausal women with ER α -positive tumors [2]. Although tamoxifen has been used to extend disease-free survival of millions of women over the last forty years, approximately 40% of these tumors eventually relapse after a 10-year continuous tamoxifen treatment [3, 4]. Besides, selective estrogen degraders (SERDs), like fulvestrant, are also commonly used for the treatment of ER (+) breast cancers. Even though there are different endocrine and targeted therapy options to treat breast cancer in clinic, drug resistance against these treatments has become the most prevalent problem due to long treatment procedure. In fact, most breast cancer deaths occur due to recurrent tumors that become more aggressive and tend to metastasize to distant organs [5]. Therefore, novel studies are needed to investigate possible mechanisms of drug resistance. Many proposed mechanisms exist to explain the mechanism of this resistance, such as dysregulations in tamoxifen metabolism, mutations to ESR1 and even specific proteins that can recognize ER-tamoxifen complexes, and alterations in protein kinase signaling [5]. Since many of these mechanisms are related with cell cycle progression and E2-related hyperactivation in cell cycle, these pathways have become the most pivotal targets for combined treatments in clinic. If we look at current clinical practices, combination of endocrine therapies with CDK4/6 inhibitors, such as Palbociclib, has become a gold standard to treat endocrine resistant breast cancers. Even though resistance and metastasis are the two main negative outcomes of endocrine treatment, the molecular mechanisms underlying these obstacles are still not clear. A significant number of studies in the last decade have addressed possible intracellular pathways that may be the reason behind endocrine resistance [6]. Dysregulation of the nuclear transport pathways are considered one of the proposed mechanisms due to causing alterations in the subcellular localization of some tumor suppressor proteins and hormone receptors [7]. As a nuclear export element, exportin 1 (XPO1) has been proposed to be used as a biomarker for the development of tamoxifen resistance. Also, the overexpression of XPO1 has been shown to be associated with tamoxifen resistance in

luminal B type breast tumors, and combined targeting of ER α and XPO1 showed promising effects to overcome tamoxifen resistance in ER (+) breast tumors [7]. The main aim of this project was to understand the molecular mechanism behind the success of targeting ER α and XPO1 together. The contribution of this research was significant because we identified a novel mechanism of endocrine resistance, which shows a clear crosstalk between nuclear export system, intracellular kinase pathways, and cellular metabolism. This mechanism specifically elucidated the metabolic impact of combinational ER α and XPO1 targeting in endocrine-resistant breast cancer cells. In addition, this project provided information about ‘deregulating cell metabolism’ during drug resistance as one of the emerging hallmarks of cancer. The major positive benefit of this study was to understand how ER α -mediated nuclear export contributes to the development of tamoxifen resistance. The delineation of this mechanism gave us an idea about the key targets of intracellular signaling during tamoxifen resistance, which we can inhibit by using our combinational targeting strategy. Further, this study allowed us to identify the metabolic alterations promoting cell survival in the endocrine-resistant tumors. Moreover, the findings obtained from this study may be applicable not only for breast cancer but also for other cancer types in which XPO1 is overexpressed, such as therapy resistant leukemia and prostate cancer. In summary, this project allowed us to extend our knowledge about intracellular regulation of drug resistance in breast cancer treatment and gave us a direction to develop more hypothesis in this field.

The overall objective of this project is to define the interaction among endocrine resistance, nuclear export system, and cellular metabolic pathways, and to evaluate the metabolic impact of combinational ER α and XPO1 targeting by using different endocrine agents and XPO1-specific Selinexor (SEL) in endocrine-resistant breast cancer cells. All research steps and their outcomes were summarized in Table 1.

Each chapter of this project was dedicated to answer several questions related to endocrine resistant in different cohort of breast cancer patients. In the first part of this research, we focused on the molecular differences between endocrine-resistant and -responsive tumors. In the second part, we investigated the molecular mechanism behind the success of ER α and XPO1 targeting in *de novo* tamoxifen-resistant breast cancer cells to mimic the situation of pre-menopausal breast cancer patients, who have never responded to tamoxifen treatment. In the last part, we concentrated on the effect of this combined treatment strategy on metastatic breast cancer cells. To do this, we aimed to study the benefit of this combined treatment in patients who developed resistance against

different endocrine agents and have advanced metastatic breast cancer profiles. Research strategies and outcomes of each aim were extended in the following paragraphs.

Aim 1 was developed to identify and characterize the role of nuclear export mechanism in tamoxifen resistance. There were three main research questions under this aim (Figure 1.1). Firstly, we investigated the link between XPO1 expression and cell proliferation rate in tamoxifen-sensitive and tamoxifen-resistant breast cancer cells. Secondly, we identified the role of XPO1 on subcellular localization of ERK5 during tamoxifen resistance development. Lastly, we determined the effect of individual and combinational ER α and XPO1 targeting on the subcellular localization of ERK5 protein in tamoxifen-resistant breast cancer cells. These results were published in a paper, which was titled as “ER α -XPO1 crosstalk controls tamoxifen sensitivity in tumors by altering ERK5 cellular localization”[7]. This study showed that XPO1 overexpression increased cell proliferation in *de novo* tamoxifen-resistant breast cancer cells. Also, we identified that nuclear localization of active kinases changes during tamoxifen resistance progression (Figure 1.2).

Aim 2 was planned as a follow-up study to understand how nuclear export system contribute to tamoxifen resistance development by testing with a novel combinational targeting strategy using 4-OHT and SEL in Luminal B type breast cancer cell lines, and to investigate the metabolic outcomes of this novel therapy. There were five main research questions under this aim (Figure 1.3). Firstly, we identified how XPO1 is associated with poor disease outcome in ER+ breast cancer cells, and we compared XPO1 expression in different breast cancer subtypes. Secondly, we investigated the effect of ER α and XPO1 targeting on gene expression profiles and the general kinase pathways in tamoxifen-resistant breast cancer cells. Thirdly, we looked at the effect of ER α and XPO1 targeting specifically on different phosphorylation sites of Akt protein. After that, we studied the effect of ER α and XPO1 targeting on general metabolic phenotypes and glycolytic and mitochondrial metabolic pathways in tamoxifen-resistant breast cancer cells. Lastly, we investigated which death pathways are activated via ER α and XPO1 targeting. The results of these research questions were published in a paper, which was titled as “Combined Targeting of Estrogen Receptor Alpha and XPO1 Prevent Akt Activation, Remodel Metabolic Pathways and Induce Autophagy to Overcome Tamoxifen Resistance”. In this study, we found that *XPO1* gene signature is upregulated specifically in Luminal B subtype of breast cancer cells, and combined targeting ER α and XPO1 modulates Akt signaling to rewire tumor cell metabolism and induce autophagy (Figure 1.4).

Aim 3 was prepared to assess the impact of other endocrine therapy options alone or in combination with Selinexor on metabolism in different endocrine resistant breast cancer cell lines and possible metastatic organ sites. There were six main research questions under this aim (Figure 1.5). Firstly, we looked at cell viability of different endocrine resistant breast cancer models under treatment of different endocrine therapy agents alone or in combination. Secondly, we determined metabolic cell phenotypes of different endocrine resistant breast cancer models. Thirdly, we monitored oncosphere formation profile/capacity of endocrine resistant breast cancer cell lines in 3D metastatic organ models. The fourth research question addressed the point of how cellular metabolic phenotype of endocrine resistant breast cancer cells change in different metastatic organ sites. Furthermore, we investigated the mechanism of single agent resistance against Selinexor. Lastly, we focused on the molecular mechanism behind the success of combined ER α and XPO1 targeting. The results of these research questions will be published in a separate research paper. Main conclusion of this part is that combined ER α and XPO1 targeting is an effective strategy to target endocrine-resistant breast cancer cells (Figure 1.6).

1.2. Table and Figures

Table 1.1. Summary of research aims and their outcomes

Type of Study	Aim	Publication
Characterization and identification of the role of XPO1 in tamoxifen resistance - Identification of the differences between endocrine-resistant and -responsive breast tumors in terms of nuclear transport system	Aim 1: To identify the role of nuclear export mechanism in tamoxifen resistance.	K. Wrobel*, Y. C. Zhao*, E. Kulkoyluoglu*, K. L. A. Chen, K. Hieronymi, J. Holloway, S. Li, T. Ray, P. S. Ray, Y. Landesman, A. E. Lipka, R. L. Smith, and Z. Madak-Erdogan, “ERα-XPO1 crosstalk controls tamoxifen sensitivity in tumors by altering ERK5 cellular localization.” Mol. Endocrinol., 2016 (*Co-first author) (published)
Identification of the role of XPO1 in <i>de novo</i> resistance	Aim 2: To understand how nuclear export system contribute to tamoxifen resistance development	Kulkoyluoglu-Cotul, E.; Smith, B.P.; Wrobel, K.; Zhao, Y.C.; Chen, K.L.A.; Hieronymi, K.; Imir, O.B.; Duong, K.; O'Callaghan, C.; Mehta, A.; Sahoo, S.; Haley, B.; Chang, H.; Landesman, Y.; Madak-Erdogan, Z. Combined Targeting of Estrogen Receptor Alpha and XPO1 Prevent Akt Activation, Remodel Metabolic Pathways and Induce Autophagy to Overcome Tamoxifen Resistance. <i>Cancers</i> 2019, <i>11</i>, 479. (published)
Identification of the role of XPO1 in metastatic organ sites	Aim 3: To assess the impact of these individual and combinational treatment strategies on metabolism in endocrine-resistant ER (+) breast cancer cells and in the body	Kulkoyluoglu-Cotul, E.; Smith, B.P.; Santaliz-Casiano, A.; Tunc E.; Zuo, Q.; Duong, K.; Ramesh R.; Park B.H.; Landesman, Y.; Madak-Erdogan, Z. Role of mitochondria and glutamine metabolism in therapy resistance: Combined targeting of estrogen receptor alpha and exportin 1 in endocrine resistant breast cancers. (<u>in progress</u>)

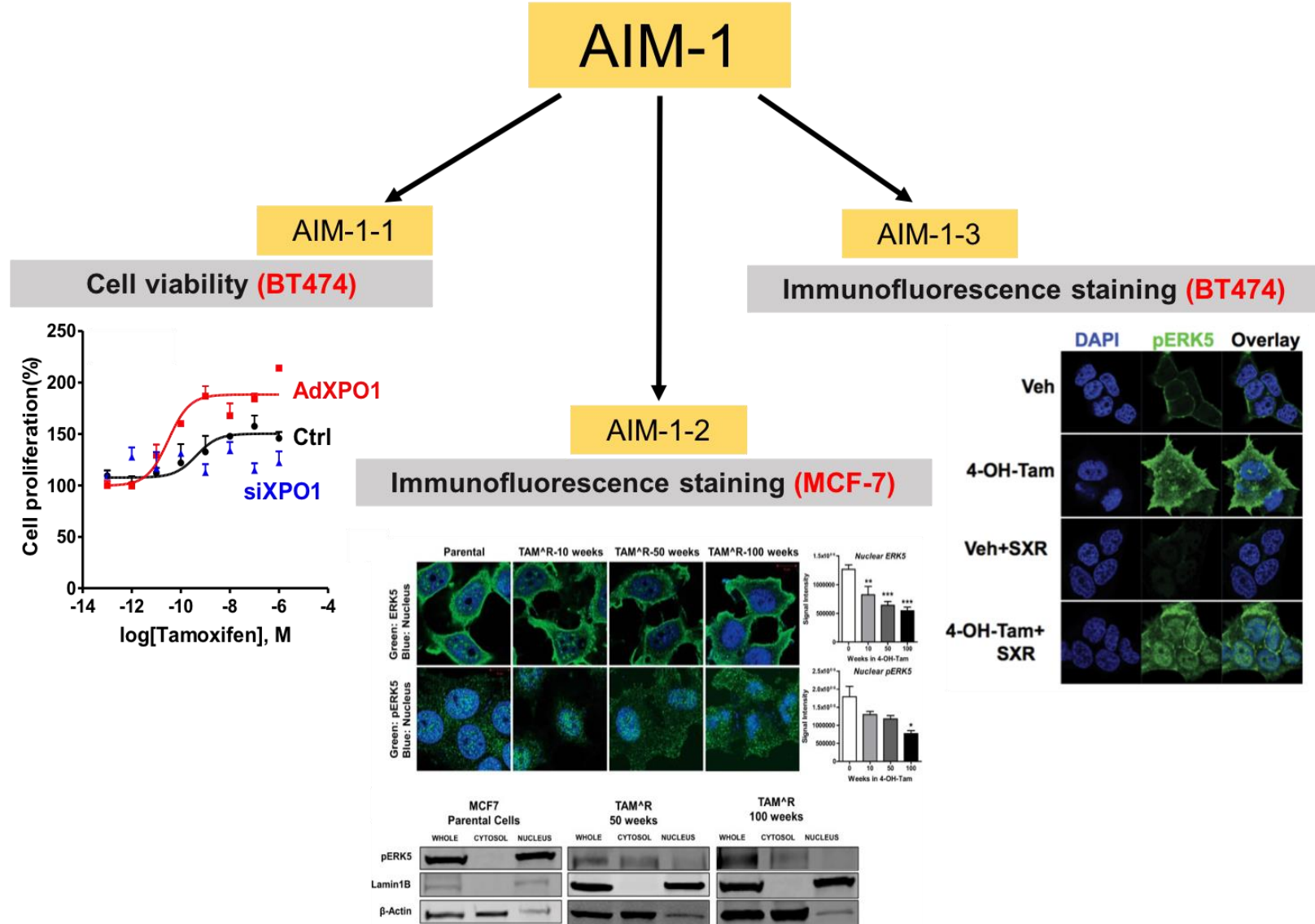


Figure 1.1. Summary of the methods and results in Aim-1.

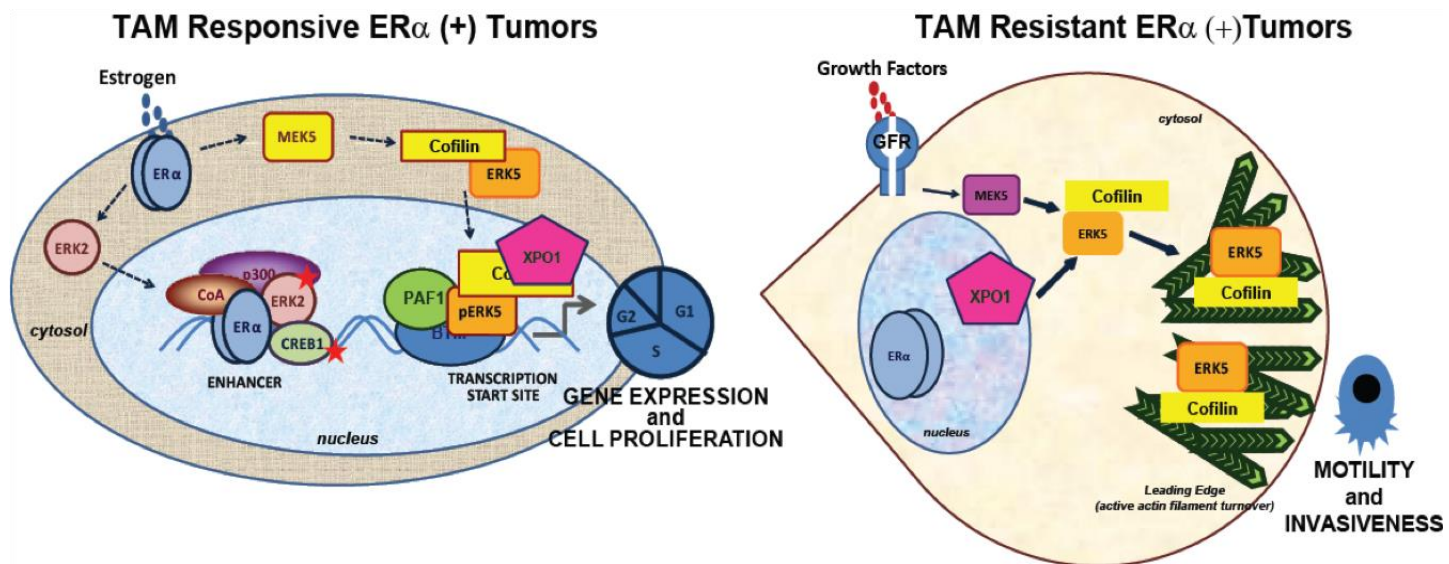
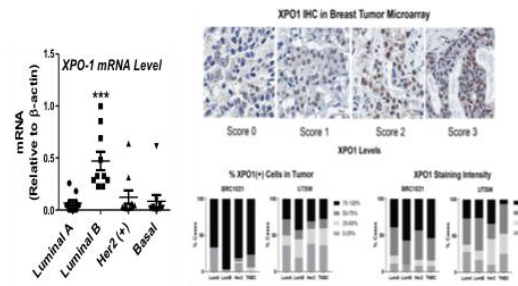


Figure 1.2. XPO1 overexpression is associated with tamoxifen resistance in breast cancer cells.

AIM-2

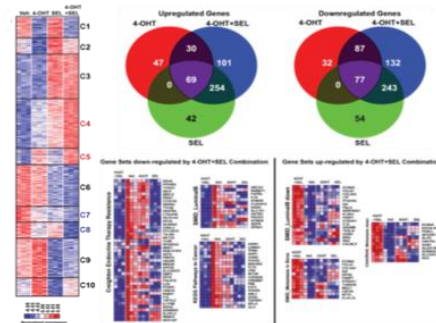
AIM-2-1

Patient tumor samples



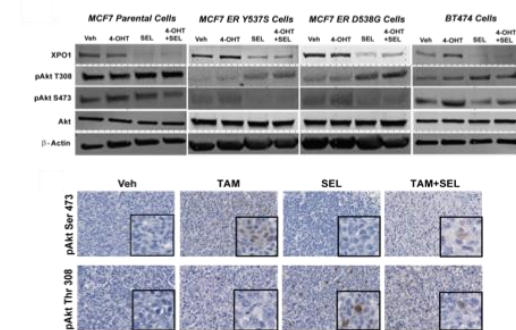
AIM-2-2

RNA sequencing (BT474)



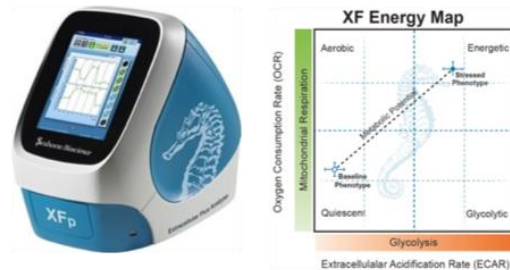
AIM-2-3

Western Blot IHC



AIM-2-4

Seahorse XFp



AIM-2-5

Western Blot (BT474) Colorimetric assays (BT474)

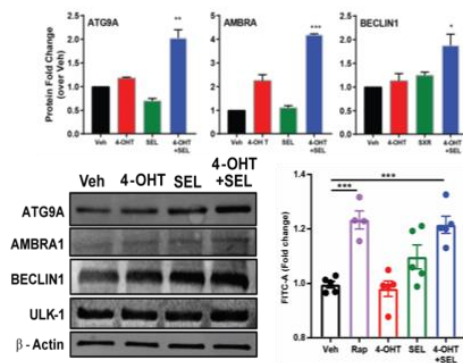


Figure 1.3. Summary of the methods and results in Aim-2.

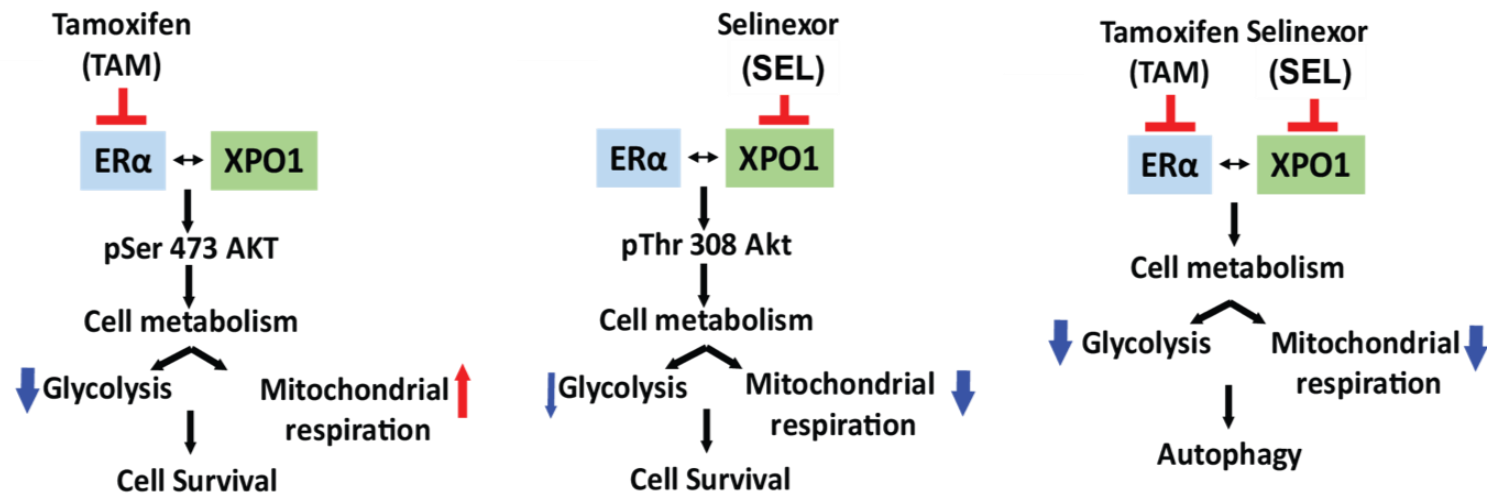


Figure 1.4. Combined targeting ERα and XPO1 modulates Akt signaling to rewire tumor cell metabolism and induce autophagy.

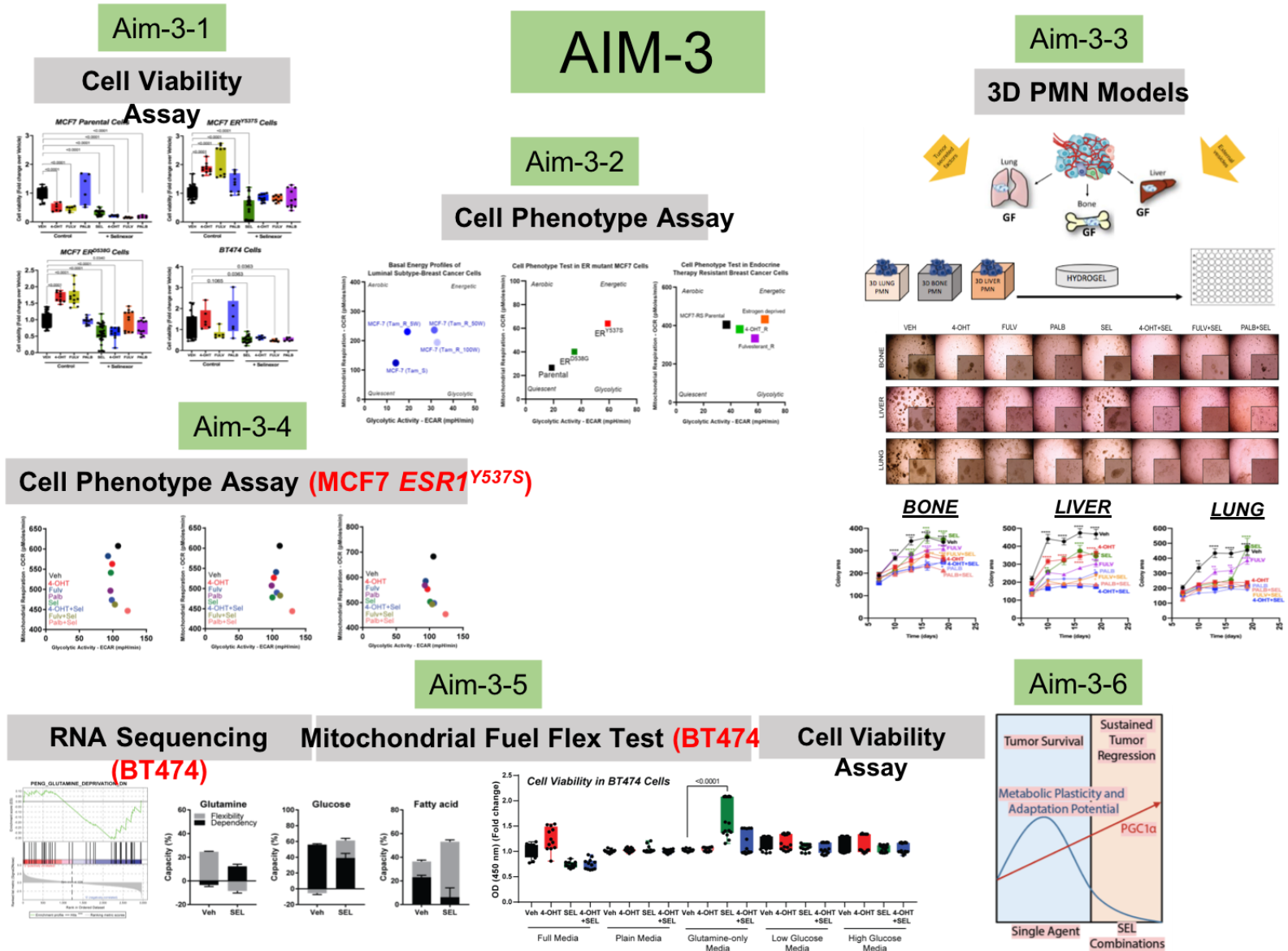


Figure 1.5. Summary of the methods and results in Aim-3.

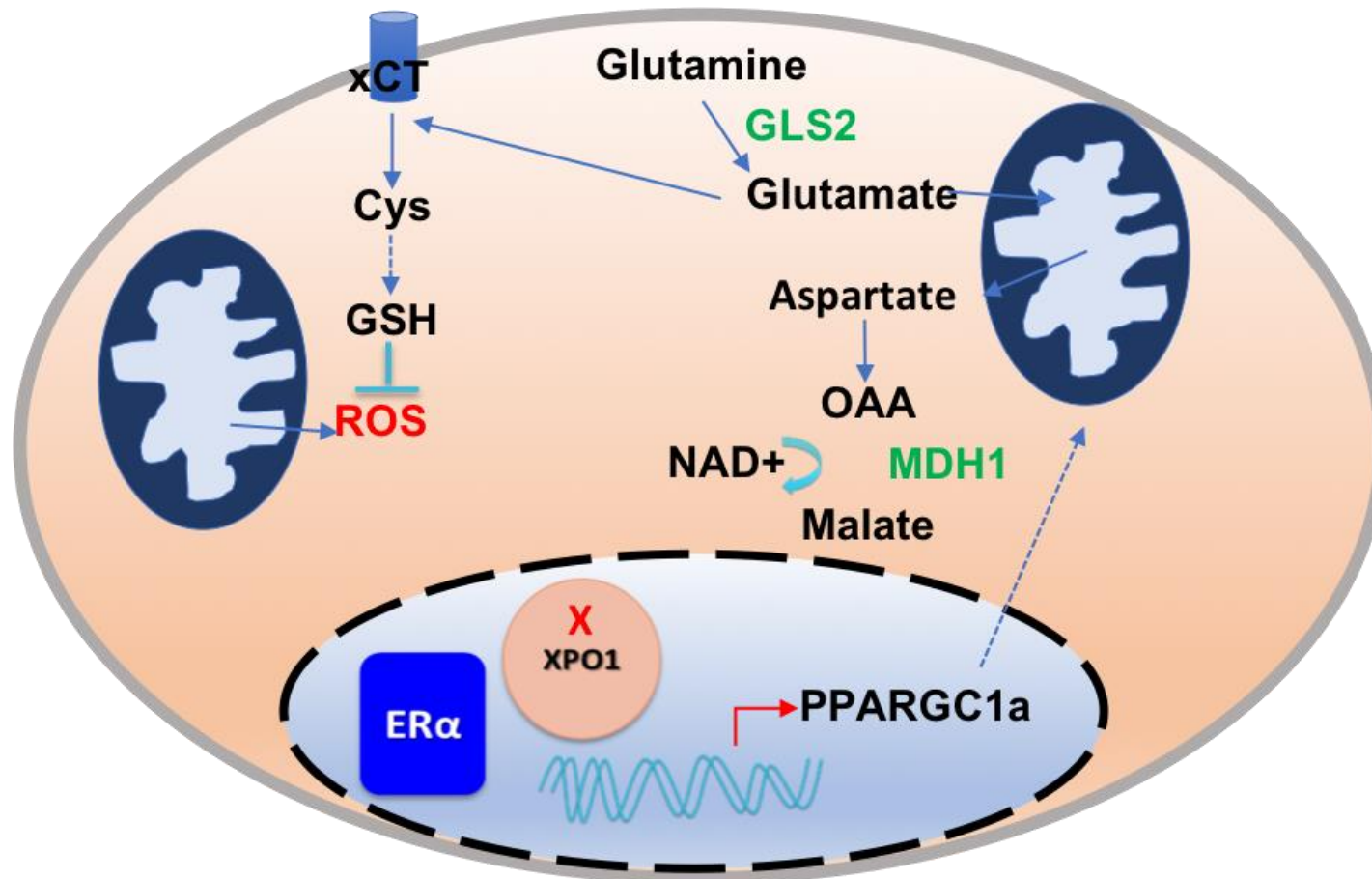


Figure 1.6. Single agent treatments are not effective due to metabolic rewiring. 4-OHT+SEL treatment is an effective strategy to target endocrine resistant breast cancer cells

1.3. References

1. S. Nilsson and J. A. Gustafsson, "Estrogen receptors: therapies targeted to receptor subtypes," *Clin Pharmacol Ther*, vol. 89, pp. 44-55, Jan 2011.
2. V. C. Jordan, "Targeting antihormone resistance in breast cancer: a simple solution," *Ann Oncol*, vol. 14, pp. 969-70, Jul 2003.
3. W. Gu, N. Dong, P. Wang, C. Shi, J. Yang, and J. Wang, "Tamoxifen resistance and metastasis of human breast cancer cells were mediated by the membrane-associated estrogen receptor ER-alpha36 signaling in vitro," *Cell Biol Toxicol*, vol. 33, pp. 183-195, Apr 2017.
4. X. Chen, X. Zha, W. Chen, T. Zhu, J. Qiu, O. D. Roe, *et al.*, "Leptin attenuates the anti-estrogen effect of tamoxifen in breast cancer," *Biomed Pharmacother*, vol. 67, pp. 22-30, Feb 2013.
5. V. K. Arora, E. Schenkein, R. Murali, S. K. Subudhi, J. Wongvipat, M. D. Balbas, *et al.*, "Glucocorticoid receptor confers resistance to antiandrogens by bypassing androgen receptor blockade," *Cell*, vol. 155, pp. 1309-22, Dec 5 2013.
6. M. Chang, "Tamoxifen resistance in breast cancer," *Biomol Ther (Seoul)*, vol. 20, pp. 256-67, May 2012.
7. K. Wrobel, Y. C. Zhao, E. Kulkoyluoglu, K. L. Chen, K. Hieronymi, J. Holloway, *et al.*, "ERalpha-XPO1 Cross Talk Controls Tamoxifen Sensitivity in Tumors by Altering ERK5 Cellular Localization," *Mol Endocrinol*, vol. 30, pp. 1029-1045, Oct 2016.

CHAPTER 2: LITERATURE REVIEW PART I:

NUCLEAR AND EXTRA NUCLEAR-INITIATED ESTROGEN RECEPTOR SIGNALING CROSSTALK AND ENDOCRINE RESISTANCE IN BREAST CANCER

2.1. Abstract

Estrogens regulate function of reproductive and non-reproductive tissues in healthy and diseased states including breast cancer. They mainly work through estrogen receptor alpha (ER α) and/or estrogen receptor beta (ER β). There are various ER α targeting agents that have been used for treatment of ER (+) breast tumors. The impact of direct nuclear activity of ER is very well characterized in ER (+) breast cancers and development and progression of endocrine resistance. Recent studies also suggested important roles for extranuclear-initiated ER α pathways, which would decrease the potency and efficiency of ER α targeting agents. In this mini-review, we will discuss the role of nuclear and extra-nuclear ER signaling and how they relate to therapy resistance in breast cancer.

Key words: Breast cancer, Estrogen receptor, 17- β Estradiol, Tamoxifen resistance

Highlights

- ER signals through nuclear and extranuclear ER-initiated signaling cascades.
- Crosstalk between two pathways regulate metabolic and developmental programs.
- Tamoxifen is still widely used in clinic to treat breast cancer.
- ER α -initiated kinase signaling play a role in therapy resistance in breast cancer.

This part is a part of publication, Kulkoyluoglu, E., Madak-Erdogan, Z., Nuclear and extranuclear-initiated estrogen receptor signaling crosstalk and endocrine resistance in breast cancer, Steroids, Volume 114, 2016, Pages 41-47, ISSN 0039-128X, <https://doi.org/10.1016/j.steroids.2016.06.007>.

2.2. Introduction

Estrogen receptor alpha (ER α) and estrogen receptor beta (ER β) are members of the Type I nuclear receptor superfamily. They regulate 17 β -estradiol (E2)-mediated transcriptional activation of responsive genes. In the last three decades, various agonists and antagonists have been designed for the treatment of ER-positive breast cancer. Although long-term usage causes resistance, tamoxifen is still the most popular endocrine agent for ER (+) breast cancer treatment. This mini-review aims to highlight recent studies that uncovered the importance of ER α and kinase signaling pathway crosstalk in therapy resistance for breast cancer.

2.3. Estrogen Receptors (ERs)

2.3.1. ER function in physiology

Estrogen receptors belong to the Type I Nuclear Receptor Super Family that comprises classical steroid receptors. There are two different forms of estrogen receptors, known as estrogen receptor alpha (ER α) and estrogen receptor beta (ER β). Both receptors can act as transcription factors or initiate extranuclear-initiated kinase signaling cascades. ER α is the first steroid receptor to be discovered by Jensen in 1958. After almost 20 years another ER was discovered [1, 2]. They are encoded by genes that are on different chromosomes, have tissue specific expression profiles, and have distinct functions. While ER α is mainly expressed in breast, uterus, prostate, brain, liver, adipose tissue and bones, ER β is widely expressed in the ovary, prostate, testes, lung, thymus, spleen, bone marrow, salivary gland and vascular epithelium. ER α and ER β usually act in opposite ways. In breast tumors, ER α expression is high, but presence of ER β correlates with better prognosis by modulating the effects of ER α [3]. Estrogens play critical roles for maintenance and development of reproductive and non-reproductive tissues since lack or excess of estrogens cause various pathological conditions human breast, ovarian, colorectal, prostate and endometrial cancers, osteoporosis, atherosclerosis, metabolic syndrome and Alzheimer's disease [4, 5]. ER α is a major player in breast cancer, and its expression correlates with better prognosis [6].

2.3.2. ER structure

ER α (NR3A1) and ER β (NR3A2) are encoded by two different genes called Esr1 (on 6q21.5) and Esr2 (on 14q.21), respectively. ER α and ER β isoforms show tissue-specific

expression and they also have been shown to form heterodimers on binding estrogen receptor elements (EREs). Although there is a high homology between DBDs (~95%) and LBDs (~50%) of ER α and ER β , N-terminal domains show only 15% sequence similarity [7]. ESR1 gene consists of 8 exons and it is located on chromosome 6q25.1. It is expressed under control of 6 tissue-specific promoters. The full-length size protein product is 66 kDa [8]. Starting from N-termini, ER α has 6 domains which are named from A to F. A/B domains are the N-terminal domain and have 180 amino acids functioning as AF-1. This region possesses a molecular recognition region (ID region), mostly carrying a random coil structure [9]. The N-termini domain can activate transcription even in the absence of the hormone by means of phosphorylation of the receptor at serine-118 and serine-167 residues by MAPK and Akt pathways, respectively. AF-1 domain can also modulate the response to SERMs together with the domain F called AF-2. Both A/B and E domains are required for full agonist action [10, 11]. C domain is referred DNA binding domain (DBD) and located between amino acid 181 and 263. It has an evolutionary conserved helix-loop-helix structure, which has two cysteine rich zinc finger motifs that interact with a palindromic (GGTCAnnnTGACC) sequence on DNA. The ER DBDs have two sub-domains which are different in term of structure and function. Proximal box, P-box, is the first sub-domain which is responsible for the interaction with the sugar-phosphate backbone on the major DNA groove of the ERE. D-box is the second sub-domain and it is important for receptor dimerization [7, 12]. Between amino acids 264 and 302, resides the D domain and carries a nuclear localization signal. This hinge region serves as a flexible connection between DBD and LBD. E/F domains between amino acid 303 and 533 are LBD and they consist of 12 helices which is critical for hormone binding and dimerization. On the far C-termini of ER, there is F domain serves as AF-2. This region works synergistically to maximize transactivation function together with AF-1 [7]. Recently, several mutations in F-domain were identified in more than 30% of the metastatic ER $_{+}$ tumors. These mutations render the receptor constitutively active and decreases inhibition of the receptor action by currently available endocrine therapies [13-17].

2.3.3. ER ligands

17 β -estradiol (E2) is synthesized from cholesterol and it is the endogenous ligand of ERs. Estrogen was purified and crystalized by Edward Doisy and Alfred Butenandt nearly simultaneously in 1929 and later 17 β -estradiol (E2) biosynthesis was shown to be achieved by

aromatase enzymes mostly in the ovaries, adipose and adrenal tissue [18]. With the discovery of the chemical structure of estrogen by using new molecular biology techniques, a variety of physiologic effects on different tissue types were also identified. These studies showed that estrogen was a good candidate to manage menopausal symptoms of women during 1970s. In these studies, it was shown that 17 β -estradiol (E2) is a steroid hormone, whose synthesis is regulated by 17-hydroxysteroid dehydrogenase. The human aromatase is encoded by CYP19A1 gene and the size of the full protein is about 123 kDa. First exon is expressed in tissue-specific manner [19]. Tissue-specific expression of aromatase regulates 17 β -estradiol (E2) synthesis in the body.

The LBD domain of ERs bind to both agonists and antagonists, but helix 12 has distinct orientations with different ligands. Upon agonist binding, including the 17 β -estradiol (E2) and the synthetic nonsteroidal estrogen diethylstilbestrol (DES) to ERs, helix 12 on LBD folds against helices 3 and 11 which allows maximal coactivator binding on TAD. However, this coactivator binding pocket is disrupted with antagonist binding such as selective estrogen receptor modulators (SERMs). In this region, long hydrophobic groove on helix 12 becomes available for corepressor binding [20, 21]. Tamoxifen and raloxifene are two SERMs and they can act either as an agonist or as an antagonist in different tissues [22]. Raloxifene acts as an ER antagonist in the breast and uterus, yet it has agonistic effects on bone structure, which provides increase in mineral density to improve menopausal osteoporotic outcomes. Tamoxifen is the most commonly used endocrine agent which is still prescribed to treat premenopausal and postmenopausal breast cancer patients. Tamoxifen has tissue specific activity; it is antagonistic in breast tissue, yet it acts as an agonist in the uterus. While it blocks AF-2, it can activate AF-1 in tissue specific manner [23]. In addition, with long-term usage, cancer cells might develop tamoxifen resistance. The molecular mechanism behind tamoxifen resistance is an active area of research by many labs and several mechanisms will be discussed in Section 3.3.

2.3.4. Post-translational modifications (PTMs) and interacting partners of ER

ER activity can be modulated by posttranslational modifications and/or differential interactions with coactivator or corepressors. [24-27]. ER α is phosphorylated at different serine residues on N-terminal transactivation domain, DBD and a Tyr residue in the LBD [28-33]. Kinases downstream of mitogen-activated protein kinase (MAPK) pathway and protein kinase pathway (PKA) increases phosphorylation of ERs [31, 34-36]. The DBD of ER is also acetylated

by p300 coactivator associated factor (PCAF) on four highly-conserved lysine residues which control the transcriptional activity of ER [37]. In addition, palmitoylation of human ER α protein on Cys-447 enables membrane association of the receptor. Studies using mice that has mutation on this residue showed that membrane-specific loss of function resulted in female infertility with abnormal ovarian function and abrogated significant vascular actions of E2 [38]. Moreover, palmitoylation affects dimerization, protein-protein interaction partners and E2-dependent degradation of the receptor as well as the phosphorylation of the ER α Ser-118 residue [39, 40]. Sumoylation is another important PTM and it usually promotes autophagy and apoptosis under stress conditions. In parallel to this function, sumoylation of ERs leads to transactivation of some cell death-related genes in presence of endogenous ligands [41]. Regulation of ER degradation is also controlled by ubiquitination. In recent articles, it has been shown that, E2-mediated ER α degradation requires ubiquitination of residues such as leucine 429 and alanine 430 on LBD [42]. A comprehensive evaluation of PTMs of ER α in breast cancer revealed several sites associated with better clinical outcome for tamoxifen therapy, whereas other phosphorylation sites were associated with poorer clinical outcome. ER α acetylation and sumoylation have also been shown to be important predictors of breast cancer outcomes [43].

Numerous ER α interacting partners have been identified in the last two decades. Steroid receptor co-activator (SRC-1) was the first nuclear receptor co-activator to be identified; it stimulates transcriptional activation by binding to AF-2 region of nuclear receptors including ER in presence of the ligand [44]. In the late 1990s, SRC-3 and receptor interacting protein 140 (RIP140) were identified as two important key co-regulators of both ER α and ER β [45, 46]. ERs also interact with other transcription factors, nuclear receptors and coregulators associated with these proteins. Vitamin D receptor interacting protein (DRIP)/thyroid hormone receptor associated proteins (TRAP) complex, nuclear receptor corepressor (NCoR) and silencing mediator for retinoid and thyroid receptor (SMRT) interact with ER_ through AF-2 domain [47 49]. Recent studies also showed that ER α physically interacts with c-MYC through AF-1 to stabilize estrogen-mediated signaling networks [50]. The retinoic acid receptor alpha (RAR α) was also shown to be in ER complex and is important for maintenance of ER-cofactor interactions. These data suggests that ER α interacts with different nuclear receptors to provide effective transcriptional activity in breast cancer cells [51].

2.4. ER activated pathways

2.4.1. Nuclear initiated pathways

In the absence of ligand, ER α binds to heat-shock proteins in the cytoplasm. In presence of the hormone, ER disassociates from HSPs, translocates into the nucleus and binds to EREs on the genome. Once ER α binds to EREs, it recruits other co-regulators to form a transcriptional regulatory complex. Then, the complex recruits basal transcription machinery and RNA Polymerase II to the proximal promoter region. Some of the genes lack any ERE-like sequences and they require another transcription factor to provide the scaffold for ER to indirectly bind to DNA. Most of the primary E2-responsive genes are activated through the direct/indirect interaction between ER and DNA. On the other hand, secondary E2-responsive genes are the ones whose expression depends on a transcription factor, which is activated by ERs [52, 53]. Many studies identified E2-regulated genes using genome-wide cDNA microarray and high-throughput RNA sequencing technologies [46, 53-62]. The majority of these analyses focused on upregulated genes and several of them characterized different mechanisms by which ER α represses transcription [63-65].

2.4.2. Extracellular initiated pathways

Estrogens are also capable of activating extranuclear-initiated kinase signaling, which contributes to gene transcription, cytoskeletal remodeling, cell proliferation and cell survival [57-59, 66, 67]. G-protein coupled ERs (GPER or GPR30) which are located on plasma membrane [68] activate MAPK and PI3K pathways in the presence of estrogens [69]. G protein coupling enables rapid estrogen activated signals to be transmitted by phospholipase C-dependent IP₃ production, which cause an increase in the level of second messengers like calcium and nitric oxide (NO) in several endometrial, granulosa, smooth muscle, duodenum cell lines [70, 71]. Recent studies have shown that GPR30 specifically binds to estrogens and is able to mediate non-genomic estrogen actions through epidermal growth factor receptor (EGFR) pathway, the Notch signaling pathway and the mitogen-activated protein kinases (MAPK) pathway [72, 73].

Most recent studies have shown that ERs control cell metabolism and cell proliferation through modulation of other transcription factors and developmental pathways. For example, ER α regulates hypoxia-inducible factor 1 (HIF-1) pathway associated with antiestrogen response in breast cancer [74]. Yu et al. have also found that ER β regulates genes targeted by Hedgehog

signaling pathway [75]. Membrane receptor actions are important in estrogen-mediated regulation of cardiovascular system and nervous system [76, 77]. For example, the membrane-initiated rapid E2 signaling is effective in hypothalamic and hippocampal neurons and this mechanism regulates energy balance, thermoregulation and proliferation in neural tissue by inducing neural protection [78, 79].

Besides secondary messenger signaling, ER α upregulates cell proliferation and cell migration through interactions with IGF-1R and mitogen-activated protein kinase (MAPK) pathway in various cancer cell lines [80-83]. The interactions between ER α localized at the plasma membrane and various specific membrane proteins, such as modulator of non-genomic activity of estrogen receptor (MNAR), Shc, EGF and IGF-1 receptors play important roles in non-genomic estrogen signaling [84-87]. Not only plasma membrane proteins, but also membrane lipids are associated with the small amount of ER α localized on the plasma membrane. Sphingosine-1 phosphate kinase (SphK1) is an enzyme which produces sphingosine-1 phosphate (Sph1) and very recent studies show that increased SphK1 expression is associated with E2-dependent activation of MAPK and intracellular Ca²⁺ mobilization in ER α -positive MCF-7 breast cancer cells [88, 89].

Endogenous ligand 17- β -E2 activates both nuclear and extranuclear-initiated ER pathways. Recent studies using estrogens conjugated to dendrimers that would stay outside the nucleus [60, 90] or modifying the ligand structure so that the ligand cannot form long-lived stable coregulator complexes [59] achieved preferential activation of these pathways. These tools were very useful and enabled researchers to show functions of extranuclear ER-initiated pathways are important in vascular protection, bone physiology and metabolic tissue function [59, 91, 92] (Figure 2.1).

2.5. The Role of Estrogen Receptor in Breast Cancer

2.5.1. Breast Cancer Incidence and Risk Factors

According to ASCO, in 2016, almost 240,000 women will be diagnosed with breast cancer and 40,000 deaths due to breast cancer will occur in the United States [93]. Due to delayed childbearing and decreased number of births, the long-term incidence of breast cancer has increased during the last decade. Yet, breast cancer mortality decreased by means of early detection and newly developed treatment strategies [94]. Unmodifiable risk factors can be sourced from both hereditary and hormonal dysfunctions. The most important genetic determinant of breast cancer is BRCA1 and BRCA2 tumor-suppressor gene mutations which are inherited in an autosomal

dominant manner. p53, PTEN and CHEK2 mutations are also other key markers of breast cancer development [95-98]. Another important risk factor is age. Statistics show that the lifetime breast cancer risk for a women increases with advancing age [99, 100]. Besides hereditary factors, hormonal factors also contribute to the risk factors of breast cancer. The effect of high estrogen and progesterone exposure in different stages of lifespan increases the risk of breast cancer [101]. However, almost 80% of breast cancers stem from modifiable factors such as obesity, high-fat intake and excess alcohol consumption [102-104].

2.5.2. Molecular Heterogeneity of Breast Cancer

Breast tumors display varying degrees of molecular heterogeneity since different subtypes are initiated from different cell types in the breast tissue [105-107]. In the clinic four main subtypes are used for classification and treatment decision of tumors: luminal A and B, HER-2 (human epidermal growth factor)/ERBB2 and triple negative/basal like. This classification is based on presence of ERs and HER-2 receptors in the tumor [108]. Almost two thirds of breast cancers are categorized under luminal type [109, 110]. The luminal A/B types are ER (+) whereas, triple negative/basal-like and HER-2 types do not express ER. Luminal A type tumors grow slowly and almost 90% of these tumors can be treated with estrogen receptor mediated targeting strategies. Compared to luminal A type, luminal B tumors are more aggressive; their progesterone receptor (PR) expression is lower and they respond poorly to hormone therapy. Gene expression profiling studies elucidated that luminal B type might display both luminal A type and HER-2 form characteristics. For example ESR1, FOXA1 and BCL2 gene expression profiles in luminal B type is similar with luminal A type. On the other hand, luminal B type tumors also have similar MK167, BIRC5 and cyclin B1 gene expressions like HER-2 subtype [111]. In clinic, luminal B type tumors are characterized by having ER+/HER2-/Ki67+ profile [112]. HER-2 oncogene is overexpressed in 70% of HER-2 type tumors which leads to activation of downstream signaling pathways. HER-2 amplified tumors have low prognosis rate because of their complex signaling features. In triple negative/basal like tumors, there are no hormone receptors (estrogen, progesterone) or HER-2 receptors, therefore they tend to be more aggressive. There are several subtypes of triple negative breast cancers. One of these subtypes is basal-like tumors. However, not all basal like tumors are triple negative [110]. Since they do not have a potential therapy target, survival rate of the patients who have triple negative tumors is lower than patients who have ER (+) tumors [113].

Mutations in ER (+) tumors are considerably more diverse within luminal A and luminal B tumors than within ER-negative subtypes. Recent studies that identified novel mutations in breast tumors showed that the overall mutation rate was lowest in luminal A tumors. PIK3CA, MAP3K1, GATA3, TP53, CDH1 and MAP2K4 are the genes are mostly mutated in luminal A subtype. Luminal B tumors also have a diverse array of mutated genes such as TP53 and PIK3CA [107]. These mutations contribute to molecular heterogeneity, and this in turn introduces new challenges as increased heterogeneity is associated with increased therapy resistance.

2.5.3. Tamoxifen and Mechanisms of therapy resistance in breast cancer

The tamoxifen (ICI46, 474) was synthesized by Dora Richardson in Arthur L. Walpole's research team in 1962 to develop anti-estrogen compounds as contraceptive pill. Later studies showed that tamoxifen was very effective in treating late stage ER-positive breast cancers [114]. Tamoxifen was the first targeted therapy for breast cancer and it improved the survival rates of many women and man who had early and late stage ER (+) breast tumors. Using gene expression analyses, researchers detected a large body of genes which are affected from endocrine therapy. These genes are related to cell proliferation, metabolism and cell invasion and inflammation and immune response [115].

Although tamoxifen has been used for years as the best treatment option for ER (+) breast cancer, studies showed that long-term tamoxifen usage eventually causes relapse in 40% of the patients. [116]. There are several hypotheses to explain the reason behind therapy resistance against tamoxifen in ER (+) breast cancers, which can be grouped under 4 main groups: alterations of ER expression and functions, metabolism of tamoxifen, alterations in co-regulatory proteins and signaling pathways and miRNAs.

The first proposed mechanism is related to direct interaction between tamoxifen and LBD of ESR1. Mutations in LBD domain of ER+ is present about 30% of patients with metastatic ER (+) breast cancer [117-122]. Specific ER mutations, such as Tyr537Ser, Tyr537Asn and Asp538Gly cause resistance to antiestrogens by changing the LBD of the ER α [17]. Some of these mutations also affected the activation status of signaling pathways, such as IGF-1 [119]. ER β also seems to be important for tamoxifen resistance. Several studies showed that certain splice variants of ER β expressions are correlated with worse outcomes in advanced breast tumors [123, 124].

Some studies suggested that transcriptional repression due to long-term tamoxifen usage might cause overpopulation of ER-negative tumor cells in tumor environment.

Metabolism of the tamoxifen in the tumor or inability of the body to produce functional metabolites are other mechanisms that were proposed for resistance to tamoxifen. In several studies about tamoxifen resistance reported that lower tamoxifen concentration inside of the tumor relative to plasma concentrations is correlated with worse outcome in breast tumors [124]. On the other hand, some studies suggested that increased concentration of certain tamoxifen metabolites can cause agonistic effects in the cancer cell [125].

The third hypothesis for tamoxifen resistance is related to the alterations in co-regulatory proteins and signaling pathways. MAPK, hedgehog, PI3K/Akt and mTOR pathways have been implicated in tamoxifen resistance. [116, 126]. For instance, some studies reported that high HER-2 levels in tamoxifen resistant tumors is associated with high expression of AIB1 (SRC-3) [127]. Moreover, high AIB1 expression is thought to be associated with a worse disease outcome in tamoxifen-treated patients because of HER-2-related agonistic effect on ER [128]. Other coactivators and corepressors, such as RIP140 [129], NCoR [130] and Nkx3-1 [131] were implicated in tamoxifen resistance since their levels correlated with tamoxifen resistance in tumor samples. In addition, some transcriptional factors (e.g. GATA3 and GREB1) were shown to play a role in regulation of ER-target genes leading to endocrine therapy resistance [132, 133]. Like various types of co-regulatory proteins, major signaling pathways also contribute to generate tamoxifen resistance. Several MAPKs (e.g. like ERK1, ERK2 and ERK3) and ribosomal S6 kinase (RSK) phosphorylate AF-1 domain of ERs and activates estrogen-dependent expression [134, 135]. The activation of growth factor signaling elements contribute to tamoxifen resistance. For instance, the direct interaction between IGF-1 receptor and membrane ERs activates estrogen signaling leading to tamoxifen resistance [80]. Furthermore, the interaction between ER α and HER2 cause phosphorylation of epidermal growth factor receptor (EGFR) [136]. Besides all these factors, E2-mediated cellular localization changes of critical signaling kinases were also found crucial in generation of tamoxifen resistance [57, 137, 138].

As an important regulators of gene expression, the role of miRNAs in tamoxifen resistance has been studied for a while. Recent studies showed that certain miRNAs are regulated by tamoxifen treatment. In fact, some studies suggested that specific miRNA expression patterns (e.g. miR-375, miR-221/222, miR-200, miR-342, miR-519a, miR-22, miR-301, miR-15a/16) can be

used as tamoxifen-resistance markers [139-146]. All of these studies concluded that miRNAs may regulate the sensitivity of breast cancer cells to tamoxifen.

2.5.4. ER (+) breast cancers and possible therapy alternatives

Currently, there are several endocrine therapy approaches to treat ER (+) breast tumors such as steroidal and non-steroidal aromatase inhibitors (AI), SERMs (e.g. raloxifen) and selective estrogen receptor down-regulators (SERDs) (e.g. fulvestrant). First of all, AIs are used to block estrogen production especially in post-menopausal women. Aromatase enzymes catalyze estrogen biosynthesis from androgens in both ovaries and adrenal glands and AIs inhibit these enzymes by competing for the natural substrate binding sites. Clinical studies showed that it is an effective strategy specifically for treating post-menopausal women whose tumors are already resistant to tamoxifen [147]. SERMs are receptor antagonists which occupy estrogen receptors and inhibit endogenous estrogen production. SERMs such as tamoxifen and raloxifen are used to treat pre-menopausal or post-menopausal patients, who have increased side-effects with AIs [148]. SERDs are also routinely used as endocrine therapy agents in clinic. Rather than endogenous estrogen inhibition, they block the effects of estrogen at the levels of the receptor by causing degradation of the receptor itself. Tamoxifen is a partial antagonist of ER_α in breast tissue and an agonist in endometrium. Unlike SERMs, SERDs like fulvestrant do not have this partial agonist activity in any other tissue [149, 150].

2.6. Concluding Remarks

Studies investigating the details of ER signaling pathways are fundamental for developing new therapeutic strategies against endocrine resistant breast cancer. Despite several different ER targeting strategies developed in the last three decades, tamoxifen is still one of the best endocrine agents. It is still widely used in premenopausal women and postmenopausal women who experience major side effects with aromatase inhibitors. One-third of these patients will have a recurrence that will decrease survival of the patient. Many labs are still working to develop strategies to overcome tamoxifen resistance in breast cancer patients. The majority of these research efforts have focused on targeting signaling pathways, such as PI3K and mTOR. However, resistance develops in this setting, too and in this case the arising tumors are even harder to manage. Therefore, novel strategies to delay or overcome tamoxifen resistance are needed.

2.7. Figure

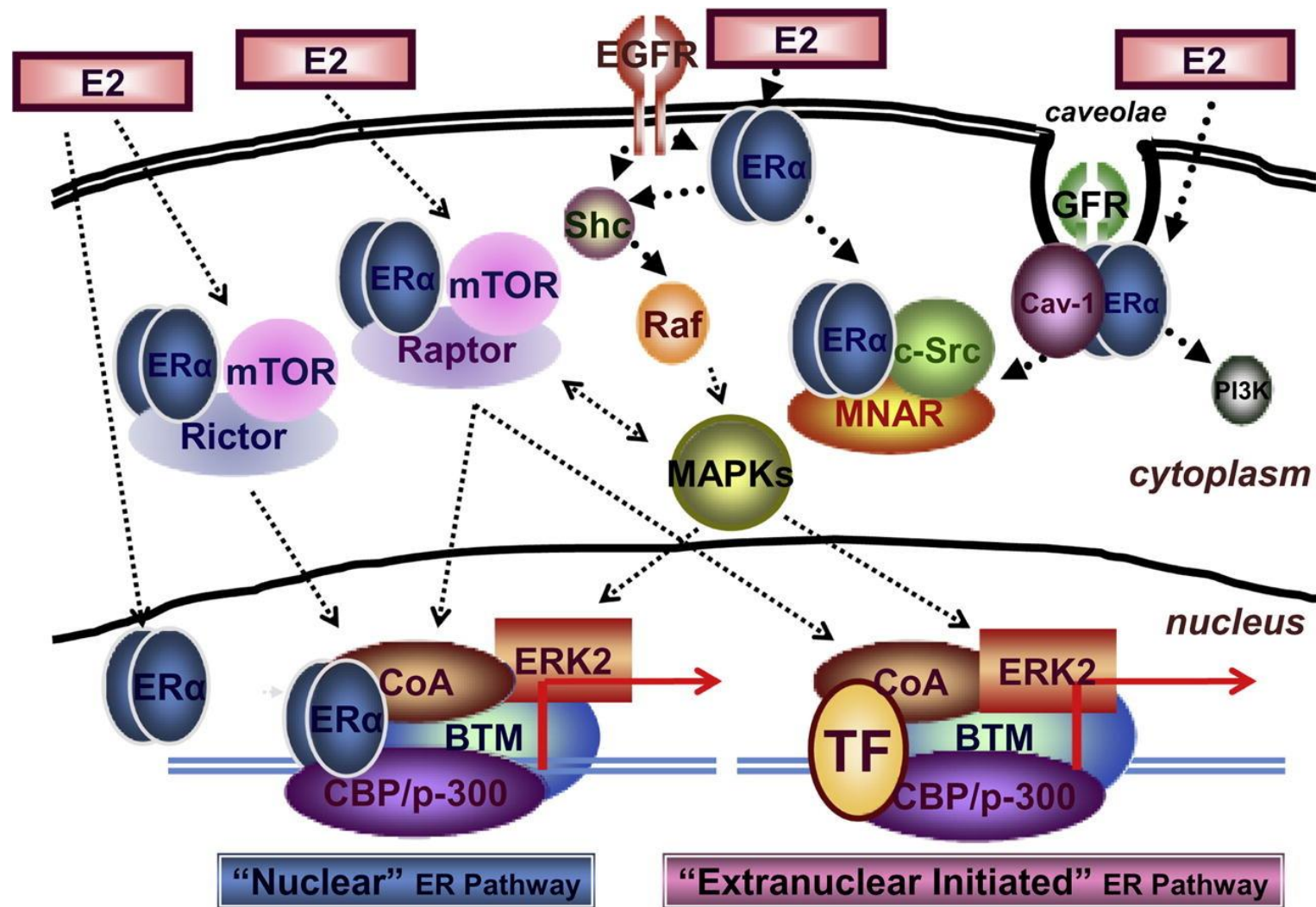


Figure 2.1. The basic estrogen signaling cascades of nuclear and extracellular initiated pathways. Estrogen receptor signaling works as other nuclear receptor signaling pathways. E2 binds to ER and then they form a homodimer structure. After that, this ligand-receptor complex can act in two ways. This system has genomic and non-genomic regulatory roles in the cells

2.8. References

1. Pedram, A., *et al.*, Membrane and nuclear estrogen receptor collaborate to suppress adipogenesis but not triglyceride content. *The FASEB Journal*, 2015.
2. Jensen, E.V. and H.I. Jacobson, Fate of Steroid Estrogens in Target Tissues. Academic Press Inc. 161-178.
3. Grober, O.M.V., *et al.*, Global analysis of estrogen receptor beta binding to breast cancer cell genome reveals an extensive interplay with estrogen receptor alpha for target gene regulation. *BMC genomics*, 2011. 12(1): p. 36-36.
4. Murphy, E., Estrogen signaling and cardiovascular disease. *Circulation Research*, 2011.109(6): p. 687-696.
5. Burns, K.a. and K.S. Korach, Estrogen receptors and human disease: An update. *Archives of Toxicology*, 2012. 86: p. 1491-1504.
6. Katzenellenbogen, B.S. and J. Frasor, Therapeutic targeting in the estrogen receptor hormonal pathway. *Seminars in Oncology*, 2004. 31: p. 28-38.
7. Kumar, R., *et al.*, The Dynamic Structure of the Estrogen Receptor. *Journal of Amino Acids*, 2011. 2011: p. 1-7.
8. Magnani, L. and M. Lupien, Chromatin and epigenetic determinants of estrogen receptor alpha (ESR1) signaling. *Molecular and cellular endocrinology*, 2014. 382(1): p. 633-41.
9. Garza, A.M.S., S.H. Khan, and R. Kumar, Site-specific phosphorylation induces functionally active conformation in the intrinsically disordered N-terminal activation function (AF1) domain of the glucocorticoid receptor. *Molecular and cellular biology*, 2010. 30(1): p. 220-30.
10. Osborne, C.K. and R. Schiff, Mechanisms of endocrine resistance in breast cancer. *Annual review of medicine*, 2011. 62: p. 233-47.
11. Zheng, L.H., *et al.*, Endocrine resistance in breast cancer. *Climacteric: the journal of the International Menopause Society*, 2013. 2014: p. 1-7.'
12. Gruber, C.J., *et al.*, Anatomy of the estrogen response element. 2004. p. 73-78.
13. Arora, Vivek K., *et al.*, Glucocorticoid Receptor Confers Resistance to Antiandrogens by Bypassing Androgen Receptor Blockade. *Cell*, 2013. 155(6): p. 1309-1322.
14. Alluri, P.G., C. Speers, and A.M. Chinnaiyan, Estrogen receptor mutations and their role in breast cancer progression. *Breast Cancer Research*, 2014. 16(6): p. 494-494.

15. Chu, D., *et al.*, ESR1 Mutations in Circulating Plasma Tumor DNA from Metastatic Breast Cancer Patients. *Clinical cancer research: an official journal of the American Association for Cancer Research*, 2015: p. 1078-0432.CCR-15-0943-.
16. Robinson, D.R., *et al.*, Activating ESR1 mutations in hormone-resistant metastatic breast cancer. *Nature genetics*, 2013. 45(12): p. 1446-51.
17. Toy, W., *et al.*, ESR1 Ligand-Binding Domain Mutations in Hormone-Resistant Breast Cancer. *Nature Genetics*, 2013. 45(12): p. 1439-45.
18. Simpson, E. and R.J. Santen, Celebrating 75 years of oestradiol. *Journal of molecular endocrinology*, 2015. 55(3): p. T1-T20.
19. Prokai, L., N.M. Rivera-Portalatin, and K. Prokai-Tatrai, Quantitative structure-activity relationships predicting the antioxidant potency of 17 β -estradiol-related polycyclic phenols to inhibit lipid peroxidation. *International Journal of Molecular Sciences*, 2013. 14(1): p. 1443-1454.
20. Li, M.J., *et al.*, Structure of estradiol metal chelate and estrogen receptor complex: the basis for designing a new class of selective estrogen receptor modulators. *Journal of Medicinal Chemistry*, 2011. 54(10): p. 3575-3580.
21. Bruning, J.B., *et al.*, Coupling of receptor conformation and ligand orientation determine graded activity. *Nature chemical biology*, 2010. 6(11): p. 837-43.
22. Waters, E.a., *et al.*, Use of tamoxifen and raloxifene for breast cancer chemoprevention in 2010. *Breast Cancer Research and Treatment*, 2012. 134(2): p. 875-880.
23. Hu, R., L. Hilakivi-Clarke, and R. Clarke, Molecular mechanisms of tamoxifen associated endometrial cancer (Review). *Oncology letters*, 2015. 9(4): p. 1495-1501.
24. Hossain, A., M.T. Kuo, and G.F. Saunders, Mir-17-5p regulates breast cancer cell proliferation by inhibiting translation of AIB1 mRNA. *Mol Cell Biol*, 2006. 26(21): p. 8191-201.
25. Yu, Z., *et al.*, A cyclin D1/microRNA 17/20 regulatory feedback loop in control of breast cancer cell proliferation. *J Cell Biol*, 2008. 182(3): p. 509-17.
26. Castellano, L., *et al.*, The estrogen receptor-alpha-induced microRNA signature regulates itself and its transcriptional response. *Proc Natl Acad Sci U S A*, 2009. 106(37): p. 15732-7.

27. Adams, B.D., H. Furneaux, and B.A. White, The micro-ribonucleic acid (miRNA) miR-206 targets the human estrogen receptor-alpha (ER alpha) and represses ER alpha messenger RNA and protein expression in breast cancer cell lines. *Mol Endocrinol*, 2007. 21(5): p. 1132-47.
28. Ali, S., *et al.*, Modulation of transcriptional activation by ligand-dependent phosphorylation of the human oestrogen receptor A/B region. *EMBO J*, 1993. 12(3): p. 1153-60.
29. Joel, P.B., A.M. Traish, and D.A. Lannigan, Estradiol and phorbol ester cause phosphorylation of serine 118 in the human estrogen receptor. *Mol Endocrinol*, 1995. 9(8): p.1041-52.
30. Lahooti, H., *et al.*, Characterization of ligand-dependent phosphorylation of the estrogen receptor. *Mol Endocrinol*, 1994. 8(2): p. 182-8.
31. Le Goff, P., *et al.*, Phosphorylation of the human estrogen receptor. Identification of hormone-regulated sites and examination of their influence on transcriptional activity. *J Biol Chem*, 1994. 269(6): p. 4458-66.
32. Arnold, S.F., *et al.*, Estradiol-binding mechanism and binding capacity of the human estrogen receptor is regulated by tyrosine phosphorylation. *Mol Endocrinol*, 1997. 11(1): p. 48-53.
33. Auricchio, F., *et al.*, Phosphorylation of estradiol receptor on tyrosine and interaction of estradiol and glucocorticoid receptors with antiphosphotyrosine antibodies. *Adv Exp Med Biol*, 1988. 231: p. 519-40.
34. Aronica, S.M. and B.S. Katzenellenbogen, Stimulation of estrogen receptor-mediated transcription and alteration in the phosphorylation state of the rat uterine estrogen receptor by estrogen, cyclic adenosine monophosphate, and insulin-like growth factor-I. *Mol Endocrinol*, 1993. 7(6): p. 743-52.
35. Bunone, G., *et al.*, Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. *EMBO J*, 1996. 15(9): p. 2174-83.
36. Chen, D., *et al.*, Phosphorylation of human estrogen receptor alpha by protein kinase A regulates dimerization. *Mol Cell Biol*, 1999. 19(2): p. 1002-15.

37. Wilson, B.J., *et al.*, An acetylation switch modulates the transcriptional activity of estrogen- related receptor alpha. *Molecular endocrinology* (Baltimore, Md.), 2010. 24(7): p. 1349-1358.
38. Adlanmerini, M., *et al.*, Mutation of the palmitoylation site of estrogen receptor α in vivo reveals tissue-specific roles for membrane versus nuclear actions. *Proceedings of the National Academy of Sciences of the United States of America*, 2014. 111(2): p. E283-90.
39. La Rosa, P., *et al.*, Palmitoylation regulates 17 β -estradiol-induced estrogen receptor- α degradation and transcriptional activity. *Molecular endocrinology* (Baltimore, Md.), 2012. 26(5): p. 762-74.
40. Marino, M., P. Ascenzi, and F. Acconcia, S-palmitoylation modulates estrogen receptor localization and functions. 2006. p. 298-303.
41. Lim, Y., *et al.*, Sumoylation regulates ER stress response by modulating calreticulin gene expression in XBP-1-dependent mode in *Caenorhabditis elegans*. *International Journal of Biochemistry and Cell Biology*, 2014. 53: p. 399-408.
42. Pesiri, V., *et al.*, Identification of an estrogen receptor α non covalent ubiquitin-binding surface: role in 17 β -estradiol-induced transcriptional activity. *Journal of cell science*, 2013. 126(Pt 12): p. 2577-82.
43. Anbalagan, M., *et al.*, Post-translational modifications of nuclear receptors and human disease. *Nuclear receptor signaling*, 2012. 10 (Figure 1): p. e001-e001.
44. Beauchemin, D., C. Lacombe, and C. Van Themsche, PAX2 is activated by estradiol in breast cancer cells of the luminal subgroup selectively, to confer a low invasive phenotype. *Molecular cancer*, 2011. 10(1): p. 148-148.
45. Madak-Erdogan, Z. and B.S. Katzenellenbogen, Aryl hydrocarbon receptor modulation of estrogen receptor α -mediated gene regulation by a multimeric chromatin complex involving the two receptors and the coregulator RIP140. *Toxicological sciences: an official journal of the Society of Toxicology*, 2012. 125(2): p. 401-11.
46. Madak-Erdogan, Z., *et al.*, Integrative genomics of gene and metabolic regulation by estrogen receptors and, and their coregulators. *Molecular Systems Biology*, 2014. 9(1): p. 676-676.
47. Konduri, S.D., *et al.*, Mechanisms of estrogen receptor antagonism toward p53 and its implications in breast cancer therapeutic response and stem cell regulation. *Proceedings of*

- the National Academy of Sciences of the United States of America*, 2010. 107(34): p. 15081-15086.
48. Ma, G., *et al.*, SRC-3 has a role in cancer other than as a nuclear receptor coactivator. *International Journal of Biological Sciences*, 2011. 7: p. 664-672.
 49. Wang, Y., *et al.*, Small molecule inhibition of the steroid receptor coactivators, SRC-3 and SRC-1. *Molecular endocrinology* (Baltimore, Md.), 2011. 25(12): p. 2041-53.
 50. Wang, C., *et al.*, Estrogen Induces c-myc Gene Expression via an Upstream Enhancer Activated by the Estrogen Receptor and the AP-1 Transcription Factor. *Molecular endocrinology* (Baltimore, Md.), 2011. 25(9): p. 1527-1538.
 51. Ross-Innes, C.S., *et al.*, Cooperative interaction between retinoic acid receptor-alpha and estrogen receptor in breast cancer. *Genes & development*, 2010. 24(2): p. 171-82.
 52. Stender, J.D., *et al.*, Genome-Wide Analysis of Estrogen Receptor _ DNA Binding and Tethering Mechanisms Identifies Runx1 as a Novel Tethering Factor in Receptor-Mediated Transcriptional Activation. *Molecular and Cellular Biology*, 2010. 30(16): p.3943-3955.
 53. O'Lone, R., *et al.*, Genomic targets of nuclear estrogen receptors. *Molecular endocrinology* (Baltimore, Md.), 2004. 18(8): p. 1859-1875.
 54. Nakamura, T., *et al.*, Estrogen Prevents Bone Loss via Estrogen Receptor Alpha and Induction of Fas Ligand in Osteoclasts. *Cell*, 2007. 130(5): p. 811-823.
 55. Kato, S., *et al.* Function of nuclear sex hormone receptors in gene regulation.
 56. Zhao, Y.C. and Z. Madak Erdogan, Systems Biology of Metabolic Regulation by Estrogen Receptor Signaling in Breast Cancer. *J Vis Exp*, 2016(109).
 57. Madak-Erdogan, Z., *et al.*, Novel roles for ERK5 and cofilin as critical mediators linking ER α -driven transcription, actin reorganization, and invasiveness in breast cancer. *Molecular cancer research: MCR*, 2014. 12(5): p. 714-27.
 58. Madak-Erdogan, Z., *et al.*, Genomic collaboration of estrogen receptor alpha and extracellular signal-regulated kinase 2 in regulating gene and proliferation programs. *Mol Cell Biol*, 2011. 31(1): p. 226-36.
 59. Madak-Erdogan, Z., *et al.*, Design of pathway preferential estrogens that provide beneficial metabolic and vascular effects without stimulating reproductive tissues. *Sci Signal*, 2016. 9(429): p. ra53.

60. Madak-Erdogan, Z., *et al.*, Nuclear and extranuclear pathway inputs in the regulation of global gene expression by estrogen receptors. *Mol Endocrinol*, 2008. 22(9): p. 2116-27.
61. Gong, P., *et al.*, Transcriptomic analysis identifies gene networks regulated by estrogen receptor alpha (ER alpha) and ER beta that control distinct effects of different botanical estrogens. *Nucl Recept Signal*, 2014. 12: p. e001.
62. Bergamaschi, A., *et al.*, The forkhead transcription factor FOXM1 promotes endocrine resistance and invasiveness in estrogen receptor-positive breast cancer by expansion of stem-like cancer cells. *Breast Cancer Res*, 2014. 16(5): p. 436.
63. Carroll, J.S., *et al.*, Genome-wide analysis of estrogen receptor binding sites. *Nature genetics*, 2006. 38(11): p. 1289-1297.
64. Carroll, J.S., *et al.*, Chromosome-Wide Mapping of Estrogen Receptor Binding Reveals Long-Range Regulation Requiring the Forkhead Protein FoxA1. *Cell*, 2005. 122(1): p. 33-43.
65. Stossi, F., Z. Madak-Erdogan, and B.S. Katzenellenbogen, Estrogen receptor alpha represses transcription of early target genes via p300 and CtBP1. *Mol Cell Biol*, 2009. 29(7): p. 1749-59.
66. Angelica, M.D. and Y. Fong, *NIH Public Access*. October, 2008. 141(4): p. 520-529.
67. Flouriot, G., *et al.*, The actin/MKL1 signaling pathway influences cell growth and gene expression through large-scale chromatin reorganization and histone post-translational modifications. *Biochemical Journal*, 2014. 461(2): p. 257-268.
68. Cheng, S.B., *et al.*, Retrograde transport of the transmembrane estrogen receptor, Gprotein-coupled-receptor-30 (GPR30/GPER) from the plasma membrane towards the nucleus. *Steroids*, 2011. 76(9): p. 892-896.
69. Marjon, N.A., *et al.*, G protein-coupled estrogen receptor (GPER) regulates mammary tumorigenesis and metastasis. 2014.
70. Song, R.X.D. and R.J. Santen, Membrane initiated estrogen signaling in breast cancer. *Biology of reproduction*, 2006. 75(1): p. 9-16.
71. Chen, D.B., *et al.*, Membrane estrogen receptor-dependent extracellular signal-regulated kinase pathway mediates acute activation of endothelial nitric oxide synthase by estrogen in uterine artery endothelial cells. *Endocrinology*, 2004. 145(1): p. 113-125.

72. Pupo, M., *et al.*, GPER activates Notch signaling in breast cancer cells and cancer associated fibroblasts (CAFs). *Int J Biochem Cell Biol*, 2014. 46: p. 56-67.
73. Filardo, E.J., *et al.*, Estrogen action via the G protein-coupled receptor, GPR30: stimulation of adenylyl cyclase and cAMP-mediated attenuation of the epidermal growth factor receptor-to-MAPK signaling axis. *Molecular endocrinology* (Baltimore, Md.), 2002. 16(1): p. 70-84.
74. Yang, J., *et al.*, Estrogen receptor- α directly regulates the hypoxia-inducible factor 1 pathway associated with antiestrogen response in breast cancer. *Proceedings of the National Academy of Sciences*, 2015. 112(49): p. 201422015-201422015.
75. Lu, Y., *et al.*, Genes targeted by the Hedgehog-signaling pathway can be regulated by Estrogen related receptor β . *BMC Molecular Biology*, 2015. 16(1): p. 19-19.
76. Lindsey, S.H. and M.C. Chappell, Evidence that the G protein-coupled membrane receptor GPR30 contributes to the cardiovascular actions of estrogen. 2011. p. 343-354.
77. Akama, K.T., *et al.*, Post-synaptic density-95 (PSD-95) binding capacity of G-proteincoupled receptor 30 (GPR30), an estrogen receptor that can be identified in hippocampal dendritic spines. *Journal of Biological Chemistry*, 2013. 288(9): p. 6438-6450.
78. Roepke, T.A., O.K. Ronnekleiv, and M.J. Kelly, Physiological consequences of membrane-initiated estrogen signaling in the brain. *Frontiers in bioscience* (Landmark edition), 2011. 16: p. 1560-73.
79. Fiocchetti, M., P. Ascenzi, and M. Marino, Neuroprotective effects of 17 β -estradiol rely on estrogen receptor membrane initiated signals. *Frontiers in Physiology*, 2012. 3 APR (April): p. 1-10.
80. Kahlert, S., *et al.*, Estrogen receptor α rapidly activates the IGF-1 receptor pathway. *Journal of Biological Chemistry*, 2000. 275(24): p. 18447-18453.
81. Gentilini, D., *et al.*, PI3K/Akt and ERK1/2 signalling pathways are involved in endometrial cell migration induced by 17 β -estradiol and growth factors. *Molecular human reproduction*, 2007. 13(5): p. 317-322.
82. Guo, R.X., *et al.*, 17 β -Estradiol activates PI3K/Akt signaling pathway by estrogen receptor (ER)-dependent and ER-independent mechanisms in endometrial cancer cells. *Journal of Steroid Biochemistry and Molecular Biology*, 2006. 99(1): p. 9-18.

83. Ye, M., *et al.*, Involvement of PI3K/Akt signaling pathway in hepatocyte growth factor induced migration of uveal melanoma cells. *Investigative ophthalmology & visual science*, 2008. 49(2): p. 497-504.
84. Barletta, F., *et al.*, Characterization of the interactions of estrogen receptor and MNAR in the activation of cSrc. *Mol Endocrinol*, 2004. 18(5): p. 1096-1108.
85. Greger, J.G., *et al.*, Phosphorylation of MNAR promotes estrogen activation of phosphatidylinositol 3-kinase. *Molecular and cellular biology*, 2007. 27(5): p. 1904-13.
86. Vadlamudi, R.K. and R. Kumar, Functional and biological properties of the nuclear receptor coregulator PELP1/MNAR. *Nuclear receptor signaling*, 2007. 5: p. e004-e004.
87. Cheskis, B.J., *et al.*, MNAR plays an important role in ERα activation of Src/MAPK and PI3K/Akt signaling pathways. *Steroids*, 2008. 73(9-10): p. 901-905.
88. Sukocheva, O. and C. Wadham, Role of sphingolipids in oestrogen signaling in breast cancer cells: An update. 2014.
89. Sukocheva, O., *et al.*, Estrogen transactivates EGFR via the sphingosine 1-phosphate receptor Edg-3: The role of sphingosine kinase-1. *Journal of Cell Biology*, 2006. 173(2): p. 301-310.
90. Harrington, W.R., *et al.*, Estrogen dendrimer conjugates that preferentially activate extranuclear, non-genomic versus genomic pathways of estrogen action. *Molecular endocrinology* (Baltimore, Md.), 2006. 20(3): p. 491-502.
91. Chambliss, K.L., *et al.*, Non-nuclear estrogen receptor α signaling promotes cardiovascular protection but not uterine or breast cancer growth in mice. *The Journal of clinical investigation*, 2010. 120(7): p. 2319-30.
92. Bartell, S.M., *et al.*, Non-Nuclear-Initiated Actions of the Estrogen Receptor Protect Cortical Bone Mass. *Molecular Endocrinology*, 2013. 27(4): p. 649-656.
93. Facts, C., American Cancer Society: Cancer Facts and Figures 2015. 2015.
94. Stuckey, A., Breast cancer: epidemiology and risk factors. *Clinical obstetrics and gynecology*, 2011. 54(1): p. 96-102.
95. Roy, R., J. Chun, and S.N. Powell, BRCA1 and BRCA2: different roles in a common pathway of genome protection. *Nature Reviews Cancer*, 2011. 12(1): p. 68-78.

96. Bourdon, J.-C., *et al.*, P53 Mutant Breast Cancer Patients Expressing P53 Γ Have As Good a Prognosis As Wild-Type P53 Breast Cancer Patients. *Breast Cancer Research*, 2011. 13(1): p. R7-R7.
97. Zhang, H.Y., *et al.*, PTEN mutation, methylation and expression in breast cancer patients. *Oncology Letters*, 2013. 6(1): p. 161-168.
98. Kuusisto, K.M., *et al.*, Screening for BRCA1, BRCA2, CHEK2, PALB2, BRIP1, RAD50, and CDH1 mutations in high-risk Finnish BRCA1/2-founder mutation-negative breast and/or ovarian cancer individuals. *Breast Cancer Research*, 2011. 13(1): p. R20-R20.
99. Kurian, A.W., *et al.*, Lifetime risks of specific breast cancer subtypes among women in four racial/ethnic groups. *Breast cancer research: BCR*, 2010. 12(6): p. R99-R99.
100. Arvold, N.D., *et al.*, Age, breast cancer subtype approximation, and local recurrence after breast-conserving therapy. *Journal of clinical oncology: official journal of the American Society of Clinical Oncology*, 2011. 29(29): p. 3885-91.
101. Brinton, J.T., *et al.*, Breast cancer risk assessment in 64,659 women at a single high volume mammography clinic. *Academic radiology*, 2012. 19(1): p. 95-9.
102. Fuentes-Mattei, E., *et al.*, Effects of Obesity on Transcriptomic Changes and Cancer Hallmarks in Estrogen Receptor-Positive Breast Cancer. *JNCI Journal of the National Cancer Institute*, 2014. 106(7): p. dju158-dju158.
103. Levine, Morgan E., *et al.*, Low Protein Intake Is Associated with a Major Reduction in IGF-1, Cancer, and Overall Mortality in the 65 and Younger but Not Older Population. *Cell Metabolism*, 2014. 19(3): p. 407-417.
104. Mobarakeh, Z.S., *et al.*, Dietary habits contributing to breast cancer risk among Iranian women. *Asian Pacific journal of cancer prevention: APJCP*, 2014. 15(21): p. 9543-7.
105. Kao, J., *et al.*, Molecular profiling of breast cancer cell lines defines relevant tumor models and provides a resource for cancer gene discovery. *PloS one*, 2009. 4(7): p. e6146- e6146.
106. Kohler, B.A., *et al.*, Annual Report to the Nation on the Status of Cancer, 1975-2011, Featuring Incidence of Breast Cancer Subtypes by Race/Ethnicity, Poverty, and State. *JNCI Journal of the National Cancer Institute*, 2015. 107(6): p. djv048-djv048.
107. Comprehensive molecular portraits of human breast tumours. *Nature*, 2012. 490(7418): p. 61-70.

108. Strehl, J.D., *et al.*, Invasive Breast Cancer: Recognition of Molecular Subtypes. *Breast care* (Basel, Switzerland), 2011. 6(4): p. 258-264.
109. Yanagawa, M., *et al.*, Luminal A and luminal B (HER2 negative) subtypes of breast cancer consist of a mixture of tumors with different genotype. *BMC research notes*, 2012. 5(1): p. 376-376.
110. Molecular Subtypes of Breast Cancer | Susan G. Komen®.
111. Creighton, C.J., The molecular profile of luminal B breast cancer. *Biologics: Targets and Therapy*, 2012. 6: p. 289-297.
112. Goldhirsch, A. *et al.*, Strategies for subtypes-dealing with the diversity of breast cancer: Highlights of the St Gallen international expert consensus on the primary therapy of early breast cancer 2011. *Annals of Oncology*, 2011. 22(8): p. 1736-1747.
113. Haque, R., *et al.*, Impact of breast cancer subtypes and treatment on survival: an analysis spanning two decades. *Cancer Epidemiol Biomarkers Prev*, 2012. 21(10): p. 1848-55.
114. Jordan, V.C., Tamoxifen (ICI46, 474) as a targeted therapy to treat and prevent breast cancer. *British journal of pharmacology*, 2006. 147 Suppl: p. S269-76.
115. De, T., *et al.*, Endocrine therapy resistance in estrogen receptor (ER)-positive breast cancer. *Drug Discovery Today*, 2016. 00(00): p. 1-8.
116. Bostner, J., *et al.*, Activation of Akt, mTOR, and the estrogen receptor as a signature to predict tamoxifen treatment benefit. *Breast cancer research and treatment*, 2013. 137(2): p. 397-406.
117. Alluri, P.G., C. Speers, and A.M. Chinnaiyan, Estrogen receptor mutations and their role in breast cancer progression. *Breast Cancer Res*, 2014. 16(6): p. 494.
118. Chu, D., *et al.*, ESR1 mutations in circulating plasma tumor DNA from metastatic breast cancer patients. *Clin Cancer Res*, 2015.
119. Jeselsohn, R., *et al.*, ESR1 mutations—a mechanism for acquired endocrine resistance in breast cancer. *Nature Reviews Clinical Oncology*, 2015. 12(10): p. 1-11.
120. Li, S., *et al.*, Endocrine-therapy-resistant ESR1 variants revealed by genomic characterization of breast-cancer-derived xenografts. *Cell Rep*, 2013. 4(6): p. 1116-30.
121. Robinson, D.R., *et al.*, Activating ESR1 mutations in hormone-resistant metastatic breast cancer. *Nat Genet*, 2013. 45(12): p. 1446-51.

122. Toy, W., *et al.*, ESR1 ligand-binding domain mutations in hormone-resistant breast cancer. *Nat Genet*, 2013. 45(12): p. 1439-45.
123. Huang, B., *et al.*, Differential expression of estrogen receptor α , β , and γ in lobular and ductal breast cancer. *PNAS*, 2014. 111(5): p. 1933-8.
124. Ohshiro, K., *et al.*, Alternate estrogen receptors promote invasion of inflammatory breast cancer cells via non-genomic signaling. *PLoS ONE*, 2012. 7(1).
125. Végvári, Á., *et al.*, Localization of tamoxifen in human breast cancer tumors by MALDI mass spectrometry imaging. *Clinical and translational medicine*, 2016. 5(1): p. 10-10.
126. Chang, M., Tamoxifen resistance in breast cancer. *Biomolecules & therapeutics*, 2012. 20(3): p. 256-67.
127. Ross, J.S., *et al.*, The HER-2 receptor and breast cancer: ten years of targeted anti-HER- 2 therapy and personalized medicine. *The oncologist*, 2009. 14(4): p. 320-368.
128. Osborne, C.K., *et al.*, Role of the estrogen receptor coactivator AIB1 (SRC-3) and HER-2/neu in tamoxifen resistance in breast cancer. *Journal of the National Cancer Institute*, 2003. 95(5): p. 353-361.
129. Rosell, M., *et al.*, Complex formation and function of estrogen receptor α in transcription requires RIP140. *Cancer Research*, 2014. 74(19): p. 5469-5479.
130. Lavinsky, R.M., *et al.*, Diverse signaling pathways modulate nuclear receptor recruitment of N-CoR and SMRT complexes. *Proceedings of the National Academy of Sciences of the United States of America*, 1998. 95(6): p. 2920-5.
131. Bhatt, S., *et al.*, OCT-4: a novel estrogen receptor- α collaborator that promotes tamoxifen resistance in breast cancer cells. *Oncogene*, 2016 (November 2015): p. 1-13.
132. Liu, H., *et al.*, Immunohistochemical evaluation of GATA3 expression in tumors and normal tissues: A useful immunomarker for breast and urothelial carcinomas. *American Journal of Clinical Pathology*, 2012. 138(1): p. 57-64.
133. Mohammed, H., *et al.*, Endogenous Purification Reveals GREB1 as a Key Estrogen Receptor Regulatory Factor. *Cell Reports*, 2013. 3(2): p. 342-349.
134. Joel, P.B., *et al.*, pp90rsk1 regulates estrogen receptor-mediated transcription through phosphorylation of Ser-167. *Molecular and cellular biology*, 1998. 18(4): p. 1978-1984.
135. Kato, S., *et al.*, Activation of the estrogen receptor through phosphorylation by mitogen activated protein kinase. *Science (New York, N.Y.)*, 1995. 270: p. 1491-1494.

136. Nass, N. and T. Kalinski, Tamoxifen resistance: From cell culture experiments towards novel biomarkers. 2015. p. 189-197.
137. Tan, D.S.P., *et al.*, Promising SINEs for Embargoing Nuclear – Cytoplasmic Export as an *Anticancer Strategy*. 2014(May): p. 527-538.
138. Menendez, J.A., *et al.*, Blockade of a key region in the extracellular domain inhibits HER2 dimerization and signaling. *Journal of the National Cancer Institute*, 2015. 107(6): p. djv090-djv090.
139. Ward, a., *et al.*, Re-expression of microRNA-375 reverses both tamoxifen resistance and accompanying EMT-like properties in breast cancer. *Oncogene*, 2012 (February 2012): p. 1173-1182.
140. Wei, Y., *et al.*, Exosomal miR-221/222 enhances tamoxifen resistance in recipient ER positive breast cancer cells. *Breast Cancer Research and Treatment*, 2014. 147(2): p. 423-431.
141. Bai, J.X., *et al.*, Tamoxifen represses miR-200 MicroRNAs and promotes epithelial-to mesenchymal transition by up-regulating c-myc in endometrial carcinoma cell lines. *Endocrinology*, 2013. 154(2): p. 635-645.
142. He, Y.J., *et al.*, miR-342 is associated with estrogen receptor- α expression and response to tamoxifen in breast cancer. *Experimental and Therapeutic Medicine*, 2013. 5(3): p. 813-818.
143. Ward, A., *et al.*, MicroRNA-519a is a novel oncomir conferring tamoxifen resistance by targeting a network of tumour-suppressor genes in ER+ breast cancer. *Journal of Pathology*, 2014. 233(4): p. 368-379.
144. Pandey, D.P. and D. Picard, miR-22 inhibits estrogen signaling by directly targeting the estrogen receptor alpha mRNA. *Molecular and cellular biology*, 2009. 29(13): p. 3783- 90.
145. Shi, W., *et al.*, MicroRNA-301 mediates proliferation and invasion in human breast cancer. *Cancer Res*, 2011. 71(8): p. 2926-2937.
146. Cittelly, D.M., *et al.*, Oncogenic HER2^{WT}16 suppresses miR-15a/16 and deregulates BCL-2 to promote endocrine resistance of breast tumors. *Carcinogenesis*, 2010. 31(12): p. 2049-2057.

147. Lin, N.U. and E.P. Winer, Advances in adjuvant endocrine therapy for postmenopausal women. *Journal of clinical oncology: official journal of the American Society of Clinical Oncology*, 2008. 26(5): p. 798-805.
148. Ma, C.X., *et al.*, Mechanisms of aromatase inhibitor resistance. *Nat Rev Cancer*, 2015. 15(5): p. 261-75.
149. Cellular and molecular mechanisms of hormonal carcinogenesis. Environmental influences. *Prog Clin Biol Res*, 1996. 394: p. xiii-xix, 1-479.
150. Osborne, C.K., A. Wakeling, and R.I. Nicholson, Fulvestrant: an oestrogen receptor antagonist with a novel mechanism of action. *Br J Cancer*, 2004. 90 Suppl 1: p. S2-6.

CHAPTER 3: LITERATURE REVIEW PART II: THE CROSSTALK BETWEEN ESTROGEN SIGNALING AND BREAST CANCER METABOLISM

3.1. Abstract

Estrogens and estrogen receptors regulate metabolism in both normal and diseased physiological states. Metabolic characteristics of intrinsic breast cancer subtypes change based on their estrogen receptor expression. The crosstalk between estrogen signaling elements and several key metabolic regulators alter metabolism in breast cancer cells, which enable tumors to rewire their metabolism to adapt to poor perfusion, transient nutrient deprivation and increased acidity. This leads to selection of drug-resistant and metastatic clones. In this review, we will discuss studies revealing the role of estrogen signaling elements in drug resistance development and metabolic adaptation during breast cancer progression.

Key words: Breast cancer, cancer metabolism, drug resistance, estrogen signaling, metabolic regulators, nutrient availability

Highlights

- Different breast cancer subtypes have different metabolic phenotypes
- Estrogen receptors modulate expression of genes important for metabolic regulation
- Extra-nuclear estrogen receptors regulate kinase signaling pathways as well as mitochondrial metabolism to impact cancer cell metabolism
- Metabolic rewiring is an inherent property of endocrine resistance albeit whether it is a driver or consequence of endocrine resistance is not clear

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3.2. Introduction

Estrogens and estrogen receptors (ERs) regulate metabolism in both normal and diseased physiological states. Despite lack of sexual reproduction, invertebrates possess ER-like proteins to regulate their metabolic processes, which shows the importance of ER signaling elements for non-reproductive functions including metabolism [1]. Metabolic changes in postmenopausal women after estrogen loss suggests that estrogen signaling regulates energy metabolism [2]. Nutrient availability is one of the important parameters for cell cycle progression and proper energy metabolism. Thus, cells constantly check the amount of nutrients in their microenvironment. Since cancer cells proliferate in an uncontrolled way, they develop different strategies to keep their proliferation rate stable even in limited nutrient conditions. This concept is called metabolic plasticity. In 2011, Hanahan and Weinberg updated their milestone review to include metabolic plasticity as an emerging cancer hallmark called ‘deregulation of cellular energetics’ [3]. Tumors reprogram their metabolism to adapt to poor perfusion (lack of enough blood supply), leading to transient nutrient deprivation, and increased acidity. During adaptation to environmental stress, drug resistant and metastatic clones are selected for [4, 5]. Given the scope of this review, we will extensively focus on the impact of estrogen signaling elements on the metabolic alterations during breast cancer progression. We caution the readers that majority of studies reported here are fairly recent and still require rigorous validation, yet they contribute to a comprehensive framework that might explain some of the clinical observations regarding ER (+) tumors.

3.2.1. Molecular Subtypes in Breast Cancer: Breast cancer classification and patient stratification is critical in terms of determining treatment strategy in clinic. Breast tumors are classified into two groups: *In situ* (20% of all cases) and invasive breast tumors (80% of all cases). Based on their location, *in situ* breast cancers are further classified into two groups: ductal carcinoma *in situ* (DCIS, about 80% of *in situ* breast cancers) and lobular carcinoma *in situ* (LCIS, about 20% of *in situ* breast cancers). About 20-50% of DCIS tumors can eventually turn to an invasive carcinoma [32]. Invasive breast cancers have the capacity to spread to other organs in the body. Estrogen Receptor (ER), Progesterone Receptor (PR) and Human epidermal growth factor receptor 2 (HER2) expression is used in the clinic to determine breast cancer subtype. In addition, expression levels of Ki67 is also utilized to decide treatment options for patients. Based on expression of these biomarkers, invasive breast tumor are divided into four main molecular

subtypes of breast cancer: Luminal A, luminal B, HER2-enriched, and triple negative (TNBC)/basal-like [33, 34]. Luminal A subtype is hormone receptor positive (HR+), HER2 negative (HER2 -), and has low Ki-67 signature. It has relative better prognosis and can be managed by endocrine therapies. Luminal B subtype is also HR (+), but it can be either HER2 (+) or HER2 (-) with higher Ki-67 expression and relatively worse prognosis and less response to endocrine agents. The HER2-enriched subtype is HR (-) and HER2 (+), and has worse prognosis compared to luminal subtypes. It is treated with HER2 targeting agents in the clinic. TNBC/basal-like subtype has no hormone receptor or HER2 expression, and it is more common in African-American women and in the individuals with *BRCA1* mutation [34]. Overall, this is the most aggressive subtype with limited targeted therapy options in the clinic. Clinical studies showed that more than 75% of all breast cancers express ER and/or PR, and approximately 10-15% of them also express HER2 [35].

3.2.2. Metabolic Genes Mutated in Different Breast Cancer Subtypes: Mutation profiles of metabolic genes are different in different breast cancer subtypes. In general, *TP53* and *MYC* mutations are most commonly found in HER2 (+) and basal-like/TNBC subtypes [10] [33], whereas, 40-50% of luminal-type tumors have mutations in PI3K/Akt/mTOR pathway elements (e.g., *PIK3CA*, *PIK3R1*, *PTEN*, *AKT1*) [34]. *PI3KCA* and *MAP3K1* are the most frequently mutated genes in this subtype [35]. On the other hand, glycolytic enzymes (e.g. *HK3*, *GPI*, *GAPDH*, *PGK1*, *ENO1*), glycolysis regulator (*PDK1*) and pentose phosphate pathway enzymes (*PGD*, *TKT*, *RPIA*) are the main metabolic genes with mutations in ER (-) breast cancers [72]. Lipid metabolism related genes (e.g., *CPT-1A* and *FASN*) are upregulated in HER2 (+) subtype, whereas *IDH1* [73] and *AKT3* mutations are the most common ones in basal like/TNBC [74] subtype.

3.3. Metabolic Differences in Breast Cancer Subtypes

There are four intrinsic molecular subtypes of breast cancer: luminal A, luminal B, HER2 (+), and basal like/triple negative breast cancer (TNBC) [6]. Each tumor subtype has a distinct proliferation rate, metastatic capacity, and metabolic phenotype and genotype (Table 3.1). Transcriptomics combined with metabolomics analyses identified altered metabolite levels and associated metabolic-pathways in different subtypes.

3.3.1. Glucose-metabolizing pathways: In a recent study, tumor samples across 33 different cancer types were analyzed and categorized based on mRNA expressions of seven major metabolic pathways. Patients with tumors that have upregulated carbohydrate, nucleotide, and vitamin/cofactor metabolism had a worse prognosis, while the ones with upregulated lipid metabolism had a better prognosis [7]. Additional studies showed that ribulose 5-phosphate, fumarate, 2-HG, glutamate/glutamine ratio, serine metabolites, kynurenine, MAG, and most of the phospholipid and sphingolipids are more abundant in TNBC subtype compared to luminal subtype [8-10]. Consistent with these results, transporters involved in macronutrient uptake and metabolic enzymes, such as GLUT1, SLC1A5, SLC7A5, GLS1, and PGDH are upregulated in TNBC [11-13]. Since preclinical studies suggests that TNBC relies more on the glucose metabolism, this also creates a metabolic vulnerability that can potentially be targeted by metformin [14, 15]. Similarly, pentose phosphate pathway intermediates, such as G6PDH and 6PGL, are also upregulated in HER2 (+) and TNBC subtypes compared to luminal ones [16, 17]. Metabolic differences are also present in a certain subtype. A recent study identified lower TCA cycle, oxidative phosphorylation in glycolysis pathways and activities in luminal A invasive lobular carcinoma subtype of tumors compared to luminal A invasive ductal carcinoma subtype of tumors [18].

3.3.2. Amino Acid-metabolizing pathways: Amino acid metabolism is also different amongst different breast cancer subtypes. MYC amplification is the main regulator of glutamine-related metabolic rewiring. MYC facilitates excess glutamine uptake by inducing the expression of glutamine transporters and glutamine-metabolizing enzymes in breast cancers [19]. This molecular mechanism is upregulated in luminal B, TNBC and HER2 (+) subtypes compared to luminal A subtypes [20]. Interestingly, inhibition of ASCT2/SLC1A5-mediated transport with pharmacological inhibitors reduces glutamine uptake only in TNBCs and not in luminal subtypes [21].

MYC also upregulates serine, glycine, tryptophan uptake and synthesis of one-carbon units, resulting in a more active TCA cycle in HER2 (+) and TNBC breast cancer subtypes [8, 22]. Functional genomics studies revealed that serine synthesis pathway is essential in breast cancer cells, and enzymes responsible for serine and glycine biosynthesis, such as PGDH, PSP, and SHMT, are expressed in ER (-) breast cancers more than ER (+) ones [13, 23]. Cancarini *et al.* demonstrated the impact of one-carbon metabolism-associated vitamins in different breast

cancer subtypes. Overall, high vitamin B intake lowered breast cancer risk in all subtypes. Notably, folate has a protective effect against ER (+) PR (+) and HER2 (-) breast cancers whereas thiamine is protective against HER2 (+) breast cancers [24].

3.3.3. Lipid-metabolizing Pathways: Due to high metabolic demand, lipid provision is essential for cancer cells. Since cell proliferation rate is directly related with intrinsic tumor biology and tumor microenvironment, genes associated with lipid metabolism are differentially expressed in different subtypes. Kim *et al.* showed that while HER2 (+) subtypes have the highest PLIN1, CPT-A1, and FASN expression, TNBC subtype tumors have the lowest expression of these genes [25]. Similarly, ACLY, FASN, and SCD1 are overexpressed in HER2 (+) subtype, whereas TNBC subtypes express less ACLY, suggesting that de novo lipid synthesis is upregulated in HER2 (+) subtype [26]. However, there are contradictory studies suggesting FASN overexpression as a targetable therapy option for TNBCs as well [27].

In addition to lipid metabolism-related enzymes, the levels of lipid metabolites also differ depending on the subtype. Several groups reported that inhibition of 27-hydroxycholesterol synthesis decreases proliferation in ER (+) breast cancers, but not in ER (-) cancers [28, 29]. Furthermore, several studies showed that various phospholipids and sphingolipids were upregulated in ER (-) subtypes relative to ER (+) ones, and higher phospholipid concentrations correlated with high tumor grade [30, 31]. Overall, these studies indicated that TNBC and HER2 (+) subtypes adapt to a more active metabolic phenotype compared to luminal subtype to sustain high metabolic demands during rapid cell proliferation.

3.4. The Crosstalk between Macronutrient Metabolism and Estrogen Signaling

3.4.1. Regulation of Glucose metabolism: In cancer cells, upregulation of key glucose transporters and glycolytic enzymes support the synthesis of the building blocks required for cell proliferation, causing a metabolic shift towards glycolysis, which is known as the ‘Warburg Effect’ [36-38]. Hypoxia-dependent upregulation of ER signaling stimulates the expression of glucose transporters (GLUT-1, GLUT-2, and GLUT-5) in breast cancer cells [37]. MCF-7 cells switch their metabolic pathways in response to 17 β -estradiol (E2) and glucose availability. High glucose level in the media activates AKT signaling and suppresses TCA cycle. [39]. E2 treatment upregulates c-Myc-hnRNP axis and expression and activity of glycolytic enzymes, such as

PFKFB3, resulting in increased F26BP and glucose uptake [40] [41]. In breast cancer cells, E2 regulates the balance between glycolysis and OXPHOS by upregulating PDH in the lack of glucose [39]. E2 also promotes addiction to PPP by upregulating G6PD enzyme, in breast cancer cells [42]. Lactate production is also upregulated by E2 in breast cancer cells [43]. HK and PFK expressions were also shown to be positively correlated with estrogen receptor expression in breast carcinomas, and inhibition of HK results in high toxicity in breast cancer cells [44-46]. In addition to two classic nuclear estrogen receptors, GPER-1 the membrane G-protein coupled ER, has been shown to control estrogen-mediated angiogenesis by upregulating PFKFB3 [47] (Figure 3.1).

3.4.2. Regulation of amino acid metabolism: Glutamine is the most abundant amino acid in the serum and preferentially consumed by breast tumors in an ER-dependent manner [11, 48]. Although there is a limited amount of studies showing the crosstalk between estrogen signaling and amino acid metabolism, clinical studies showed that ER (+) tumors were glutamine-enriched and glutamate-reduced compared to ER (-) breast tumors [11, 12, 49]. Specifically, enzymes responsible for glutamine synthesis (GS, GDH) are upregulated in ER (+) breast cancer cells relative to ER (-) ones [50, 51]. Conversely, glutamine uptake and enzymes related with glutamine catabolism (GLS) were shown to be upregulated in ER (-) breast tumors [52]. Accordingly, glutaminase inhibitor treatment was reported to have a better antitumor effects on ER (-) breast cancer cells [53]. Besides glutamine, dietary arginine supplementation and upregulated ASL levels are associated with high cell proliferation rate in breast tumors, and inhibition of ASL activity was shown to inhibit ER (-) breast cancer growth [54]. Asparagine is directed to TCA cycle during adaptation to glutamine depletion in metastatic ER (-) murine breast cancer cells [55, 56]. Leucine deprivation inhibits cell proliferation and induces apoptosis in ER (+) breast cancers by decreasing FASN expression [57]. These results suggest that there is a complex crosstalk between estrogen signaling and amino acid synthesis pathways (Figure 3.1).

3.4.3. Regulation of lipid metabolism: Clinical observations showed that higher levels of E2 lead to decreased fat accumulation, whereas ovariectomy, anti-estrogen therapy or menopause has the inverse effect in females [58]. Both E2 treatment and ER α /ER β overexpression suppress lipogenesis and triglyceride accumulation via competitive binding to PPAR γ in adipose tissue and various hormone-related cancers [59-61]. These effects are due to upregulation of leptin and

STAT-3 genes in the liver, and XBP in endocrine-related cancers [62-64]. Additionally, estradiol downregulates CD36, a transporter for free fatty acids, expression in breast cancer cells [65]. FASN and ACC-1 are overexpressed in breast cancer cells compared to normal cells, and their inhibition is proposed as a potential therapy in breast cancers [66, 67]. Also, inhibition of SCD-1 was shown to inhibit breast tumor growth [68]. In addition to changes in lipid metabolism pathways, specific free fatty acids were shown to impact energy status and cell viability in breast cancer cells. Anacardic acid but not oleic acid or salicylic acid inhibited cell viability in ER(+) cells by reducing cellular respiration[69]. Lastly, biosynthesis of choline, which is a vitamin-like essential nutrient and an important element in the plasma membrane, was found to be regulated by ER α [70].

The effect of estrogen signaling on lipid metabolism is not unidirectional. Inhibition of FASN suppresses E2-stimulated cell growth and survival in breast cancer cells via activation of apoptotic pathways and inactivation of oncogenic PI3K/Akt pathway [71]. Many groups showed that 27-hydroxycholesterol could act as a ligand for ER and promotes cell proliferation via ER signaling in breast cancers [28, 29]. In conclusion, there is a bidirectional signaling between estrogen signaling and lipid metabolism. More studies are required to elucidate the molecular mechanism behind this crosstalk (Figure 3.1).

3.5. The Crosstalk between Metabolic Regulators and Estrogen Signaling

Estrogen signaling globally affects organ systems and any dysregulation in estrogen signaling may cause major pathologic conditions. At molecular level, this global effect of estrogen signaling can be explained with its complex crosstalk with other major cellular pathways (Figure 3.2).

3.5.1. ER-HIF1 α crosstalk: Hypoxia-inducible factor (HIF) is a central regulator of oxygen homeostasis in the cell. As the tumor grows, due to diffusion limit of oxygen, the tumor core becomes hypoxic. This lack of oxygen activates a transcriptional complex involving HIF-1 α , deregulating numerous genes that are crucial for metabolic adaptation, angiogenesis, and eventually metastasis [75]. High HIF-1 α expression in tumors is associated with poor disease outcome and higher mortality in breast cancer patients [76]. Recently, HIF-1 α was shown to be a

transcriptional target of ER α and high HIF-1 α expression, conferring tamoxifen resistance in ER α (+) tumors [77]. In return, hypoxia response reduces ER α expression and cell proliferation [78]. Other studies showed that HIF-1 α and ER α transcriptionally regulate a common group of genes [79]. In addition to ER α , GPER-1, which mediate the non-genomic effects of estrogens, promote HIF-dependent transcription and stimulate glycolysis in endocrine-regulated cells [79, 80]. This well-established cross-talk enables new therapeutic approaches to treat hypoxic ER (+) breast cancers [78, 81].

3.5.2. ER-Ras/Raf/MAPK crosstalk: Ras/Raf/MAPK pathway is one of the most studied signal transduction pathway in the cell, which acts as a highway for the communication of intracellular and extracellular environment. The oncogenic role of Ras/Raf/MAPK pathway is very well studied in breast cancers, however, the role of ER α in this pathway is still elusive. Studies comparing the expression levels of the members of Ras/Raf/MAPK pathway in different breast cancer subtypes reported that patient samples with overexpression of Ras and MAPK proteins are more invasive and they have lower ER α expression [82]. Consistent with this data, most of the transgenic *in vivo* models overexpressing Ras develop ER (-) breast tumors. Ando *et al.* recently developed an *in vivo* breast cancer mouse model expressing a constitutively active Ki-Ras in the mammary tissues. This model developed ER (+) adenocarcinomas with luminal A subtype breast cancer characteristics [83]. FASN activation by mutant KRAS was shown in lung cancer, and similar effects of Ras/MAPK pathway activation in ER (+) breast cancer remains to be explored [84].

3.5.3. ER-PI3K/Akt/mTOR crosstalk: PI3K/Akt/mTOR pathway is the key regulator of all cellular characteristics which are essential for the vitality of a cell, such as proliferation, metabolism, motility, survival and apoptosis. Components of PI3K/AKT/mTOR pathway, including PIK3CA, PTEN, PIK3R1 and AKT1 are mutated in nearly 25% of breast tumors and are associated with drug responses in ER (+) and ER (-) tumors [85-87]. Estrogens stimulate this pathway to regulate migratory and invasive characteristics of ER (+) tumors [88, 89]. In return, mTOR signaling regulates the expression level and activity of ER α . Also, mTOR acts as a co-regulator for ER α [90, 91]. In one mode of action, PI3K pathway regulates ER-dependent transcription in breast cancer cells through KMT2D epigenetic regulator, and its inhibition activated the methyltransferase activity of KMTD2, leading to the activation of ER [92]. PI3K

inhibition increases ER level and activity [86, 92], and PI3K inhibitor-endocrine agent combinations were tested with minor success in clinical trials to treat women with endocrine-resistant disease (NCT01339442, NCT02273973) [93, 94]. There are ongoing clinical trials with next generation PI3K inhibitors (NCT01971515, NCT03056755, NCT02684032 and NCT02437318) in advanced hormone receptor positive breast cancers and most recently positive results were reported from these trials [95, 96].

3.5.4. ER-p53 crosstalk: p53 is the most well-known tumor suppressor protein in the cell. This pathway responds to extracellular and intracellular stress conditions and controls cell cycle checkpoints. ERs and p53 directly regulate expression of each other. Further, E2-induced cell proliferation was shown to be regulated by p53 or via direct interaction between ER α homodimers and p53 hetero-complex [97, 98]. Recent studies showed that the ER α -p53-mediated transcriptional regulation was attenuated by ER β due to a physical interaction between ER β and p53 [99]. Somatic loss of ER β accelerates the formation of p53-deficient mammary tumors, suggesting a protective role of ER β against breast tumorigenesis [100].

3.5.5. ER-c-MYC crosstalk: c-MYC is a pathway that regulates cell growth and proliferation. More recently, it was shown to orchestrate metabolic pathways, which supply nutrients and other required elements to activate DNA replication and cell division. c-MYC is a proto-oncogene and a direct target and co-regulator of ER α in breast cancer [101]. The upregulation of c-MYC and its downstream effectors are associated with poor disease outcome, high metastatic capacity, and endocrine resistance in breast tumors [102]. ER α and c-MYC act synergistically to induce cell proliferation [103, 104]. A recent study found that ER and HER2 (+) crosstalk regulates glutamine metabolism through c-MYC in AI-resistant cells [105].

3.6. Metabolic Alterations in Endocrine-Resistant Breast Tumors

SERMs, SERDs, and aromatase inhibitors are the first-in-line treatment strategy for ER (+) tumors [106]. However, one-third of all breast tumors eventually develop a resistance to these treatments. Drug-resistant and metastatic clones are selected as tumors reprogram their metabolism to adapt to poor perfusion, transient nutrient deprivation, and increased acidity. Identification of the crosstalk between estrogen receptor signaling and endocrine resistance-driven metabolic

alterations is critical to overcome resistance. Moreover, the distinct metabolic programs define metastatic organ sites in breast cancer cells, for example, activation of Akt and glycolytic pathways were seen in liver specific metastases emphasizing the importance of unique metabolic pathway adaptations in cancer cells [107].

Metabolic enzymes, which facilitate reactions in the committed steps of glycolysis, are misregulated and are potential targets in recurrent breast tumors. PFKFB3 is an activator of PFK1, which is the key regulator of the second committed step in glycolysis. Several groups showed that targeting PFKFB3 may be a potential strategy to overcome endocrine resistance [47, 108]. Inhibition of HK2, which facilitates the first committed step of glycolysis, is associated with retarded growth of tamoxifen-resistant breast tumors. Tamoxifen resistance is driven by HIF-1 α hyperactivation via modulation of Akt/mTOR and/or AMPK signaling pathways [109]. Of note, a novel epigenetic modulator NSD2 gene is upregulated in tamoxifen-resistant cells, leading to methylation of promoters of key glucose metabolic enzymes such as HK2 and G6PDH as well as TIGAR [110]. These results reveal that endocrine resistance is promoted by multiple mechanisms inducing glycolytic flux in breast cancers.

Mitochondrial respiration rates are higher in endocrine-resistant cells compared to parental controls. In tamoxifen-responsive cells, 4-hydroxy-tamoxifen (4-OHT) treatment reduces mitochondrial activity [111]. Elevated ER α expression increases NRF-1 and TFAM expressions, leading to increased OXPHOS activity in endocrine resistant tumors [112, 113]. In addition, tamoxifen-stimulated mitochondrial ER β has an antagonist role in breast cancer cells by increasing ROS concentrations in the mitochondria [114]. Also, enhanced mitochondrial function and oxidative stress lead to tamoxifen resistance in MCF-7 breast cancer cells, and GCLC and NQO1 were suggested as potential biomarkers to target mitochondrial activation in tamoxifen resistance [115]. A recent biomarker study proposed more than 60 genes, including mitochondrial ribosomal and mitochondrial complex proteins, as novel mitochondrial biomarkers to predict early treatment failure and recurrence in patients who were treated with tamoxifen [116]. Horizontal transfer and packaging of mitochondrial DNA are significantly associated with therapy resistance development. Additionally, mitochondrial DNA acts as an oncogenic signal in cancer-stem cells by sustaining OXPHOS-dependent endocrine therapy resistance [117]. Loss of one of the key tumor suppressor genes, *RB1*, is associated with repressed mitochondrial protein translation and

OXPHOS, leading to highly aggressive metastatic breast cancers with high OXPHOS activity [118].

Tumor-associated cells in the tumor microenvironment and fuel sources used for mitochondrial respiration are crucial in endocrine resistance. Cancer-associated fibroblasts (CAFs) enable cancer cells to survive by providing lactate and ketone bodies to enhance their mitochondrial activity. Inhibition of mitochondrial activity with metformin and arsenic trioxide (ATO) overcomes fibroblast-induced tamoxifen resistance in ER (+) breast cancer cell lines [119]. Additionally, increased PI3K/Akt pathway activity in CAFs facilitate multidrug resistance in both ER (+) and ER (-) breast tumors by exporting GPERs outside of the nucleus with a nuclear exporter called CRM1 (XPO1). This mechanism was suggested to sustain excess pyruvate and lactate concentrations due to enhanced glycolysis and mitochondrial activity during endocrine resistance development [120]. Of note, AFs also drive trastuzumab resistance in HER2 (+) breast cancers through expanding NF κ B, JAK/STAT, and PI3K/Akt pathways with increasing IL-6 expression [121].

Drug-resistant breast cancer cells have a distinct amino acid signature compared to non-resistant ones. The ratio between glutamine and glutamate levels is used as a biomarker for tumor aggressiveness and endocrine resistance in breast cancer cells [12, 105]. Aromatase inhibitor resistant breast cancer cell lines upregulate transporters involved in glutamine uptake [12, 105]. Also, tamoxifen-resistant cell lines are more sensitive to cysteine levels compared to tamoxifen-sensitive ones [122]. Doxycycline-resistant MCF-7 breast cancer cells have higher levels of cysteine/methionine-regulating enzymes (such as CBS, MTHFR, and BHMT) than doxycycline-sensitive ones. These studies highlight the success of the combination treatment strategies involving hormonal/targeted therapy agents with the enzymes targeting amino acid biosynthesis pathways.

Lipid metabolism is also altered during the development of drug resistance. Inhibition of ACC-1 via leptin and TGF β signaling causes elevation of acetyl-CoA, leading to recurrence and metastasis in breast cancer cells [123]. Furthermore, recent metabolic-profiling data from several breast cancer studies revealed that nucleic acid and cholesterol synthesis pathways are activated during tamoxifen resistance in breast cancer cells. Consistent with this data, combining cholesterol-lowering medicines with endocrine treatment was shown to improve metabolic outcomes in endocrine-resistant breast cancer cells [124]. Also, neutral lipids, lipid droplets, and

free cholesterol get accumulated in tamoxifen-resistant breast cancer cells compared to non-resistant ones [125]. In addition, differences in lipid metabolic pathways provide novel vulnerabilities in ER (+) tumors that might be targeted with already existing inhibitors [126].

3.7. Measurement of Metabolomics in Liquid Biopsies

Emerging field of liquid biopsies to diagnose patients, follow-up treatment responses and monitor recurrence is gaining popularity in the clinic. The main idea of the liquid biopsy is to collect blood, saliva or urine samples from the patients to detect circulating tumor cells (CTCs) and/or other circulating factors (e.g. metabolites, DNA or RNA). Following collection of the samples, metabolites in these samples can be analyzed with a wide-array of techniques such as, sequencing, nuclear magnetic resonance (NMR) or liquid-or-gas chromatography (LC-GC). This approach can be used for early detection and genotyping of cancer cells [127], monitoring the metabolic effects of a treatment strategy, predicting drug response or resistance [128], metastatic capacity [129], or detecting certain targetable mutations for therapy selection in breast cancers. For example, comparison of plasma samples from breast cancer patients and healthy individuals showed that high levels of cortisol, glutamine, L-Arginine, linoleic acid, L-Lysine, L-valine, uric acid, tyrosine and phenylalanine were associated with breast cancer [130] [131]

High levels of histidine, acetoacetate, glycerol, pyruvate, glycoproteins, mannose, glutamate and phenylalanine were reported to be associated with metastatic phenotype in breast cancers [129]. Recently, several clinical trials used identification of *PI3KCA* mutations in circulating tumor material to predict outcomes of inhibitor treatments targeting PI3K pathway [132].

3.8. Concluding Remarks and Future Perspectives

Estrogen signaling has a significant impact on how breast cancer cells rewire their metabolic pathways to meet their high energy demand during proliferation. These alterations can be seen in all aspects of macronutrients' metabolism, including pathways associated with glucose, glutamine, and fatty acid metabolism. Early differences in metabolic phenotypes might inform clinicians about recurrence and have potential to be used as biomarkers in the clinic to aid in the therapy decisions of breast cancer. Liquid biopsy for circulating markers of metabolic phenotype of tumors offer early detection, monitoring and prediction of therapy responses in clinic.

Understanding how ERs regulate these pathways is crucial as this new knowledge will reveal new vulnerabilities to overcome drug resistance in different breast cancer subtypes. Specifically, the crosstalk between estrogen signaling elements and key metabolic regulators should be elucidated with more studies to better target ER (+) breast cancers by combining endocrine therapy options with metabolic inhibitors.

3.9. Table and Figures

Table 3.1: Molecular Differences in Different Breast Cancer Subtypes

	Luminal A subtype	Luminal B subtype	HER2 (+) subtype	Basal like/TNBC
Glucose metabolism			G6PDH and 6PGL are upregulated in HER2 (+) subtypes compared to luminal ones [16, 17].	Ribulose 5-phosphate, fumarate, 2-HG, glutamate/glutamine ratio, serine metabolites, kynurenine, and MAG are more abundant in TNBC subtype compared to luminal subtype [7]. GLUT1, SLC1A5, SLC7A5, GLS1 and PGDH are upregulated in TNBC [11-13]. G6PDH and 6PGL are upregulated in TNBC subtypes compared to luminal ones [16, 17].
Amino acid metabolism		MYC is overexpressed in luminal B, compared to luminal A [19].	MYC is overexpressed in HER2 (+) compared to luminal A [19]. MYC-related serine, glycine, tryptophan uptake and synthesis of one-carbon units result in a more active TCA cycle in HER2 (+) [8, 21].	MYC is overexpressed in luminal B, HER2 (+), and TNBC compared to luminal A [19]. Inhibition of ASCT2/SLC1A5-mediated glutamine uptake is more effective in TNBCs than in luminal subtypes [20]. MYC-related serine, glycine, tryptophan uptake and synthesis of one-carbon units result in a more active TCA cycle in TNBC [8, 21]. Enzymes, responsible for serine-glycine biosynthesis, such as PGDH, PSP and SHMT are expressed more in ER- subtypes compared to ER (+) subtypes [13, 22]
Fatty acid metabolism	Inhibition of 27-hydroxycholesterol synthesis decreases proliferation in ER (+) cancers, not in ER (-) cancers [27, 28] *.	Inhibition of 27-hydroxycholesterol synthesis decreases proliferation in ER (+) cancers, not in ER (-) cancers [27, 28] *.	PLIN1, CPT-A1, ACLY, SCD1 and FASN expressions are the highest in HER2 (+) subtype [24, 25]. Phospholipid and sphingolipid levels are high in ER (-) breast cancers [7, 29, 30] *.	PLIN1, CPT-A1, ACLY and FASN expressions are the lowest in TNBC subtype [24, 25]. Phospholipid and sphingolipid levels are high in ER (-) breast cancers [7, 29, 30] *.

Table 3.1 (cont.): Molecular Differences in Different Breast Cancer Subtypes

Macronutrient metabolism	High vitamin B levels lowered risk of breast cancers in all subtypes [23]. Folate has a protective effect against ER (+) PR (+) HER2 (-) subtypes [23] *.	High vitamin B levels lowered risk of breast cancers in all subtypes [23]. Folate has a protective effect against ER (+) PR (+) HER2 (-) subtypes [23] *.	High vitamin B levels lowered risk of breast cancers in all subtypes [23]. Thiamine is protective against HER2 (+) subtypes [23].	High vitamin B levels lowered risk of breast cancers in all subtypes [23].
Metabolic Gene Mutations	<i>PIK3CA, PIK3R1, PTEN, AKT1, MAP3K1</i> [34, 35]	<i>PIK3CA, PIK3R1, PTEN, AKT1, MAP3K1</i> [34, 35]	<i>TP53, MYC</i> [10, 33] <i>HK3,GPI, GAPDH, PGK1, ENO1, PDK1, PGD, TKT, RPIA</i> [72] <i>CPT-A1, FASN</i> [26]	<i>TP53, MYC</i> [10, 33] <i>HK3,GPI, GAPDH, PGK1, ENO1, PDK1, PGD, TKT, RPIA</i> [72] <i>IDH1, AKT3</i> [73, 74]

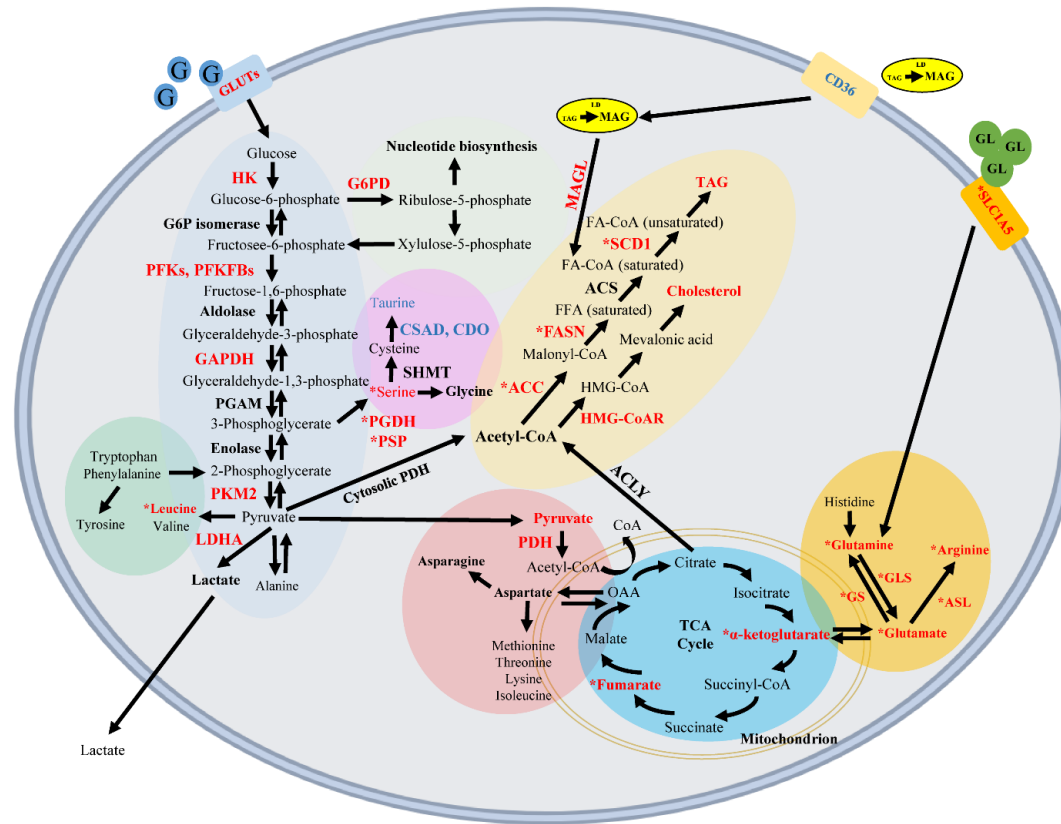


Figure 3.1: Metabolic enzymes, metabolites and macronutrient transporters regulated by estrogen signaling. The enzymes and macronutrient transporters, which are upregulated by estrogen signaling and high metabolite levels are shown with red characters. The enzymes and macronutrient transporters, which are downregulated by estrogen signaling macronutrient transporters and low metabolite levels are shown with blue characters. *High levels and expressions of metabolic enzymes, metabolites and macronutrient transporters in breast cancers. ACC: Acetyl-CoA carboxylase, ACS: Acetyl-CoA synthases, ACLY: ATP citrate lyase, ATP S.: ATP synthase, ASL: Argininosuccinate lyase, CD-36: Cluster of differentiation 36, CSAD: Cysteine sulfinic acid decarboxylase, CDO: Cysteine dioxygenase, FASN: Fatty acid synthase, FFA: Free fatty acid, G: Glucose, GL: Glutamine, GAPDH: Glyceraldehyde-3-phosphate dehydrogenase, G6P isomerase: Glucose-6-phosphate isomerase, G6PD: Glucose-6-phosphate dehydrogenase, GLUTs: Glucose transporters, GSL-1: Glutaminase-1, GS: Glutamine synthase, HK: Hexokinase, HMG-CoA: 3-Hydroxy-3-methylglutaryl-CoA, HMG-CoAR: 3-Hydroxy-3-methylglutaryl-CoA reductase, LDHA: Lactate dehydrogenase-A, LD: Lipid droplet, MAG: Monoacylglycerol, MAGL: Monoacylglycerol lipase, OAA: Oxaloacetate, PDH: Pyruvate dehydrogenase, PFKs: Phosphofructokinase, PFKBPs: Phosphofructokinase fructose biphosphatases, PGAM: Phosphoglycerate mutase, PGDH: Phosphoglycerate dehydrogenase, PKM2: Pyruvate kinase isoenzyme M2, PSP: Phosphoserine phosphatase, Q: Quinone, SCD1: Stearoyl-CoA desaturase-1, SLC1A5: Solute carrier family 1 member 5, TAG: Triacylglycerol

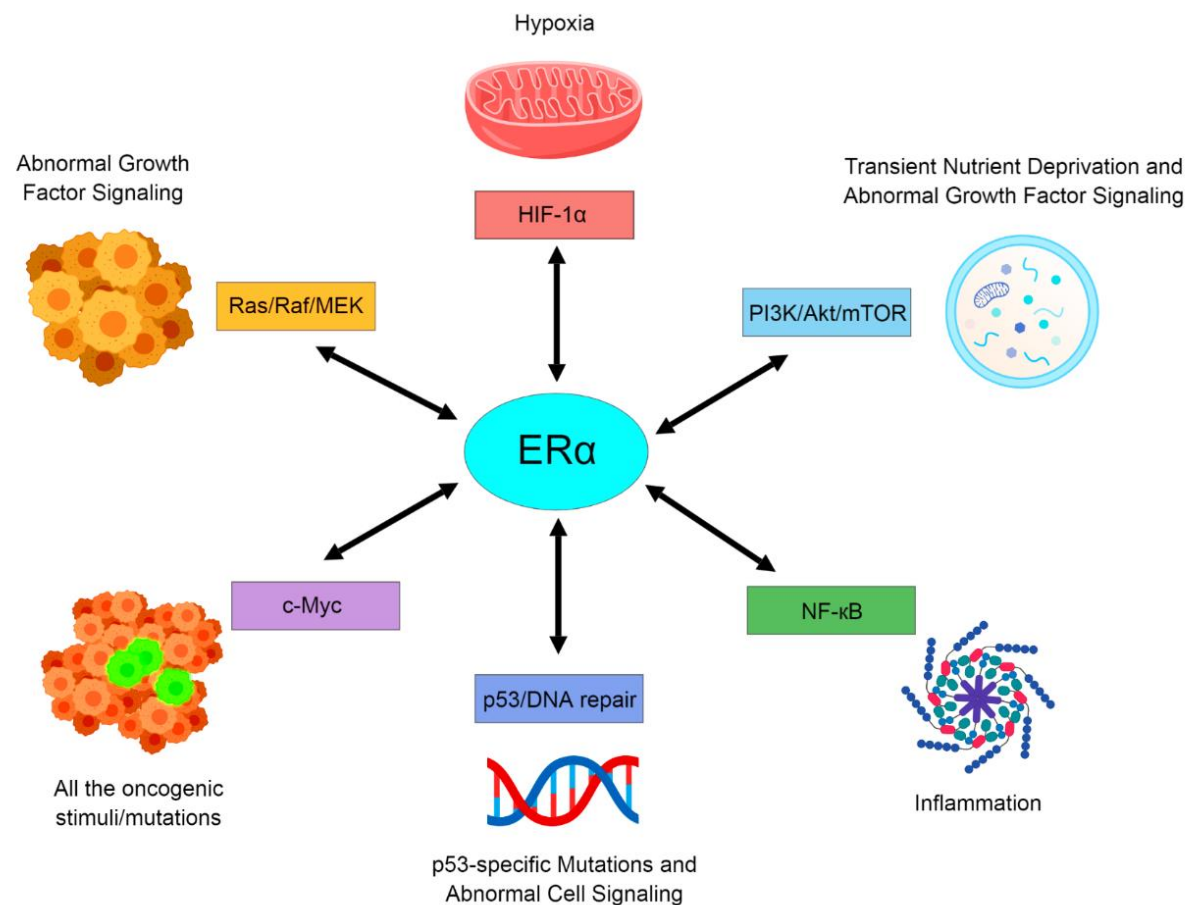


Figure 3.2: Abnormal estrogen signaling stimulates cell proliferation and growth in the mammary tissue. This situation triggers various stress stimuli in a solid tumor, such as poor perfusion, transient nutrient deprivation and increased acidity. All these external factors activate different stress-associated pathways in the cell and eventually promotes cell proliferation and creates hypoxia in the core of the tumor. In addition, ER-dependent regulator mechanisms is not unidirectional. These pathways also regulate genomic, extra-nuclear and post-translational regulation of ERα, and upregulates several downstream targets to promote pathways associated with various hallmarks of cancer such as angiogenesis, metabolic deregulation, drug resistance and angiogenesis.

3.10. References

1. G. V. Markov, J. Gutierrez-Mazariegos, D. Pitrat, I. M. L. Billas, F. Bonneton, D. Moras, *et al.*, "Origin of an ancient hormone/receptor couple revealed by resurrection of an ancestral estrogen," *Science Advances*, vol. 3, 2017.
2. F. Mauvais-Jarvis, D. J. Clegg, and A. L. Hevener, "The role of estrogens in control of energy balance and glucose homeostasis," vol. 34, ed, 2013, pp. 309-338.
3. D. Hanahan and R. A. Weinberg, "Hallmarks of cancer: The next generation. Vol. 144, Cell. 2011. p. 646–74. llmarks of cancer: The next generation," vol. 144, ed, 2011, pp. 646-674.
4. M. Mimeault and S. K. Batra, "Hypoxia-inducing factors as master regulators of stemness properties and altered metabolism of cancer- and metastasis-initiating cells," *Journal of Cellular and Molecular Medicine*, vol. 17, pp. 30-54, 2013.
5. D. Verduzco, M. Lloyd, L. Xu, A. Ibrahim-Hashim, Y. Balagurunathan, R. A. Gatenby, *et al.*, "Intermittent hypoxia selects for genotypes and phenotypes that increase survival, invasion, and therapy resistance," *PLoS ONE*, vol. 10, 2015.
6. H. P. Sinn and H. Kreipe, "A brief overview of the WHO classification of breast tumors, 4th edition, focusing on issues and updates from the 3rd edition," vol. 8, ed, 2013, pp. 149-154.
7. X. Peng, Z. Chen, F. Farshidfar, X. Xu, P. L. Lorenzi, Y. Wang, *et al.*, "Molecular Characterization and Clinical Relevance of Metabolic Expression Subtypes in Human Cancers," vol. 23, pp. 255-269, 2018.
8. J. W. Locasale, "Serine, glycine and one-carbon units: Cancer metabolism in full circle," vol. 13, ed, 2013, pp. 572-583.
9. V. Cappelletti, E. Iorio, P. Miodini, M. Silvestri, M. Dugo, and M. G. Daidone, "Metabolic Footprints and Molecular Subtypes in Breast Cancer," *Disease Markers*, vol. 2017, 2017.
10. A. Terunuma, N. Putluri, P. Mishra, E. A. Mathé, T. H. Dorsey, M. Yi, *et al.*, "MYC-driven accumulation of 2-hydroxyglutarate is associated with breast cancer prognosis," *Journal of Clinical Investigation*, vol. 124, pp. 398-412, 2014.
11. J. Budczies, S. F. Brockmüller, B. M. Müller, D. K. Barupal, C. Richter-Ehrenstein, A. Kleine-Tebbe, *et al.*, "Comparative metabolomics of estrogen receptor positive and

- estrogen receptor negative breast cancer: Alterations in glutamine and beta-alanine metabolism," *Journal of Proteomics*, vol. 94, pp. 279-288, 2013.
12. J. Budczies, B. M. Pfitzner, B. Györfy, K. J. Winzer, C. Radke, M. Dietel, *et al.*, "Glutamate enrichment as new diagnostic opportunity in breast cancer," *International Journal of Cancer*, vol. 136, pp. 1619-1628, 2015.
 13. S. K. Kim, W. H. Jung, and J. S. Koo, "Differential expression of enzymes associated with serine/glycine metabolism in different breast cancer subtypes," *PLoS ONE*, vol. 9, 2014.
 14. S. Oh, H. Kim, K. Nam, and I. Shin, "Glut1 promotes cell proliferation, migration and invasion by regulating epidermal growth factor receptor and integrin signaling in triple-negative breast cancer cells," *BMB reports*, vol. 50, pp. 132-137, 2017.
 15. R. Wahdan-Alaswad, Z. Fan, S. M. Edgerton, B. Liu, X. S. Deng, S. S. Arnadottir, *et al.*, "Glucose promotes breast cancer aggression and reduces metformin efficacy," *Cell Cycle*, vol. 12, pp. 3759-3769, 2013.
 16. J. Choi, E. S. Kim, and J. S. Koo, "Expression of Pentose Phosphate Pathway-Related Proteins in Breast Cancer," *Dis Markers*, vol. 2018, p. 9369358, 2018.
 17. K. C. Patra and N. Hay, "The pentose phosphate pathway and cancer," ed, 2014.
 18. T. Du, L. Zhu, K. M. Levine, N. Tasdemir, A. V. Lee, D. A. A. Vignali, *et al.*, "Invasive lobular and ductal breast carcinoma differ in immune response, protein translation efficiency and metabolism," *Scientific Reports*, vol. 8, p. 7205, 2018/05/08 2018.
 19. M. Yue, J. Jiang, P. Gao, H. Liu, and G. Qing, "Oncogenic MYC Activates a Feedforward Regulatory Loop Promoting Essential Amino Acid Metabolism and Tumorigenesis," *Cell Rep*, vol. 21, pp. 3819-3832, Dec 26 2017.
 20. M. L. Craze, H. Cheung, N. Jewa, N. D. M. Coimbra, D. Soria, R. El-Ansari, *et al.*, "MYC regulation of glutamine-proline regulatory axis is key in luminal B breast cancer," *Br J Cancer*, vol. 118, pp. 258-265, Jan 2018.
 21. M. van Geldermalsen, Q. Wang, R. Nagarajah, A. D. Marshall, A. Thoeng, D. Gao, *et al.*, "ASCT2/SLC1A5 controls glutamine uptake and tumour growth in triple-negative basal-like breast cancer," *Oncogene*, vol. 35, pp. 3201-8, Jun 16 2016.
 22. S. Kim, D. H. Kim, W. H. Jung, and J. S. Koo, "Expression of glutamine metabolism-related proteins according to molecular subtype of breast cancer," *Endocrine-Related Cancer*, vol. 20, pp. 339-348, 2013.

23. R. Possemato, K. M. Marks, Y. D. Shaul, M. E. Pacold, D. Kim, K. Birsoy, *et al.*, "Functional genomics reveal that the serine synthesis pathway is essential in breast cancer," *Nature*, vol. 476, pp. 346-350, 2011.
24. I. Cancarini, V. Krogh, C. Agnoli, S. Grioni, G. Matullo, V. Pala, *et al.*, "Micronutrients involved in one-carbon metabolism and risk of breast cancer subtypes," *PLoS ONE*, vol. 10, 2015.
25. S. Kim, Y. Lee, and J. S. Koo, "Differential expression of lipid metabolism-related proteins in different breast cancer subtypes," *PLoS ONE*, vol. 10, 2015.
26. M. E. Monaco, "Fatty acid metabolism in breast cancer subtypes," *Oncotarget*, vol. 8, 2017.
27. A. Giro-Perafita, A. Sarrats, F. Perez-Bueno, G. Oliveras, M. Buxo, J. Brunet, *et al.*, "Fatty acid synthase expression and its association with clinico-histopathological features in triple-negative breast cancer," *Oncotarget*, vol. 8, pp. 74391-74405, Sep 26 2017.
28. E. R. Nelson, S. E. Wardell, J. S. Jasper, S. Park, S. Suchindran, M. K. Howe, *et al.*, "27-Hydroxycholesterol links hypercholesterolemia and breast cancer pathophysiology," *Science*, vol. 342, pp. 1094-1098, 2013.
29. Q. Wu, T. Ishikawa, R. Sirianni, H. Tang, J. G. McDonald, I. S. Yuhanna, *et al.*, "27-Hydroxycholesterol promotes cell-autonomous, ER-positive breast cancer growth," *Cell Reports*, vol. 5, pp. 637-645, 2013.
30. M. Kawashima, N. Iwamoto, N. Kawaguchi-Sakita, M. Sugimoto, T. Ueno, Y. Mikami, *et al.*, "High-resolution imaging mass spectrometry reveals detailed spatial distribution of phosphatidylinositols in human breast cancer," *Cancer Science*, vol. 104, pp. 1372-1379, 2013.
31. M. He, S. Guo, and Z. Li, "In situ characterizing membrane lipid phenotype of breast cancer cells using mass spectrometry profiling," *Sci Rep*, vol. 5, pp. 11298-11298, 2015.
32. "Highlights in breast cancer from the 2015 American Society of Clinical Oncology annual meeting," *Clin Adv Hematol Oncol*, vol. 13, pp. 423-7, Jul 2015.
33. C. Kandath, M. D. McLellan, F. Vandin, K. Ye, B. Niu, C. Lu, *et al.*, "Mutational landscape and significance across 12 major cancer types," *Nature*, vol. 502, pp. 333-339, Oct 17 2013.
34. P. Mishra and S. Ambs, "Metabolic Signatures of Human Breast Cancer," *Mol Cell Oncol*, vol. 2, Jul-Sep 2015.

35. T. Sorlie, "Molecular portraits of breast cancer: tumour subtypes as distinct disease entities," *Eur J Cancer*, vol. 40, pp. 2667-75, Dec 2004.
36. R. V. Simões, I. S. Serganova, N. Kruchevsky, A. Leftin, A. A. Shestov, H. T. Thaler, *et al.*, "Metabolic Plasticity of Metastatic Breast Cancer Cells: Adaptation to Changes in the Microenvironment," *Neoplasia*, vol. 17, pp. 671-684, 2015.
37. I. Hamann, D. Kryszewski, D. Glubrecht, V. Bouvet, A. Marshall, L. Vos, *et al.*, "Expression and function of hexose transporters GLUT1 , GLUT2 , and GLUT5 in breast cancer — effects of hypoxia," 2018.
38. E. A. Wellberg, S. Johnson, J. Finlay-Schultz, A. S. Lewis, K. L. Terrell, C. A. Sartorius, *et al.*, "The glucose transporter GLUT1 is required for ErbB2-induced mammary tumorigenesis," *Breast Cancer Research*, vol. 18, 2016.
39. F. O'Mahony, M. Razandi, A. Pedram, B. J. Harvey, and E. R. Levin, "Estrogen Modulates Metabolic Pathway Adaptation to Available Glucose in Breast Cancer Cells," *Molecular Endocrinology*, vol. 26, pp. 2058-2070, 2012.
40. Y. Imbert-Fernandez, B. F. Clem, J. O'Neal, D. A. Kerr, R. Spaulding, L. Lanceta, *et al.*, "Estradiol stimulates glucose metabolism via 6-phosphofructo-2-kinase (PFKFB3)," *Journal of Biological Chemistry*, vol. 289, pp. 9440-9448, 2014.
41. S. A. Salama, M. A. Mohammad, C. R. Diaz-Arrastia, M. W. Kamel, G. S. Kilic, B. T. Ndofor, *et al.*, "Estradiol-17 β upregulates pyruvate kinase M2 expression to coactivate estrogen receptor- α and to integrate metabolic reprogramming with the mitogenic response in endometrial cells," *Journal of Clinical Endocrinology and Metabolism*, vol. 99, pp. 3790-3799, 2014.
42. Y. Sun, X. Gu, E. Zhang, M. A. Park, A. M. Pereira, S. Wang, *et al.*, "Estradiol promotes pentose phosphate pathway addiction and cell survival via reactivation of Akt in mTORC1 hyperactive cells," *Cell Death Dis*, vol. 5, p. e1231, May 15 2014.
43. R. E. Burke, S. C. Harris, and W. L. McGuire, "Lactate dehydrogenase in estrogen-responsive human breast cancer cells," *Cancer Res*, vol. 38, pp. 2773-6, Sep 1978.
44. A. Sato-Tadano, T. Suzuki, M. Amari, K. Takagi, Y. Miki, K. Tamaki, *et al.*, "Hexokinase II in breast carcinoma: a potent prognostic factor associated with hypoxia-inducible factor-1 α and Ki-67," *Cancer Sci*, vol. 104, pp. 1380-8, Oct 2013.

45. R. G. Coelho, I. C. Calaca, D. M. Celestrini, A. H. Correia-Carneiro, M. M. Costa, P. Zancan, *et al.*, "Hexokinase and phosphofructokinase activity and intracellular distribution correlate with aggressiveness and invasiveness of human breast carcinoma," *Oncotarget*, vol. 6, pp. 29375-87, Oct 6 2015.
46. Y. Wu, M. Sarkissyan, E. McGhee, S. Lee, and J. V. Vadgama, "Combined inhibition of glycolysis and AMPK induces synergistic breast cancer cell killing," *Breast Cancer Research and Treatment*, vol. 151, pp. 529-539, 2015.
47. A. Trenti, S. Tedesco, C. Boscaro, N. Ferri, A. Cignarella, L. Trevisi, *et al.*, "The glycolytic enzyme PFKFB3 is involved in estrogen-mediated angiogenesis via GPER1," *Journal of Pharmacology and Experimental Therapeutics*, vol. 1, pp. jpet.116.238212-jpet.116.238212, 2017.
48. R. C. Geck and A. Toker, "Nonessential amino acid metabolism in breast cancer," vol. 62, ed, 2016, pp. 11-17.
49. A. J. Bott, I. C. Peng, Y. Fan, B. Faubert, L. Zhao, J. Li, *et al.*, "Oncogenic Myc Induces Expression of Glutamine Synthetase through Promoter Demethylation," *Cell Metab*, vol. 22, pp. 1068-77, Dec 1 2015.
50. S. Tyanova, R. Albrechtsen, P. Kronqvist, J. Cox, M. Mann, and T. Geiger, "Proteomic maps of breast cancer subtypes," *Nat Commun*, vol. 7, p. 10259, Jan 4 2016.
51. J. B. Spinelli, H. Yoon, A. E. Ringel, S. Jeanfavre, C. B. Clish, and M. C. Haigis, "Metabolic recycling of ammonia via glutamate dehydrogenase supports breast cancer biomass," *Science*, vol. 358, pp. 941-946, Nov 17 2017.
52. L. Jerby, L. Wolf, C. Denkert, G. Y. Stein, M. Hilvo, M. Oresic, *et al.*, "Metabolic associations of reduced proliferation and oxidative stress in advanced breast cancer," *Cancer Res*, vol. 72, pp. 5712-20, Nov 15 2012.
53. M. I. Gross, S. D. Demo, J. B. Dennison, L. Chen, T. Chernov-Rogan, B. Goyal, *et al.*, "Antitumor activity of the glutaminase inhibitor CB-839 in triple-negative breast cancer," *Mol Cancer Ther*, vol. 13, pp. 890-901, Apr 2014.
54. H. L. Huang, W. C. Chen, H. P. Hsu, C. Y. Cho, Y. H. Hung, C. Y. Wang, *et al.*, "Argininosuccinate lyase is a potential therapeutic target in breast cancer," *Oncol Rep*, vol. 34, pp. 3131-9, Dec 2015.

55. S. R. V. Knott, E. Wagenblast, S. Khan, S. Y. Kim, M. Soto, M. Wagner, *et al.*, "Asparagine bioavailability governs metastasis in a model of breast cancer," *Nature*, vol. 554, pp. 378-381, Feb 15 2018.
56. J. Zhang, J. Fan, S. Venneti, J. R. Cross, T. Takagi, B. Bhinder, *et al.*, "Asparagine plays a critical role in regulating cellular adaptation to glutamine depletion," *Mol Cell*, vol. 56, pp. 205-218, Oct 23 2014.
57. F. Xiao, C. Wang, H. Yin, J. Yu, S. Chen, J. Fang, *et al.*, "Leucine deprivation inhibits proliferation and induces apoptosis of human breast cancer cells via fatty acid synthase," *Oncotarget*, vol. 7, pp. 63679-63689, Sep 27 2016.
58. M. A. Quinn, X. Xu, M. Ronfani, and J. A. Cidlowski, "Estrogen Deficiency Promotes Hepatic Steatosis via a Glucocorticoid Receptor-Dependent Mechanism in Mice," *Cell Reports*, vol. 22, pp. 2601-2614, 2018.
59. Y. M. Lin, B. K. Velmurugan, Y. L. Yeh, C. C. Tu, T. J. Ho, T. Y. Lai, *et al.*, "Activation of estrogen receptors with E2 downregulates peroxisome proliferator-activated receptor γ in hepatocellular carcinoma," *Oncology Reports*, vol. 30, pp. 3027-3031, 2013.
60. J. M. Hall and M. L. Robinson, "Peroxisome Proliferator-Activated Receptor γ as a Therapeutic Target in Human Breast Cancer," *Journal of Steroids & Hormonal Science*, vol. 6, 2015.
61. G. Zhang, X. Hou, and S. Gao, "Stimulation of peroxisome proliferator-activated receptor γ inhibits estrogen receptor α transcriptional activity in endometrial carcinoma cells," *Oncology Reports*, vol. 33, pp. 1227-1234, 2015.
62. E. Choi, W. Kim, S. K. Joo, S. Park, J. H. Park, and Y. K. Kang, "Expression patterns of STAT3 , ERK and estrogen-receptor α are associated with development and histologic severity of hepatic steatosis : a retrospective study," pp. 1-9, 2018.
63. M. Shen and H. Shi, "Estradiol and estrogen receptor agonists oppose oncogenic actions of leptin in HepG2 cells," *PLoS ONE*, vol. 11, 2016.
64. S. Sengupta, C. G. Sharma, and V. C. Jordan, "Estrogen regulation of X-box binding protein-1 and its role in estrogen induced growth of breast and endometrial cancer cells," *Horm Mol Biol Clin Investig*, vol. 2, pp. 235-243, Aug 1 2010.
65. I. P. Uray, Y. Liang, and S. M. Hyder, "Estradiol down-regulates CD36 expression in human breast cancer cells," *Cancer Lett*, vol. 207, pp. 101-7, Apr 15 2004.

66. Y. Wang, F. P. Kuhajda, J. N. Li, E. S. Pizer, W. F. Han, L. J. Sokoll, *et al.*, "Fatty acid synthase (FAS) expression in human breast cancer cell culture supernatants and in breast cancer patients," *Cancer Lett*, vol. 167, pp. 99-104, Jun 10 2001.
67. Y. Yang, P. J. Morin, W. F. Han, T. Chen, D. M. Bornman, E. W. Gabrielson, *et al.*, "Regulation of fatty acid synthase expression in breast cancer by sterol regulatory element binding protein-1c," *Exp Cell Res*, vol. 282, pp. 132-7, Jan 15 2003.
68. D. Luyimbazi, A. Akcakanat, P. F. McAuliffe, L. Zhang, G. Singh, A. M. Gonzalez-Angulo, *et al.*, "Rapamycin regulates stearyl CoA desaturase 1 expression in breast cancer," *Mol Cancer Ther*, vol. 9, pp. 2770-84, Oct 2010.
69. B. N. Radde, N. Alizadeh-Rad, S. M. Price, D. J. Schultz, and C. M. Klinge, "Anacardic Acid, Salicylic Acid, and Oleic Acid Differentially Alter Cellular Bioenergetic Function in Breast Cancer Cells," *Journal of Cellular Biochemistry*, vol. 117, pp. 2521-2532, 2016.
70. M. Jia, T. Andreassen, L. Jensen, T. F. Bathen, I. Sinha, H. Gao, *et al.*, "Estrogen Receptor alpha Promotes Breast Cancer by Reprogramming Choline Metabolism," *Cancer Res*, vol. 76, pp. 5634-5646, Oct 1 2016.
71. J. A. Menendez and R. Lupu, "Fatty acid synthase regulates estrogen receptor- α signaling in breast cancer cells," *Oncogenesis*, vol. 6, 2017.
72. H. Harami-Papp, L. S. Pongor, G. Munkacsy, G. Horvath, A. M. Nagy, A. Ambrus, *et al.*, "TP53 mutation hits energy metabolism and increases glycolysis in breast cancer," *Oncotarget*, vol. 7, pp. 67183-67195, Oct 11 2016.
73. K. Eason and A. Sadanandam, "Molecular or Metabolic Reprograming: What Triggers Tumor Subtypes?," *Cancer Res*, vol. 76, pp. 5195-200, Sep 15 2016.
74. S. Banerji, K. Cibulskis, C. Rangel-Escareno, K. K. Brown, S. L. Carter, A. M. Frederick, *et al.*, "Sequence analysis of mutations and translocations across breast cancer subtypes," *Nature*, vol. 486, pp. 405-9, Jun 20 2012.
75. S. Koyasu, M. Kobayashi, Y. Goto, M. Hiraoka, and H. Harada, "Regulatory mechanisms of HIF-1 activity: Two decades of knowledge," *Cancer Science*, pp. 1-12, 2017.
76. D. Generali, A. Berruti, M. P. Brizzi, L. Campo, S. Bonardi, S. Wigfield, *et al.*, "Hypoxia-inducible factor-1alpha expression predicts a poor response to primary chemoendocrine therapy and disease-free survival in primary human breast cancer," *Clin Cancer Res*, vol. 12, pp. 4562-8, Aug 1 2006.

77. J. Yang, A. AlTahan, D. T. Jones, F. M. Buffa, E. Bridges, R. B. Interiano, *et al.*, "Estrogen receptor- α directly regulates the hypoxia-inducible factor 1 pathway associated with antiestrogen response in breast cancer," *Proceedings of the National Academy of Sciences*, vol. 112, pp. 201422015-201422015, 2015.
78. M. Padro, R. J. Louie, B. V. Lananna, A. J. Krieg, L. A. Timmerman, and D. A. Chan, "Genome-independent hypoxic repression of estrogen receptor alpha in breast cancer cells," *BMC Cancer*, vol. 17, p. 203, Mar 20 2017.
79. J. Yang, A. M. Jubb, L. Pike, F. M. Buffa, H. Turley, D. Baban, *et al.*, "The histone demethylase JMJD2B is regulated by estrogen receptor alpha and hypoxia, and is a key mediator of estrogen induced growth," *Cancer Res*, vol. 70, pp. 6456-66, Aug 15 2010.
80. L. Zhang, W. Xiong, N. Li, H. Liu, H. He, Y. Du, *et al.*, "Estrogen stabilizes hypoxia-inducible factor 1alpha through G protein-coupled estrogen receptor 1 in eutopic endometrium of endometriosis," *Fertil Steril*, vol. 107, pp. 439-447, Feb 2017.
81. J. Meehan, C. Ward, A. Turnbull, J. Bukowski-Wills, A. J. Finch, E. J. Jarman, *et al.*, "Inhibition of pH regulation as a therapeutic strategy in hypoxic human breast cancer cells," *Oncotarget*, pp. 1-19, 2017.
82. A. Loboda, M. Nebozhyn, R. Klinghoffer, J. Frazier, M. Chastain, W. Arthur, *et al.*, "A gene expression signature of RAS pathway dependence predicts response to PI3K and RAS pathway inhibitors and expands the population of RAS pathway activated tumors," *BMC Med Genomics*, vol. 3, p. 26, Jun 30 2010.
83. S. Ando, R. Malivindi, S. Catalano, P. Rizza, I. Barone, S. Panza, *et al.*, "Conditional expression of Ki-Ras(G12V) in the mammary epithelium of transgenic mice induces estrogen receptor alpha (ERalpha)-positive adenocarcinoma," *Oncogene*, vol. 36, pp. 6420-6431, Nov 16 2017.
84. J. D. Stender, J. C. Nwachukwu, I. Kastrati, Y. Kim, T. Strid, M. Yakir, *et al.*, "Structural and Molecular Mechanisms of Cytokine-Mediated Endocrine Resistance in Human Breast Cancer Cells," *Mol Cell*, vol. 65, pp. 1122-1135 e5, Mar 16 2017.
85. S. Yndestad, E. Austreid, I. R. Svanberg, S. Knappskog, P. E. Lønning, and H. P. Eikesdal, "Activation of Akt characterizes estrogen receptor positive human breast cancers which respond to anthracyclines," *Oncotarget*, vol. 8, pp. 41227-41241, 2017.

86. A. Bosch, Z. Li, A. Bergamaschi, H. Ellis, E. Toska, A. Prat, *et al.*, "PI3K inhibition results in enhanced estrogen receptor function and dependence in hormone receptor-positive breast cancer," *Science Translational Medicine*, vol. 7, 2015.
87. J. Bostner, E. Karlsson, M. J. Pandiyan, H. Westman, L. Skoog, T. Fornander, *et al.*, "Activation of Akt, mTOR, and the estrogen receptor as a signature to predict tamoxifen treatment benefit," *Breast cancer research and treatment*, vol. 137, pp. 397-406, 2013.
88. K. Zhou, P. Sun, Y. Zhang, X. You, P. Li, and T. Wang, "Estrogen stimulated migration and invasion of estrogen receptor-negative breast cancer cells involves an ezrin-dependent crosstalk between G protein-coupled receptor 30 and estrogen receptor beta signaling," *Steroids*, vol. 111, pp. 113-120, 2016.
89. G. Zhang, "Upregulation of estrogen receptor mediates migration, invasion and proliferation of endometrial carcinoma cells by regulating the PI3K/AKT/mTOR pathway," *Oncology Reports*, pp. 1175-1182, 2013.
90. A. Alayev, R. S. Salamon, S. M. Berger, N. S. Schwartz, R. Cuesta, R. B. Snyder, *et al.*, "mTORC1 directly phosphorylates and activates ERalpha upon estrogen stimulation," *Oncogene*, vol. 35, pp. 3535-43, Jul 7 2016.
91. A. Shrivastav, M. Bruce, D. Jaksic, T. Bader, S. Seekallu, C. Penner, *et al.*, "The mechanistic target for rapamycin pathway is related to the phosphorylation score for estrogen receptor-alpha in human breast tumors in vivo," *Breast Cancer Res*, vol. 16, p. R49, May 22 2014.
92. E. Toska, H. U. Osmanbeyoglu, P. Castel, C. Chan, R. C. Hendrickson, M. Elkabets, *et al.*, "PI3K pathway regulates ER-dependent transcription in breast cancer through the epigenetic regulator KMT2D," *Science*, vol. 355, pp. 1324-1330, Mar 24 2017.
93. S. A. Hurvitz, F. Andre, Z. Jiang, Z. Shao, M. S. Mano, S. P. Neciosup, *et al.*, "Combination of everolimus with trastuzumab plus paclitaxel as first-line treatment for patients with HER2-positive advanced breast cancer (BOLERO-1): A phase 3, randomised, double-blind, multicentre trial," *The Lancet Oncology*, vol. 16, pp. 816-829, 2015.
94. H. Chow, P. M. Ghosh, R. Devere White, C. P. Evans, M. A. Dall'Era, S. A. Yap, *et al.*, "A phase 2 clinical trial of everolimus plus bicalutamide for castration-resistant prostate cancer," *Cancer*, vol. 122, pp. 1897-1904, 2016.

95. F. Andre, M. Campone, E. M. Ciruelos, H. Iwata, S. Loibl, H. S. Rugo, *et al.*, "SOLAR-1: A phase III study of alpelisib + fulvestrant in men and postmenopausal women with HR+/HER2– advanced breast cancer (BC) progressing on or after prior aromatase inhibitor therapy," *Journal of Clinical Oncology*, vol. 34, pp. TPS618-TPS618, 2016.
96. I. A. Mayer, V. G. Abramson, L. Formisano, J. M. Balko, M. V. Estrada, M. E. Sanders, *et al.*, "A Phase Ib Study of Alpelisib (BYL719), a PI3K α -Specific Inhibitor, with Letrozole in ER⁺/HER2[–] Metastatic Breast Cancer," *Clinical Cancer Research*, vol. 23, pp. 26-34, 2017.
97. W. Liu, S. D. Konduri, S. Bansal, B. K. Nayak, S. A. Rajasekaran, S. M. Karuppayil, *et al.*, "Estrogen receptor-alpha binds p53 tumor suppressor protein directly and represses its function," *J Biol Chem*, vol. 281, pp. 9837-40, Apr 14 2006.
98. S. T. Bailey, H. Shin, T. Westerling, X. S. Liu, and M. Brown, "Estrogen receptor prevents p53-dependent apoptosis in breast cancer," *Proc Natl Acad Sci U S A*, vol. 109, pp. 18060-5, Oct 30 2012.
99. W. Lu and B. S. Katzenellenbogen, "Estrogen Receptor-beta Modulation of the ERalpha-p53 Loop Regulating Gene Expression, Proliferation, and Apoptosis in Breast Cancer," *Horm Cancer*, vol. 8, pp. 230-242, Aug 2017.
100. I. Bado, F. Nikolos, G. Rajapaksa, W. Wu, J. Castaneda, S. Krishnamurthy, *et al.*, "Somatic loss of estrogen receptor beta and p53 synergize to induce breast tumorigenesis," *Breast Cancer Res*, vol. 19, p. 79, Jul 3 2017.
101. A. S. Cheng, V. X. Jin, M. Fan, L. T. Smith, S. Liyanarachchi, P. S. Yan, *et al.*, "Combinatorial analysis of transcription factor partners reveals recruitment of c-MYC to estrogen receptor-alpha responsive promoters," *Mol Cell*, vol. 21, pp. 393-404, Feb 3 2006.
102. S. Sengupta, M. C. Biarnes, and V. C. Jordan, "Cyclin dependent kinase-9 mediated transcriptional de-regulation of cMYC as a critical determinant of endocrine-therapy resistance in breast cancers," *Breast Cancer Res Treat*, vol. 143, pp. 113-24, Jan 2014.
103. R. Cericatto, A. Pozzobon, D. M. Morsch, C. H. Menke, I. S. Brum, and P. M. Spritzer, "Estrogen receptor-alpha, bcl-2 and c-myc gene expression in fibroadenomas and adjacent normal breast: association with nodule size, hormonal and reproductive features," *Steroids*, vol. 70, pp. 153-60, Mar 2005.

104. C. Wang, J. A. Mayer, A. Mazumdar, K. Fertuck, H. Kim, M. Brown, *et al.*, "Estrogen induces c-myc gene expression via an upstream enhancer activated by the estrogen receptor and the AP-1 transcription factor," *Mol Endocrinol*, vol. 25, pp. 1527-38, Sep 2011.
105. Z. Chen, Y. Wang, C. Warden, and S. Chen, "Cross-talk between ER and HER2 regulates c-MYC-mediated glutamine metabolism in aromatase inhibitor resistant breast cancer cells," *J Steroid Biochem Mol Biol*, vol. 149, pp. 118-27, May 2015.
106. H. Pan, R. Gray, J. Braybrooke, C. Davies, C. Taylor, P. McGale, *et al.*, "20-Year Risks of Breast-Cancer Recurrence after Stopping Endocrine Therapy at 5 Years," *N Engl J Med*, vol. 377, pp. 1836-1846, Nov 9 2017.
107. F. Dupuy, S. Tabaries, S. Andrzejewski, Z. Dong, J. Blagih, M. G. Annis, *et al.*, "PDK1-Dependent Metabolic Reprogramming Dictates Metastatic Potential in Breast Cancer," *Cell Metab*, vol. 22, pp. 577-89, Oct 6 2015.
108. Q. Yang and P. Hou, "Targeting PFKFB3 in the Endothelium for Cancer Therapy," vol. 23, ed, 2017, pp. 197-200.
109. Y. M. Woo, Y. Shin, E. J. Lee, S. Lee, S. H. Jeong, H. K. Kong, *et al.*, "Inhibition of aerobic glycolysis represses Akt/mTOR/HIF-1 α axis and restores tamoxifen sensitivity in antiestrogen-resistant breast cancer cells," *PLoS ONE*, vol. 10, pp. 1-18, 2015.
110. J. Wang, Z. Duan, Z. Nugent, J. X. Zou, A. D. Borowsky, Y. Zhang, *et al.*, "Reprogramming metabolism by histone methyltransferase NSD2 drives endocrine resistance via coordinated activation of pentose phosphate pathway enzymes," *Cancer Letters*, vol. 378, pp. 69-79, 2016.
111. Brandie N. Radde, Margarita M. Ivanova, Huy X. Mai, Joshua K. Salabei, Bradford G. Hill, and Carolyn M. Klinge, "Bioenergetic differences between MCF-7 and T47D breast cancer cells and their regulation by oestradiol and tamoxifen," *Biochemical Journal*, vol. 465, pp. 49-61, 2015.
112. B. N. Radde, M. M. Ivanova, H. X. Mai, N. Alizadeh-Rad, K. Piell, P. Van Hoose, *et al.*, "Nuclear respiratory factor-1 and bioenergetics in tamoxifen-resistant breast cancer cells," *Experimental Cell Research*, vol. 347, pp. 222-231, 2016.
113. M. M. Ivanova, B. N. Radde, J. Son, F. F. Mehta, S. H. Chung, and C. M. Klinge, "Estradiol and tamoxifen regulate NRF-1 and mitochondrial function in mouse mammary gland and uterus," *Journal of Molecular Endocrinology*, vol. 51, pp. 233-246, 2013.

114. M. Razandi, a. Pedram, V. C. Jordan, S. Fuqua, and E. R. Levin, "Tamoxifen regulates cell fate through mitochondrial estrogen receptor beta in breast cancer," *Oncogene*, vol. 32, pp. 3274-85, 2013.
115. M. Fiorillo, F. Sotgia, D. Sisci, A. R. Cappello, and M. P. Lisanti, "Mitochondrial "power" drives tamoxifen resistance: NQO1 and GCLC are new therapeutic targets in breast cancer," *Oncotarget*, vol. 8, pp. 20309-20327, 2017.
116. F. Sotgia, M. Fiorillo, and M. P. Lisanti, "Mitochondrial markers predict recurrence, metastasis and tamoxifen-resistance in breast cancer patients: Early detection of treatment failure with companion diagnostics," *Oncotarget*, vol. 8, pp. 68730-68745, 2017.
117. P. Sansone, C. Savini, I. Kurelac, Q. Chang, L. B. Amato, A. Strillacci, *et al.*, "Packaging and transfer of mitochondrial DNA via exosomes regulate escape from dormancy in hormonal therapy-resistant breast cancer," *Proceedings of the National Academy of Sciences*, pp. 201704862-201704862, 2017.
118. E. Zacksenhaus, M. Shrestha, J. C. Liu, I. Vorobieva, P. E. D. Chung, Y. J. Ju, *et al.*, "Mitochondrial OXPHOS Induced by RB1 Deficiency in Breast Cancer: Implications for Anabolic Metabolism, Stemness, and Metastasis," vol. 3, ed, 2017, pp. 768-779.
119. U. E. Martinez-Outschoorn, A. Goldberg, Z. Lin, Y. H. Ko, N. Flomenberg, C. Wang, *et al.*, "Anti-estrogen resistance in breast cancer is induced by the tumor microenvironment and can be overcome by inhibiting mitochondrial function in epithelial cancer cells," *Cancer Biology and Therapy*, vol. 12, pp. 924-938, 2011.
120. T. Yu, G. Yang, Y. Hou, X. Tang, C. Wu, X. A. Wu, *et al.*, "Cytoplasmic GPER translocation in cancer-associated fibroblasts mediates cAMP/PKA/CREB/glycolytic axis to confer tumor cells with multidrug resistance," *Oncogene*, vol. 36, pp. 2131-2145, 2017.
121. Y. Mao, Y. Zhang, Q. Qu, M. Zhao, Y. Lou, J. Liu, *et al.*, "Cancer-associated fibroblasts induce trastuzumab resistance in HER2 positive breast cancer cells," *Mol. BioSyst.*, vol. 11, pp. 1029-1040, 2015.
122. C. S. Ryu, H. C. Kwak, J. Y. Lee, S. J. Oh, N. T. T. Phuong, K. W. Kang, *et al.*, "Elevation of cysteine consumption in tamoxifen-resistant MCF-7 cells," *Biochemical Pharmacology*, vol. 85, pp. 197-206, 2013.

123. M. Rios Garcia, B. Steinbauer, K. Srivastava, M. Singhal, F. Mattijssen, A. Maida, *et al.*, "Acetyl-CoA Carboxylase 1-Dependent Protein Acetylation Controls Breast Cancer Metastasis and Recurrence," *Cell Metabolism*, vol. 26, pp. 842-855.e5, 2017.
124. S. Borgquist, A. Giobbie-Hurder, T. P. Ahern, J. E. Garber, M. Colleoni, I. Láng, *et al.*, "Cholesterol, cholesterol-lowering medication use, and breast cancer outcome in the BIG 1-98 study," *Journal of Clinical Oncology*, vol. 35, pp. 1179-1188, 2017.
125. S. Hultsch, M. Kankainen, L. Paavolainen, R.-m. Kovanen, and E. Ikonen, "Association of tamoxifen resistance and lipid reprogramming in breast cancer," 2018.
126. V. Brovkovich, Y. Izhar, J. M. Danes, O. Dubrovskiy, I. T. Sakallioglu, L. M. Morrow, *et al.*, "Fatostatin induces pro- and anti-apoptotic lipid accumulation in breast cancer," *Oncogenesis*, vol. 7, p. 66, 2018/08/24 2018.
127. E. Heitzer, S. Perakis, J. B. Geigl, and M. R. Speicher, "The potential of liquid biopsies for the early detection of cancer," *NPJ Precis Oncol*, vol. 1, p. 36, 2017.
128. S. Wei, L. Liu, J. Zhang, J. Bowers, G. A. Gowda, H. Seeger, *et al.*, "Metabolomics approach for predicting response to neoadjuvant chemotherapy for breast cancer," *Mol Oncol*, vol. 7, pp. 297-307, Jun 2013.
129. E. Jobard, C. Pontoizeau, B. J. Blaise, T. Bachelot, B. Elena-Herrmann, and O. Tredan, "A serum nuclear magnetic resonance-based metabolomic signature of advanced metastatic human breast cancer," *Cancer Lett*, vol. 343, pp. 33-41, Feb 1 2014.
130. J. Budczies, C. Denkert, B. M. Muller, S. F. Brockmoller, F. Klauschen, B. Györffy, *et al.*, "Remodeling of central metabolism in invasive breast cancer compared to normal breast tissue - a GC-TOFMS based metabolomics study," *BMC Genomics*, vol. 13, p. 334, Jul 23 2012.
131. M. Jove, R. Collado, J. L. Quiles, M. C. Ramirez-Tortosa, J. Sol, M. Ruiz-Sanjuan, *et al.*, "A plasma metabolomic signature discloses human breast cancer," *Oncotarget*, vol. 8, pp. 19522-19533, Mar 21 2017.
132. A. R. Kodahl, S. Ehmsen, N. Pallisgaard, A. M. B. Jylling, J. D. Jensen, A. V. Laenkholm, *et al.*, "Correlation between circulating cell-free PIK3CA tumor DNA levels and treatment response in patients with PIK3CA-mutated metastatic breast cancer," *Mol Oncol*, vol. 12, pp. 925-935, Jun 2018.

CHAPTER 4: HYPOTHESIS AND OBJECTIVE

4.1. Long-term goal

The **long-term goal** of this research is to develop effective treatment strategies to overcome endocrine resistance in ER (+) breast cancer.

4.2. Overall objective of the project

The overall objective of this project is to define the interaction among endocrine resistance, nuclear export system, and cellular metabolic pathways, and to evaluate the metabolic impact of combinational ER α and XPO1 targeting by using combination of endocrine therapy agents and a XPO1-specific small molecule inhibitor (Selinexor-SEL) in different endocrine resistant ER (+) breast cancer cell lines.

4.3. Central hypothesis

Our **central hypothesis** is that combined targeting of ER α and XPO1 remodel metabolic pathways to induce cell death and overcome tamoxifen resistance

4.4. Specific aims

The central hypothesis of this work was evaluated with fourteen specific aims under three main parts. In the first part, we focused on the characterization of XPO1 protein in tamoxifen resistance. In the second part, we tried to elucidate the mechanism behind combined targeting of ER α and XPO1 at a molecular level. We investigated which intracellular pathways are affected in tamoxifen resistant breast cancer cells from this combined treatment. In the third part, we expanded our focus area and assessed the effect of XPO1 targeting in metastatic breast cancer cells in combination with other endocrine therapy agents. To do this, we used 3D cell culture models to mimic actual metastatic breast tumor microenvironment. Also, we investigated the molecular mechanism behind the success of combined ER α -XPO1 targeting.

PART I: To identify the role of nuclear export mechanism in tamoxifen resistance.

- Aim1-1: Investigate the link between XPO1 expression and cell proliferation rate in tamoxifen-resistant breast cancer cells.
- Aim1-2: Identify the role of XPO1 on subcellular localization of ERK5 during tamoxifen resistance development.
- Aim1-3: Determine the effect of individual and combinational ER α and XPO1 targeting on the subcellular localization of ERK5 protein in tamoxifen-resistant breast cancer cells

PART II: To understand how nuclear export system contribute to tamoxifen resistance development by testing with a novel combinational targeting strategy using 4-OHT and SEL in Luminal B type breast cancer cell lines, and to investigate the metabolic outcomes of this novel therapy.

- Aim-2-1: Identify how XPO1 is associated with poor disease outcome in ER+ breast cancer cells. Compare XPO1 expression in different breast cancer subtypes.
- Aim-2-2: Identify the effect of ER α and XPO1 targeting on gene expression profiles and the general kinase pathways in tamoxifen-resistant breast cancer cells.
- Aim-2-3: Identify the effect of ER α and XPO1 targeting specifically on different phosphorylation sites of Akt protein.
- Aim-2-4: Identify the effect of ER α and XPO1 targeting on general metabolic phenotypes and glycolytic and mitochondrial metabolic pathways in tamoxifen-resistant breast cancer cells.
- Aim-2-5: Investigate which death pathways are activated via ER α and XPO1 targeting.

Part III: To assess the impact of other endocrine therapy options alone or in combination with Selinexor on metabolism in different endocrine resistant breast cancer cell lines and possible metastatic organ sites.

- Aim-3-1: Determine cell viability of different endocrine resistant breast cancer models under treatment of different endocrine therapy agents alone or in combination.
- Aim-3-2: Determine metabolic cell phenotypes of different endocrine resistant breast cancer models.

- Aim-3-3: Determine oncosphere formation profile/capacity of endocrine resistant breast cancer cell lines in 3D metastatic organ models.
- Aim-3-4: Investigate how cellular metabolic phenotype of endocrine resistant breast cancer cells change in different metastatic organ sites.
- Aim-3-5: Investigate why Selinexor alone is not successful.
- Aim-3-6: Investigate the mechanism behind the success of combined ER α -XPO1 targeting by combining different endocrine therapy agents in combination with Selinexor.

CHAPTER 5: EXPERIMENTAL PART I:

ER α -XPO1 CROSSTALK CONTROLS TAMOXIFEN SENSITIVITY IN TUMORS BY ALTERING ERK5 CELLULAR LOCALIZATION

5.1. Abstract

Most breast cancer deaths occur in women with recurrent, estrogen receptor (ER)- α (+), metastatic tumors. There is a critical need for therapeutic approaches that include novel, targetable mechanism-based strategies by which ER α (+) tumors can be resensitized to endocrine therapies. The objective of this study was to validate a group of nuclear transport genes as potential biomarkers to predict the risk of endocrine therapy failure and to evaluate the inhibition of XPO1, one of these genes as a novel means to enhance the effectiveness of endocrine therapies. Using advanced statistical methods, we found that expression levels of several of nuclear transport genes including XPO1 were associated with poor survival and predicted recurrence of tamoxifen-treated breast tumors in human breast cancer gene expression data sets. In mechanistic studies we showed that the expression of XPO1 determined the cellular localization of the key signaling proteins and the response to tamoxifen. We demonstrated that combined targeting of XPO1 and ER α in several tamoxifen-resistant cell lines and tumor xenografts with the XPO1 inhibitor, Selinexor, and tamoxifen restored tamoxifen sensitivity and prevented recurrence in vivo. The nuclear transport pathways have not previously been implicated in the development of endocrine resistance, and given the need for better strategies for selecting patients to receive endocrine modulatory reagents and improving therapy response of relapsed ER α (+) tumors, our findings show great promise for uncovering the role these pathways play in reducing cancer recurrences.

Key words: Endocrine resistance, Breast cancer, Glutamine deprivation, Combined drug targeting

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*authors equally contributed.

5.2. Introduction

The nuclear hormone estrogen receptor (ER) α is present in approximately 75% of human breast cancers and is considered one of the most critical predictive factor of breast cancer prognosis [1]. ER α is targeted by endocrine therapies, which are generally well tolerated [1]. Tamoxifen is one of the most effective therapeutics when a patient is diagnosed with ER-positive (ER α [+]) breast cancer. Although several recent trials showed that a combination of aromatase inhibitors (AIs) and ovarian suppression was effective in premenopausal breast cancer treatment, AIs also have significant adverse side effects in some postmenopausal women, such as increased joint pain, bone fractures, and cardiovascular disease risk [1–3] and the American Society for Clinical Oncology still recommends the use of tamoxifen for premenopausal women [3]. Therefore, tamoxifen remains an important endocrine therapy agent in both pre- and postmenopausal women and is expected to continue to be widely used for some time.

The benefit of endocrine therapies is limited, as demonstrated by tumor recurrence in 30% of ER α (+) patients. The recurrence of cancer in ER α -negative (ER α [–]) patients is higher in the first 5 years after the diagnosis, yet for ER α (+) patients, there is a consistent long-term risk of death due to recurrent breast cancer and even a greater risk after 7 years [3]. In fact, most breast cancer deaths occur in women with recurrent ER α (+) metastatic tumors [5, 6]. Recurrence appears to result from the up-regulation of cytoplasmic-initiated/ER α -dependent kinase pathways that provide an alternative mechanism to support tumor cell proliferation and survival and renders tumor cells resistant to endocrine therapies [7–11]. Hormonal regulation of breast cancer involves crucial inputs from estrogens, acting through ERs, and growth factors, operating through growth factor receptors that regulate downstream protein kinase pathways. The relative regulatory inputs to/from these two pathways are thought to underlie the degree to which the cancers remain more indolent and responsive to endocrine therapies vs the acquisition of resistance to these therapies. In the latter situation, the up-regulation of protein kinases serves as a hallmark of endocrine-resistant breast cancer [12,–15]. Also, whereas the presence of ER α is usually associated with a more favorable prognosis [16,–18], it is increasingly appreciated that not all ER α (+) breast cancer patients have a good outcome. A significant subset of patients with ER α (+) breast cancers, specifically those patients characterized as having a luminal B molecular subtype, also have high levels of protein kinase activity, do not respond to tamoxifen as efficiently, and have much less favorable disease-free survival, despite often being treated with additional chemotherapy [19,–22].

ERK5, a member of the MAPK family, is present in most human breast tumors and is overexpressed in approximately 20% of these tumors [23]. It was also recently identified in a kinase screening study as a major factor that regulates circulating tumor cell invasiveness [24]. We have recently identified ER α as the key factor responsible for the activation and regulation of the subcellular localization of ERK5 [25]. In this previous work, we showed that its nuclear localization is abrogated by treatment with ERK5 or ER α inhibitors. Based on this information, we used a combinatorial approach in which we took advantage of our tamoxifen-sensitive and tamoxifen-resistant cell culture models, an animal model and data from patient samples to delineate the role of nuclear transport pathways, particularly exportin 1 (XPO1), in tamoxifen sensitivity and endocrine therapy resistance. We identified high levels of XPO1, the major nuclear exporter of the tumor suppressor proteins, as a biomarker for tamoxifen resistance and evaluated its inhibition as a novel means to enhance the effectiveness of endocrine therapies. Our findings suggest that a higher expression of selected nuclear export pathway proteins results in decreased residence times of important nuclear factors that might be involved in proper transcriptional responses to tamoxifen treatment, thus conferring resistance to tamoxifen. Enhanced export to the cytoplasm results in key proteins communicating with other components of the cancer cell machinery involved in cell motility and enhanced kinase signaling, which function to increase aggressiveness of these cells. Our results show that inhibition of nuclear export machinery would improve therapy responsiveness and delay the development of hormone targeted treatment resistance and recurrence.

5.3. Materials and Methods

5.3.1. Cell culture, adenovirus, small interfering RNA, and ligand treatments

MCF-7 cells were grown in MEM with non-essential amino acids (NEAA) salts (Sigma), supplemented with 10% calf serum (HyClone) and 100 μ g/mL penicillin/streptomycin (Invitrogen) [26]. T47D cells were grown in DMEM with 10% calf serum (HyClone) and 100 μ g/mL penicillin/streptomycin (Invitrogen). BT474 was cultured in American Type Culture Collection-recommended Hybri-care medium with 10% inactivated fetal bovine serum (FBS), sodium bicarbonate, and antibiotics. MDA-MB-468 and MDA-MB-134 cells were grown in Leibovitz's medium with 20% calf serum (HyClone) and 100 μ g/mL penicillin/streptomycin (Invitrogen). HCC1500 cells were cultured in American Type Culture Collection-formulated

RPMI 1640 media with 10% FBS, sodium bicarbonate, and antibiotics. All cell lines were obtained from American Type Culture Collection. For experiments with 4-OHT treatment, the cells were maintained in the corresponding phenol red-free medium for at least 3 days and were then seeded at a density of 3×10^5 cells per 10-cm tissue culture dish (Corning) for 2 days before adenovirus infection. Recombinant adenoviruses were obtained from Applied Biological Materials Inc and were used to generate the MCF-7 cells expressing dominant-negative ERK5 or the overexpression XPO1 (AdXPO1) as described previously [25]. Adenovirus with a cytomegalovirus construct was used as an infection control (AdCMV).

5.3.2. Immunofluorescence microscopy and data analysis

MCF-7, MCF-7 TAM R, and BT474 cells were treated with Veh (0.1% EtOH) or 1 μ M 4-OHT in the presence or absence of 100 nM SEL for the indicated times. Cells were then washed in PBS and fixed on glass coverslips in 4% paraformaldehyde for 30 minutes and washed two times for 5 minutes in PBS. After incubation in acetone for 5 minutes, another PBS wash was performed and then cells were incubated with antibodies against XPO1 (Santa Cruz Biotechnology; 1:500), ER α (Santa Cruz Biotechnology; 1:1000), ERK5 (Bethyl; 1:2000), or phospho-ERK5 (Upstate, Millipore; 1:500). The next day, the cells were incubated with goat anti-mouse Alexa 568 or goat anti-rabbit Alexa 488 secondary antibodies. These slides were mounted and stained using Prolong Gold antifade with DAPI (Molecular Probes) to identify the nuclei.

BT474 xenograft samples were paraffin embedded and sectioned (4–5 μ m). After rehydration, antigen retrieval, and blocking, the slides were incubated with XPO1 antibody (Santa Cruz Biotechnology; 1:100). The next day, the slides were incubated with goat anti-mouse Alexa 568 secondary antibody. These slides were mounted, and stained using Prolong Gold antifade with 4,6-diamidino-2-phenylindole (DAPI) (Molecular Probes) to identify the nuclei.

Samples were imaged using a 63 $\times$ /1.4 oil DIC M27 objective in a Zeiss LSM 700 or 710 laser-scanning confocal microscope (Carl Zeiss). The images were obtained in a sequential manner using a 488-Ar (10 mW) laser line for phosphorylated ERK5 (pERK5) signal (500–550 nm emission) and 555 nm (10 mW) laser line for ER α (600–650 nm emission). The individual channels were obtained using a sequential scanning mode to prevent bleed-through of the

excitation signal. Laser power, gain, and offset were kept constant across the samples and scanned in a high resolution format of 512×512 or 1024×1024 pixels with two/four frame averaging.

Further quantification of the images was performed in Fiji software (<http://fiji.sc/wiki/index.php/Fiji>). Briefly, images were converted to eight bits for segmentation for each channel. Images for all channels were background subtracted using a rolling-ball method, with a pixel size of 100. Images were segmented using the DAPI channel. The DAPI images were contrast enhanced using the Otsu algorithm. To split touching nuclei and produce the final nuclear masks, the watershed algorithm was used. The resulting objects that had an area of less than 20 pixels and were close to edges were considered noise and were discarded. The DAPI image was selected as the mask, and the signal from pERK5 and/or ERK5 signal was quantified in the nucleus. Three frames per treatment were quantified. Experiments were repeated two times.

5.3.3. Cell Proliferation, cell cycle progression, invasion, motility, and soft agar assays

For proliferation assays, cells were seeded on 96-well plates at a confluency 2000 cells/well (except MDA-MB-134: 5000 cells/well and HCC-1500: 7000 cells/well) in no-phenol red media with 5% charcoal-dextran (CD) FBS. Cells were treated on day 2 and day 5 with indicated concentrations of (Z)-4-hydroxytamoxifen (Sigma-Aldrich; number H7904) and/or SXR (Selleckchem; number S7252). On day 7 a water-soluble tetrazolium-1 (WST-1) assay (Roche) was used to quantify the cell proliferation ratio. Absorbance was measured at 450 nm using the BioTek Cytation5 plate reader.

5.3.4. Western blot analysis in cell lines

On day 1 the cells were seeded on 10-cm plates at a concentration of 100 000 per plate in no-phenol red media with 5% CD FBS. The media were changed on day 3. On day 6 whole-cell lysates were collected in radioimmunoprecipitation assay buffer (Thermo Scientific) with $1\times$ Complete protease inhibitor (Roche). Samples were sonicated and boiled in sodium dodecyl sulfate loading buffer and then resolved on precast gels (Bio-Rad Laboratories) and transferred to a nitrocellulose membrane. Membrane was blocked in Odyssey blocking buffer (LI-COR). The following antibodies were used: pERK5 Thr218/Tyr 220 (number 3371; Cell Signaling), Lamin B1 (ab16048; Abcam), NUP153 (A301-789A), KPNA3 (A301-626A), NUP205 (A303-935A), RANGAP1 (A302-026A), KPNA2 (A300-483A), XPO1 (A300-469A), ERK5 (A302-656A), all

from Bethyl Laboratories, and ERK2 antibody (D-2 sc-1647; Santa Cruz Biotechnology) were used in 1:1000 dilution. β -Actin (Sigma; SAB1305546) was used in 1:10 000 dilution. The secondary antibodies were obtained from Odyssey and were used at a working concentration of 1:10 000. The images of the membranes are obtained by a LI-COR Odyssey CLx infrared imaging device and software. To compare the levels of nuclear receptor signature genes in different cell lines, we normalized the signal from the signature protein to the β -actin signal. The average values and SEM from three experiments were reported.

For the preparation of nuclear/cytoplasmic fractions, cells were seeded at 2×10^6 cells per 10-cm dishes. The media were changed at day 2. At day 4, the cells were treated with Veh (0.1% EtOH) or 1 μ M 4-OHT for 45 minutes. Whole-cell samples were suspended in cytosolic extraction buffer (10 mM HEPES, pH 7.9; 10 mM KCl; 0.1 mM EDTA; 0.1% Nonidet P-40; and protease/phosphatase inhibitors), and they were centrifuged at 12700 rpm for 10 minutes at 4°C. Cytosolic fractions were transferred into a fresh tube and kept on ice. The pellets were washed with cytosolic extraction buffer without Nonidet P-40 and phosphatase/protease inhibitors three times and dissolved in lysis buffer (0.5 M EDTA; 1 M Tris HCl; pH 8; 10% sodium dodecyl sulfate; 10% Empigen; and phosphatase/protease inhibitors). After a 10-minute incubation on ice, the nuclear fractions were sonicated once at 20% amplitude for 5 seconds and centrifuged at 12700 rpm for 10 minutes. Supernatants (nuclear fractions) were transferred into fresh tubes.

5.4. Results

5.4.1. ERK5 nuclear localization is reduced during the course of tamoxifen resistance

To characterize the state of the cellular localization of active ERK5 upon 4-OH-Tam treatment, we monitored the localization of ERK5 and pERK5 in a cell culture model of breast cancer endocrine resistance. We grew the initially tamoxifen-responsive MCF-7 cells in continuous 4-OHT treatment for 100 weeks. We and others have earlier demonstrated that this model mimics the development of endocrine resistance and for the characterization of molecular changes associated with the tamoxifen-resistant phenotype [26]. When we monitored the localization of ERK5 in cells that were in 4-OHT for 10, 50, and 100 weeks, we found that ERK5 and pERK5 localization to the nucleus was lost progressively compared with parental MCF-7 cells. (Figure 5.1.A). This was consistent with an increased cytosolic localization of pERK5 as the resistance progressed (Figure 5.1.B).

5.4.2. XPO1 overexpression in MCF-7 and BT474 cell increase ERK5 activation

Overexpression of XPO1 using an adenovirus system resulted in at least a 2-fold increase in the level of XPO1 mRNA and protein in MCF7 and BT474 cells increased active ERK5 levels in both of the cell lines (Figure 5.2.A). Moreover, XPO1 overexpression increased the proliferation of both cell lines, which was blocked by the XPO1 inhibitor (Figure 5.2.B).

5.4.3. 4-OHT treatment increases nuclear pERK5 in MCF-7 cells, but cytoplasmic pERK5 in BT474 cells.

To test whether the 4-OHT-mediated localization of pERK5 was different in tamoxifen-resistant and -sensitive cell lines, we treated MCF-7 cells and BT474 cells with Veh or 4-OHT for 45 minutes and then isolated cytoplasmic or nuclear fractions. Western blot analysis of each fraction revealed that 4-OHT treatment resulted in an increase in nuclear pERK5 in MCF-7 cells, whereas active ERK5 was in cytoplasm in BT474 cells after 4-OHT treatment (Figure 5.3.A). To test whether this localization disparity in two cell lines was due to XPO1 activity, we treated BT474 cells with Veh or 4-OHT in the presence or absence of SEL for 45 minutes and then subjected cells to immunofluorescence analysis. This experiment showed that in the presence of 4-OH-TAM, active ERK5 was completely extranuclear and treatment of the cells with the SXR relocated ERK5 back into the nucleus (Figure 5.3.B).

5.5. Discussion

In our studies, we found that as ER α (+) breast tumors acquire resistance to tamoxifen, a group of nuclear transport proteins including XPO1 will be upregulated, increasing ERK5 export from the nucleus. Thus, ER α , which is in the nucleus, will not have the partners to elicit proper transcriptional responses to tamoxifen and ERK5, which partners with other cytoplasmic proteins, will now contribute to tumorigenicity and tamoxifen resistance (Figure 5.4). The development of tamoxifen resistance is a major limitation to the effectiveness of treatment of hormone-responsive breast cancer. Whereas ER α presence is usually associated with a more favorable prognosis, it is increasingly appreciated that not all ER α (+) breast cancer patients have a good outcome. A significant subset of patients with ER α (+) breast cancers, such as those patients characterized as luminal B cancers that contain ER but also have high levels of protein kinase activity, have a much

less favorable disease-free survival. Blocking the activity of ER using selective ER modulators such as tamoxifen or raloxifene, or the ER-degrading agents fulvestrant or AIs, which reduce estrogen production, has proven highly effective in targeted treatment of hormone-responsive breast cancers [1, 27, 28].

Our approach was built upon our initial findings that estrogens increase the nuclear localization of key signaling molecules like ERK5, and the absence of ER α renders ERK5 extranuclear [25]. When outside the nucleus, ERK5 enhances the actin cytoskeleton reorganization and thus contributes to cell aggressiveness and motility, which are characteristics of breast cancers that are resistant to endocrine therapies. This study validated the hypothesis that nuclear export proteins level could be used as a marker for the risk of recurrence. Establishing XPO1 as a target for inhibition would enhance the effectiveness of endocrine therapies by maintaining tamoxifen sensitivity. Targeting the localization of key signaling molecules to cellular localizations in which they can be more efficiently used by ER α to increase efficiency of tamoxifen or other endocrine agents has a promise of higher efficacy and lower toxicity.

Cancer cells of different tumor types have been shown to be more sensitive to XPO1 inhibition than normal cells, including myeloma, in which ratjadone, another XPO1 inhibitor, is shown to be selective and kill myeloma but not normal cells [29, 30]. The inhibition of XPO1 in cervical cancer using another small molecule inhibitor, LMB, demonstrated the higher sensitivity of XPO1 inhibition in the cancer vs the normal cells [31]. XPO1 inhibitors might resensitize tamoxifen-responsive tumors to tamoxifen by modulating localization of these other factors as well. This could have a broad translational importance in the prevention and treatment of late-stage cancers. For example, in cancers in which ERK5 is localized to the cytoplasm, cellular aggressiveness can be downregulated by the pharmacological inhibition of XPO1, which results in a decreased nuclear export, thus allowing the return of ERK5 into the nucleus, in which it contributes to transcription and effective tamoxifen responsiveness. Thus, important advances in the therapy of late-stage disease and avoidance of complications associated with broad kinase inhibitors could ultimately be expected. Furthermore, our findings might be applicable to other cancers, including therapy-resistant leukemia, prostate cancers, and triple-negative breast cancer, for which highly selective XPO1 inhibitors are already in clinical trials [31, 32, 33].

Our research represents a new and substantive departure from the status quo by shifting the focus to modulating the localization of key proteins rather than modulating their actual activity. Most current research efforts in the therapy resistance field have focused on a delineation of the underlying mechanisms that lead to increased activity of selective signaling pathways. Undoubtedly, interrogating and targeting the end-point kinases in tumors is highly relevant, and these studies led to the development of combination therapies involving phosphatidylinositol 3-kinase inhibitors or mammalian target of rapamycin pathway inhibitors together with endocrine agents. However, resistance to these combination therapies also occurs, and in such cases, the cancer that develops is considerably more aggressive due to hyperactivation of compensatory mitogenic signaling pathways [34]. Moreover, these kinase inhibitors have many adverse side effects. More recently, ER α mutations that decrease the sensitivity of the receptor to selective ER modulators and selective estrogen receptor degraders were identified in more than 30% of the metastatic, but not primary, tumors [35,–39]. However, in two-thirds of ER-positive metastatic tumors, the mechanism of therapy resistance is not attributable to ER mutation, and alterations often cannot be targeted effectively.

Our findings strongly suggest that this approach will be effective in allowing the relocalization of key proteins, such as ERK5, to the nucleus to improve transcriptional response to tamoxifen that would otherwise function to regulate invasiveness and aggressiveness in the cytoplasm. In summary, our study reported here is the first attempt in the field to define the causal role of the nuclear export pathways in tamoxifen resistance and explore the feasibility of targeting these pathways to improve the response to tamoxifen and decrease the risk of recurrence.

5.6. Figures

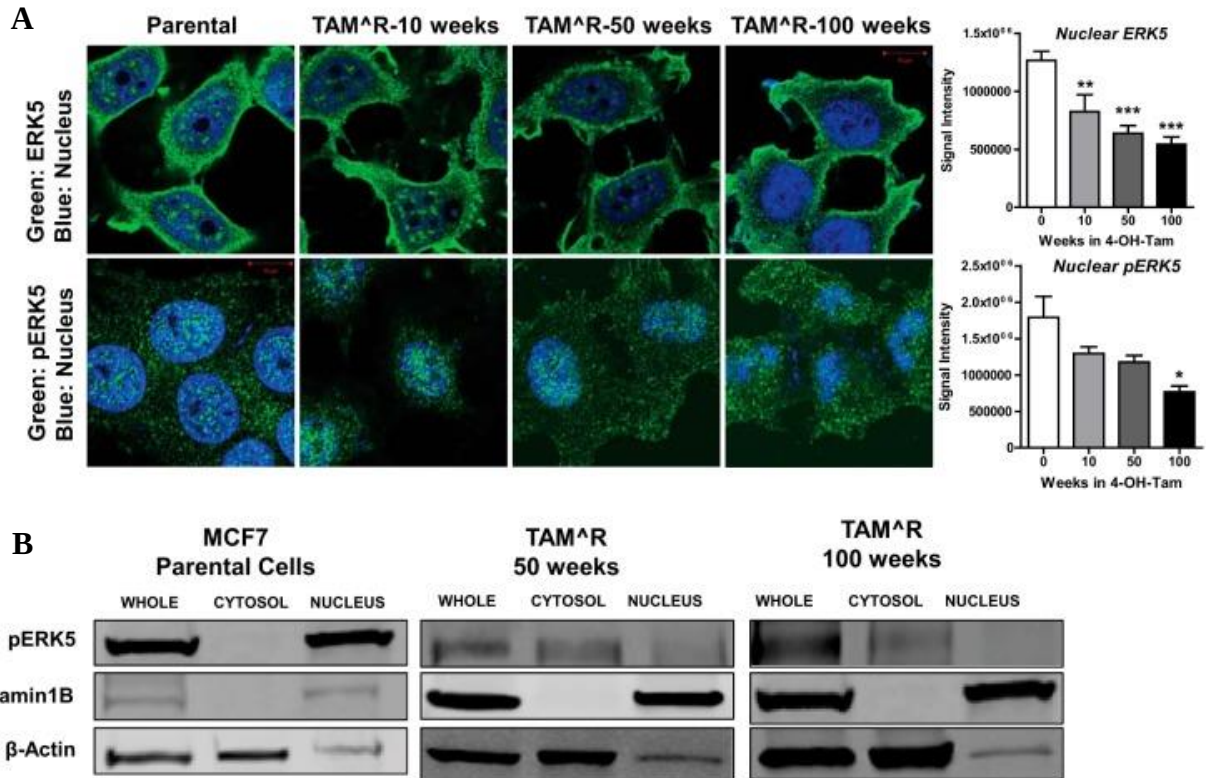


Figure 5.1: ERK5 nuclear localization is lost in MCF-7 cells that are tamoxifen resistant. A) ERK5 (upper panel) and pERK5 (lower panel) immunostaining after 45 minutes of 4-OH-TAM treatment in tamoxifen-sensitive MCF-7 cells and tamoxifen-resistant MCF-7 TAM R cells that were kept in 4-OH-TAM for the indicated times. MCF-7 cells or MCF-7 TAM R cells at different stages of resistance progression were treated with 1 μ M 4-OH-TAM for 45 minutes, and immunofluorescence microscopy was performed with an antibody specific to ERK5 or pERK5. Nuclei were stained with DAPI. Three fields per slide were quantified ($n = 3$). A one-way ANOVA model was fitted to assess the contribution of tamoxifen resistance progression on 4-OH-TAM treatment-induced ERK5 or pERK5 nuclear localization. When the main effects were statistically significant at $\alpha = .05$, pairwise t tests with a Newman-Keuls correction were used to identify the time that ERK5 or pERK5 localization was significantly different from parental MCF-7 cells. *, $P < .05$; **, $P < .01$; ***, $P < .001$; ****, $P < .0001$. **B)** Nuclear localization of pERK5 decreases as tamoxifen resistance progresses. MCF-7 parental cells or MCF-7 TAM R cells that were kept in media containing 4-OH-TAM for 50 or 100 weeks were fractionated, and whole-cell lysate and cytosolic or nuclear fractions were subjected to Western blot analysis using pERK5, Lamin1b (as nuclear fraction marker), and β -actin (as cytosolic fraction marker) antibodies. **C,** ERK5 activity regulated migratory potential in MCF-7 TAM R cells. MCF-7 TAM R cells were infected with AdCMV or dominant-negative AdERK5 for 24 hours and then seeded on the upper chamber of a transwell system for migration assays

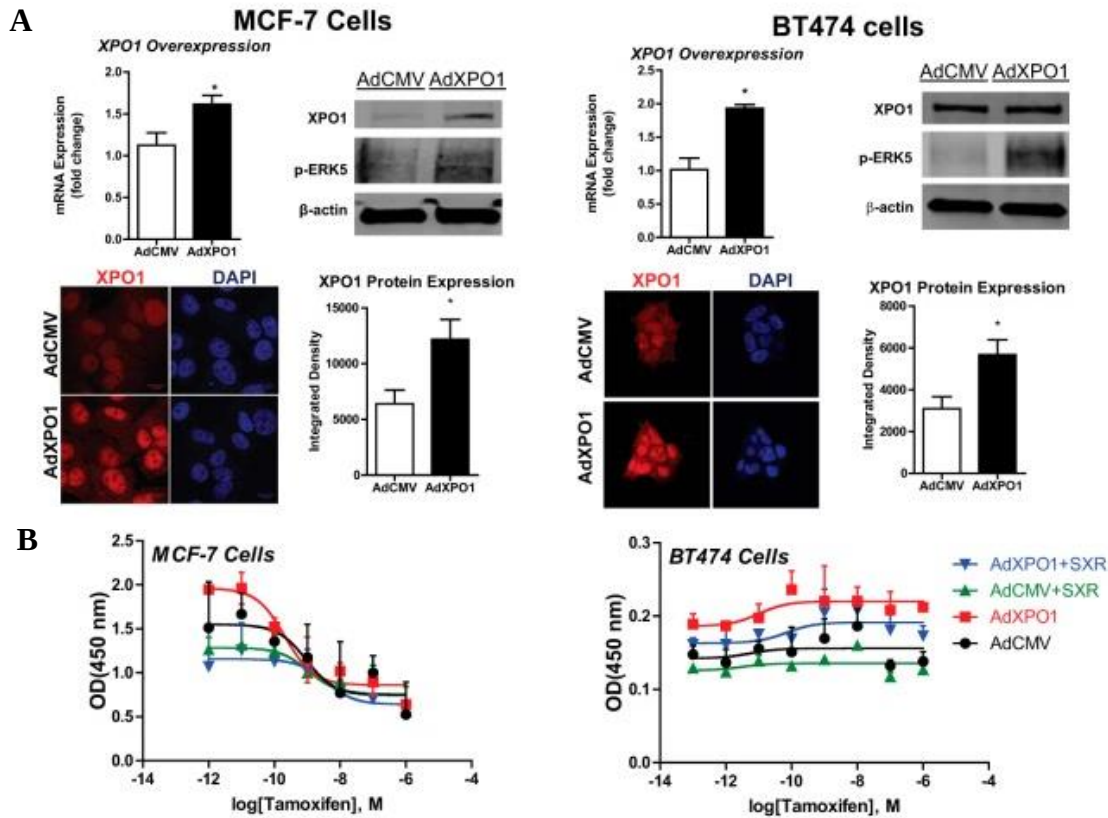


Figure 5.2: XPO1 levels increase cell proliferation of tamoxifen-resistant cell lines in the presence of 4-OHT.

A) XPO1 overexpression in MCF-7 and BT474 cell increase ERK5 activation. MCF7- cells (left panel) or BT474 cells (right panel) were infected with AdCMV as control or AdXPO1. *XPO1* mRNA overexpression was detected by qPCR. XPO1 protein overexpression was assessed by Western blot of immunofluorescence analysis. A *t* test was applied to assess whether AdXPO1 virus infection resulted in statistically significant overexpression of XPO1 in each cell line. *, $P < .05$ **B)** XPO1 overexpression in MCF-7 and BT474 cells increase cell proliferation. MCF-7 cells (left panel) and BT474 cells (right panel) were infected with AdCMV as control or AdXPO1, and then cells were treated with increasing doses of 4-OH-TAM in the presence or absence of 100 nM SEL.

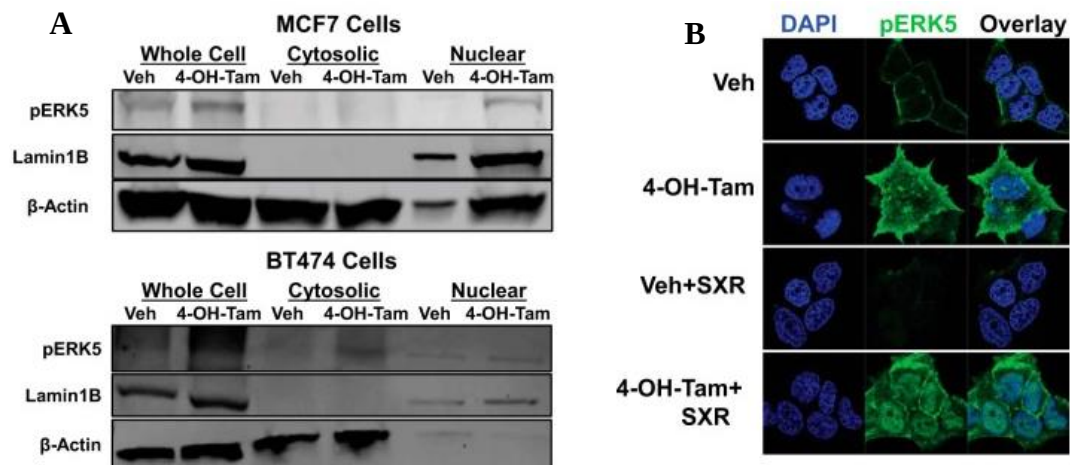


Figure 5.3: Impact of XPO1 activity on subcellular localization of pERK5. A) 4-OHT treatment increases nuclear pERK5 in MCF-7 cells but cytoplasmic pERK5 in BT474 cells. MCF7- cells and BT474 cells were treated with Veh (0.1% EtOH) or 1 μ M 4-OH-TAM for 45 minutes. Nuclear and cytoplasmic fractions were prepared as described. The increase in pERK5 levels in nuclear or cytoplasmic fractions were assessed by Western blot analysis. The experiment was repeated three times and representative results are displayed. **B)** XPO1 inhibition relocalizes pERK5 into the nucleus in the presence of 4-OHT in BT474 cells. BT474 cells were treated with Veh (0.1% EtOH) or 1 μ M 4-OHT in the presence or absence of 100 nM SEL for 45 minutes. Localization of pERK5 was monitored using immunofluorescence analysis. Ctrl, control.

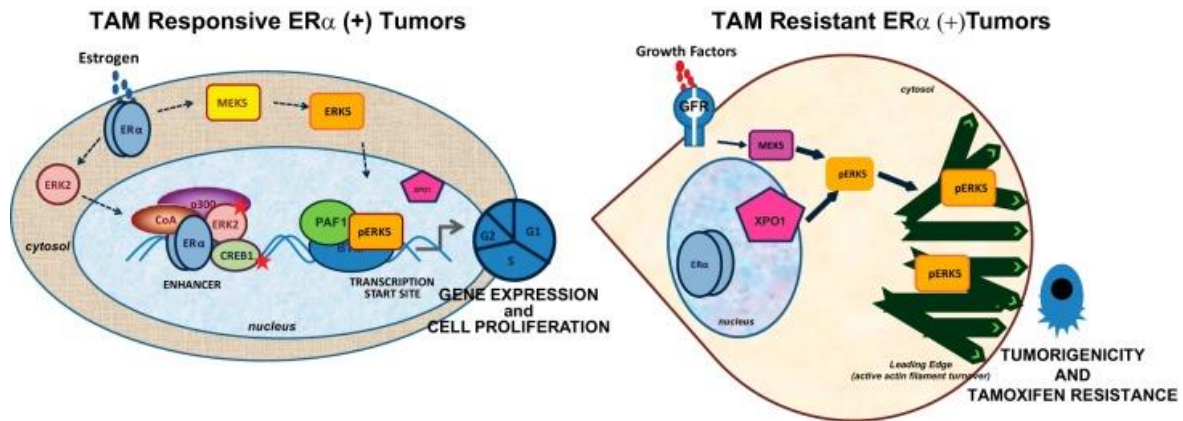


Figure 5.4: Model. As ERα (+) breast tumors acquire resistance to tamoxifen, a group of nuclear transport proteins including XPO1 will be upregulated, increasing ERK5 export from nucleus. Thus, 1) ERα, which is in the nucleus, will not have the partners to elicit proper transcriptional responses to tamoxifen and 2) ERK5, which partners with other cytoplasmic proteins, will now contribute to increased tumorigenicity and tamoxifen resistance.

5.7. References

1. Katzenellenbogen BS, Frasor J. Therapeutic targeting in the estrogen receptor hormonal pathway. *Semin Oncol.* 2004;31: 28–38.
2. Howell A, Cuzick J, Baum M, *et al.* Results of the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial after completion of 5 years' adjuvant treatment for breast cancer. *Lancet.* 2005; 365:60–62.
3. Burstein HJ, Temin S, Anderson H, *et al.* Adjuvant endocrine therapy for women with hormone receptor-positive breast cancer: American Society of Clinical Oncology clinical practice guideline focused update. *J Clin Oncol.* 2014; 32(21):2255–2269.
4. Ross-Innes CS, Stark R, Teschendorff AE, *et al.* Differential oestrogen receptor binding is associated with clinical outcome in breast cancer. *Nature.* 2012; 481: 389–393.
5. Jatoi I, Chen BE, Anderson WF, Rosenberg PS. Breast cancer mortality trends in the United States according to estrogen receptor status and age at diagnosis. *J Clin Oncol.* 2007; 25: 1683–1690.
6. Yu KD, Wu J, Shen ZZ, Shao ZM. Hazard of breast cancer-specific mortality among women with estrogen receptor-positive breast cancer after five years from diagnosis: implication for extended endocrine therapy. *J Clin Endocrinol Metab.* 2012; 97: E2201–E2209.
7. Massarweh S, Schiff R. Resistance to endocrine therapy in breast cancer: exploiting estrogen receptor/growth factor signaling crosstalk. *Endocr Relat Cancer.* 2006; 13 (suppl 1):S15–S24.
8. Schiff R, Massarweh SA, Shou J, *et al.* Advanced concepts in estrogen receptor biology and breast cancer endocrine resistance: implicated role of growth factor signaling and estrogen receptor coregulators. *Cancer Chemother Pharmacol.* 2005; 56 (suppl 1):10–20.
9. Creighton CJ, Casa A, Lazard Z, *et al.* Insulin-like growth factor-I activates gene transcription programs strongly associated with poor breast cancer prognosis. *J Clin Oncol.* 2008; 26: 4078–4085.
10. Sengupta S, Schiff R, Katzenellenbogen BS. Post-transcriptional regulation of chemokine receptor CXCR4 by estrogen in HER2 overexpressing, estrogen receptor-positive breast cancer cells. *Breast Cancer Res Treat.* 2009; 117:243–251.

11. Arpino G, Wiechmann L, Osborne CK, Schiff R. Cross talk between the estrogen receptor and the HER tyrosine kinase receptor family: molecular mechanism and clinical implications for endocrine therapy resistance. *Endocr Rev.* 2008; 29:217–233.
12. Jordan VC, O'Malley BW. Selective estrogen-receptor modulators and anti-hormonal resistance in breast cancer. *J Clin Oncol.* 2007;25: 5815–5824.
13. McGlynn LM, Kirkegaard T, Edwards J, *et al.* Ras/Raf-1/MAPK pathway mediates response to tamoxifen but not chemotherapy in breast cancer patients. *Clin Cancer Res.* 2009;15: 1487–1495.
14. Santen RJ, Song RX, McPherson R, *et al.* The role of mitogen-activated protein (MAP) kinase in breast cancer. *J Steroid Biochem Mol Biol.* 2002; 80:239–256.
15. Sivaraman VS, Wang H, Nuovo GJ, Malbon CC. Hyperexpression of mitogen-activated protein kinase in human breast cancer. *J Clin Invest.* 1997;99: 1478–1483.
16. Parker JS, Mullins M, Cheang MC, *et al.* Supervised risk predictor of breast cancer based on intrinsic subtypes. *J Clin Oncol.* 2009; 27: 1160–1167.
17. Ribelles N, Perez-Villa L, Jerez JM, *et al.* Pattern of recurrence of early breast cancer is different according to intrinsic subtype and proliferation index. *Breast Cancer Res.* 2013; 15: R98.
18. Voduc KD, Cheang MC, Tyldesley S, Gelmon K, Nielsen TO, Kennecke H. Breast cancer subtypes and the risk of local and regional relapse. *J Clin Oncol.* 2010; 28:1684–1691.
19. Ades F, Zardavas D, Bozovic-Spasojevic I, *et al.* Luminal B breast cancer: molecular characterization, clinical management, and future perspectives. *J Clin Oncol.* 2014; 32: 2794–2803.
20. Creighton CJ. The molecular profile of luminal B breast cancer. *Biologics.* 2012;6: 289–297.
21. Loi S, Sotiriou C, Haibe-Kains B, *et al.* Gene expression profiling identifies activated growth factor signaling in poor prognosis (Luminal-B) estrogen receptor positive breast cancer. *BMC Med Genomics.* 2009; 2: 37.
22. Tran B, Bedard PL. Luminal-B breast cancer and novel therapeutic targets. *Breast Cancer Res.* 2011; 13: 221.

23. Montero JC, Ocana A, Abad M, Ortiz-Ruiz MJ, Pandiella A, Esparis-Ogando A. Expression of Erk5 in early stage breast cancer and association with disease free survival identifies this kinase as a potential therapeutic target. *PLoS One*. 2009; 4: e5565.
24. Javaid S, Zhang J, Smolen GA, et al. MAPK7 regulates EMT features and modulates the generation of CTCs. *Mol Cancer Res*. 2015;13:934–943.
25. Madak-Erdogan Z, Ventrella R, Petry L, Katzenellenbogen BS. Novel roles for ERK5 and cofilin as critical mediators linking ER α -driven transcription, actin reorganization, and invasiveness in breast cancer. *Mol Cancer Res*. 2014; 12: 714–727.
26. Bergamaschi A, Madak-Erdogan Z, Kim YJ, Choi YL, Lu H, Katzenellenbogen BS. The forkhead transcription factor FOXM1 promotes endocrine resistance and invasiveness in estrogen receptor-positive breast cancer by expansion of stem-like cancer cells. *Breast Cancer Res*. 2014;16:436.
27. Jordan VC, O'Malley BW. Selective estrogen-receptor modulators and antihormonal resistance in breast cancer. *J Clin Oncol*. 2007;25:5815–5824.
28. Santen RJ, Brodie H, Simpson ER, Siiteri PK, Brodie A. History of aromatase: saga of an important biological mediator and therapeutic target. *Endocr Rev*. 2009;30:343–375.
29. Salas Fragomeni RA, Chung HW, Landesman Y, et al. CRM1 and BRAF inhibition synergize and induce tumor regression in BRAF-mutant melanoma. *Mol Cancer Ther*. 2013;12:1171–1179.
30. Turner JG, Marchion DC, Dawson JL, et al. Human multiple myeloma cells are sensitized to topoisomerase II inhibitors by CRM1 inhibition. *Cancer Res*. 2009;69:6899–6905.
31. Abdul Razak AR, Mau-Soerensen M, Gabrail NY, et al. First-in-class, first-in-human phase I study of selinexor, a selective inhibitor of nuclear export, in patients with advanced solid tumors. *J Clin Oncol*. In press.
32. van der Watt PJ, Maske CP, Hendricks DT, et al. The Karyopherin proteins, Crm1 and Karyopherin β 1, are overexpressed in cervical cancer and are critical for cancer cell survival and proliferation. *Int J Cancer*. 2009;124:1829–1840.
33. Cheng Y, Holloway MP, Nguyen K, et al. XPO1 (CRM1) inhibition represses STAT3 activation to drive a survivin-dependent oncogenic switch in triple-negative breast cancer. *Mol Cancer Ther*. 2014;13:675–686.

34. Dees EC, Carey LA. Improving endocrine therapy for breast cancer: it's not that simple. *J Clin Oncol*. 2013;31:171–173.
35. Alluri PG, Speers C, Chinnaiyan AM. Estrogen receptor mutations and their role in breast cancer progression. *Breast Cancer Res*. 2014;16:494.
36. Chu D, Paoletti C, Gersch C, *et al*. ESR1 mutations in circulating plasma tumor DNA from metastatic breast cancer patients. *Clin Cancer Res*. 2016;22(4):993–999.
37. Li S, Shen D, Shao J, *et al*. Endocrine-therapy-resistant ESR1 variants revealed by genomic characterization of breast-cancer-derived xenografts. *Cell Rep*. 2013;4:1116–1130.
38. Robinson DR, Wu YM, Vats P, *et al*. Activating ESR1 mutations in hormone-resistant metastatic breast cancer. *Nat Genet*. 2013;45:1446–1451.
39. Toy W, Shen Y, Won H, *et al*. ESR1 ligand-binding domain mutations in hormone-resistant breast cancer. *Nat Genet*. 2013;45:1439–1445.

CHAPTER 6: EXPERIMENTAL PART II:

COMBINED TARGETING OF ESTROGEN RECEPTOR ALPHA AND XPO1 PREVENT AKT ACTIVATION, REMODEL METABOLIC PATHWAYS AND INDUCE AUTOPHAGY TO OVERCOME TAMOXIFEN RESISTANCE

6.1. Abstract

A majority of breast cancer specific deaths in women with ER α (+) tumors occur due to metastases that are resistant to endocrine therapy. There is a critical need for novel therapeutic approaches to resensitize recurrent ER α (+) tumors to endocrine therapies. The objective of this study was to elucidate mechanisms of improved effectiveness of combined targeting of ER α and the nuclear transport protein XPO1 in overcoming endocrine resistance. Selinexor (SEL), an XPO1 antagonist, has been evaluated in multiple late stage clinical trials in patients with relapsed and /or refractory hematological and solid tumor malignancies. Our transcriptomics analysis showed that 4-Hydroxytamoxifen (4-OHT), SEL alone or their combination induced differential Akt signaling- and metabolism-associated gene expression profiles. Western blot analysis in endocrine resistant cell lines and xenograft models validated differential Akt phosphorylation. Using the Seahorse metabolic profiler, we showed that ER α -XPO1 targeting changed the metabolic phenotype of TAM-resistant breast cancer cells from an energetic to a quiescent profile. This finding demonstrated that combined targeting of XPO1 and ER α rewired the metabolic pathways and shut down both glycolytic and mitochondrial pathways that would eventually lead to autophagy. Remodeling metabolic pathways to regenerate new vulnerabilities in endocrine resistant breast tumors is novel, and given the need for better strategies to improve therapy response in relapsed ER α (+) tumors, our findings show great promise for uncovering the role that ER α -XPO1 crosstalk plays in reducing cancer recurrences.

Keywords: Breast Cancer; Endocrine resistance; Nuclear Transport Pathways; XPO1; ER α ; Metabolic rewiring

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6.2. Introduction

The nuclear hormone receptor estrogen receptor alpha (ER α) is present in approximately 70% of both early and late stage human breast cancers [1, 2]. ER α is targeted by endocrine therapies that are well tolerated and provide long-term disease-free survival if patients have localized disease [1]. Unfortunately, 30% of patients with ER positive (ER (+)) disease experience recurrence and metastasis, and there is a consistent long-term risk of death due to recurrent breast cancer with an even greater risk after 7 years [3, 4, 5, 6, 7]. Moreover, the 5-year relative survival of patients with ER(+) metastatic disease is 24%- almost none are cured and each year more women with recurrent ER (+) metastatic tumors die compared to women with ER (-) tumors [2, 8, 9].

Endocrine therapy is regarded as an effective treatment option for ER (+) metastatic cancer. Unfortunately, endocrine-resistance develops during the course of initial and subsequent endocrine treatment in almost all patients and occurs through various mechanisms including the mutational alterations in the ESR1 gene sequence, dysregulation in signaling pathways (i.e. Her2 signaling) and changes in drug uptake and metabolism [10]. Endocrine-resistant patients require increasingly toxic therapies. These are not optimal in terms of pharmacological properties due to poor tolerance and side-effects that decrease the quality of the patient's life. Thus, endocrine resistance remains a significant clinical problem. A novel therapeutic approach to resensitize ER (+) metastatic tumors to endocrine therapies together with methods to select patients likely to benefit from this approach is needed. Without new strategies, many patients with ER (+) tumors will continue to face diminished prospects for long-term survival being prescribed regimens that decrease their quality of life without receiving clinical benefit.

Tamoxifen (TAM) remains the “first-in-line” endocrine therapy and is regarded as one of the most effective treatments for patients diagnosed with ER (+) breast cancer. While several recent trials showed that aromatase inhibitors (AIs) in combination with ovarian suppression were effective in premenopausal breast cancer treatment, the American Society for Clinical Oncology (ASCO) continues to recommend TAM for premenopausal women [5]. This may be attributed to the observation that AIs may also have significant adverse side effects in some postmenopausal women, such as increased joint pain, bone fractures, and risk of cardiovascular disease [1, 5, 11]. Therefore, TAM remains as an important endocrine therapeutic agent in both pre- and postmenopausal women and is expected to continue to be widely used for some time.

In our previously published study which sought to understand the mechanism of TAM resistance in a preclinical setting, we identified a group of nuclear transport proteins, including exportin 1 (XPO1), which were upregulated in TAM-resistant cell lines and tumors [12]. Importantly, XPO1 upregulation caused nuclear export of ERK5, which is necessary in the nucleus for proper transcriptional response of ER α to TAM. Moreover, when ERK5 translocates to the cytoplasm, it can partner with other cytoplasmic proteins, including actin reorganization proteins, which contribute to cancer cell motility. However, it is not known whether XPO1 activates other mechanisms that may be targeted by therapies in current use, specifically for endocrine therapy resistant and ER (+) breast cancers.

Based on previous studies [12, 13, 14], we hypothesized that ER α and XPO1 work together to drive TAM-resistance, and that combined targeting would more effectively sustain tumor regression than targeting either protein alone. Specifically, we showed that XPO1 mRNA levels were higher in Luminal B subtype of tumors which are more refractory to endocrine treatments, and that high XPO1 expression values were associated with a poor outcome in all women who were treated with TAM [12]. Therefore, XPO1 could be an excellent therapeutic target in breast cancer as XPO1 is already targeted in other therapy-resistant cancers with the highly specific small molecule inhibitor SEL, which is an orally active and well-tolerated drug [15, 16]. Our rationale is that targeting XPO1 together with ER α is clinically relevant and combining TAM with SEL potentially offers higher efficacy, specificity, and lower toxicity for the treatment of endocrine resistant, recurrent ER (+) breast cancer.

To evaluate the effects of combining TAM (4-OHT) with SEL, in the present study we used transcriptome analysis and found that the combination differentially modulated Akt signaling-associated gene expression as well as glycolytic and mitochondrial gene expression programs. Because of differential changes in Akt phosphorylation with 4-OHT and/or SEL, we hypothesized that metabolic pathways associated with Akt activity and consequently the metabolic profile of breast cancer cells would change in the presence of 4-OHT and SEL. We demonstrated that combined targeting of XPO1 and ER α in TAM-resistant cell lines shuts down metabolic pathways that would eventually lead to autophagy. Our studies provide a novel mechanism of action of combined targeting of ER α and XPO1 to overcome TAM resistance in recurrent ER α (+) breast cancer.

6.3. Materials and Methods

6.3.1. Cell Culture and Ligand Treatments

All cell lines were obtained from American Type Culture Collection. MCF-7 cells were cultured in Improved Minimal Essential Medium (MEM) with NEAA salts (Sigma, St Louis, MO), supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT), 100 µg/ml penicillin/streptomycin (Invitrogen, Carlsbad, CA) and 1500 mg/L sodium bicarbonate (Gibco, MA). *Acquired resistance* was studied from resistance progression cell lines previously derived and characterized by long-term exposure of parental MCF-7 cells to 4-OH-Tam (MCF-7 Tam^R) [17, 18]. These cells retain ERα expression, do not require E2 for growth, are not growth inhibited by SERMs and 4-OH-Tam stimulates their growth.[17, 18]. Human breast cancer cell lines BT474, HCC-1500 and MDA-MB-134, which are 4-OH-Tam-resistant, were used as a model of *de novo resistance* [18, 19, 20]. BT474 were cultured in ATCC recommended Hybri-care medium with 10% inactivated FBS, sodium bicarbonate and antibiotics. MDA-MB-134 cells were cultured in Leibovitz's medium with 20% FBS (HyClone), and 100 g/ml penicillin/streptomycin (Invitrogen). HCC 1500 cells were cultured in ATCC-formulated RPMI1640 media with 10% FBS, sodium bicarbonate and antibiotics. Before ligand treatments, cells were grown in the corresponding phenol red-free media at least for three days.

For isobologram analysis, BT474 and MCF-7 Tam^R cells were seeded at a density of 2×10^3 cells/well in a 96-well plate, and were grown overnight in IMEM media without phenol red. On day 1, cells were treated with different doses of 4-OHT (from 10^{-9} M to 10^{-5} M) (Sigma) and SEL (from 10^{-9} M to 10^{-5} M) (Selleckchem) alone and in combination. Treatment was repeated again on day 4. On day 7, WST1 assay (Roche) was used to quantify cell proliferation ratio. Absorbance was measured at 450 nm using BioTek, Cytation5 plate reader. IC50 and isobologram calculations were made based on literature [*] by using Microsoft® Office Excel and statistical analyses were done by using Graphpad® Prism8 software. All experiment conditions had six technical repeats and experiments were repeated at least for three times.

6.3.2. RNA Sequencing Analysis

RNASeq analysis was performed as previously described [21, 22, 23]. Briefly, BT474 cells were treated with Vehicle (Veh, 0.5% EtOH), 10^{-6} M 4-OHT (Sigma, St. Louis, MO), 10^{-7} M Selinexor (SEL) (Selleckchem, Houston, TX) or 4-OHT+SEL combination for 24 hours.

Concentrations of ligands are based on our previously published study [18] and clinical data [24, 25, 26]. Total RNA was extracted with TRIzol reagent (Life Technologies) according to manufacturer protocol and cleaned using QIAGEN clean-up kit. RNA quality was assessed using bioanalyzer. Total RNA from each sample (3 per treatment group) were sequenced at the UIUC sequencing center, and data was generated in Fastqc file format to compare the expressions between the four treatment groups.

Preprocessing and Quality Control: Fastqc files containing raw RNA sequencing data were trimmed using Trimmomatic (version 0.36) [27]. Next, the reads were mapped to the Homo sapiens reference genome (GRCh37) from Ensembl [28] database and aligned using the STAR alignment tool (Version 2.5.3) [29]. After this, the read counts were generated from SUBREAD (Version 1.5.2) [30] and featured counts were exported for statistical analysis in R. Quality control and normalization was conducted in R using edgeR (Version 3.20.9) [31].

Statistical Analysis and DEGs: Statistical analysis was conducted in R using limma (Version 3.34.9) [32, 33]. Empirical Bayesian statistics were conducted on the fitted model of the contrast matrix. Differentially expressed genes were then determined by fold-change and p-value with Benjamini and Hochberg [34] multiple test correction for each gene, for each treatment relative to the vehicle control. We considered genes with fold-change >1.5 and p-value < 0.05 as statistically significant, differentially expressed. Cluster3 software was used for clustering the differentially expressed genes. Data was visualized using Treeview Java. PCA analysis was performed using StrandNGS (Version 3.1.1). GSEA [35, 36] analysis was used to identify GO terms associated with different treatments.

6.3.3. *In vivo* xenograft study, immunohistochemistry staining (IHC) and data analysis

All experiments involving animals were conducted with protocols approved by the University of Illinois at Urbana-Champaign and by the National Institutes of Health standards for use and care of animals (IACUC Protocol 14193). Tumor xenograft studies were performed using the BT474 cell line in immunocompromised female mice as previously described [12, 37, 38]. Briefly, 6-week-old BALB/c athymic, ovariectomized, nude female mice from Taconic Biosciences were used. After one week of acclimatization, 0.72 mg, 60-day release E2 pellets from Innovative Research of America were implanted subcutaneously to maintain a uniform level of estrogen. The next day 2.5×10^7 BT474 cells resuspended in 50% PBS and 50% Matrigel were

injected subcutaneously into both right and left flank of each mouse. Once the tumor size reached 200 mm³, five animals were randomized to each treatment group. Half of the mice were implanted with vehicle pellets and the other half were implanted with 25 mg, 60-day release TAM pellets from Innovative Research of America. Each group was randomized to Vehicle or SEL injection (20 mg/kg). Concentration was selected based on clinically relevant dose [24, 25, 26]. Biweekly injections were performed (Monday and Friday) for 4 weeks. Each mouse was housed individually. Animals were monitored daily by the veterinarians for any signs of starvation, dehydration, stress, and pain. Total weight, food intake, and tumor size was monitored using a digital caliper biweekly. Tumors were removed from euthanized mice at the end of the experiment or at the time when tumor size reached 1000 mm³ and were fixed in 10% neutral-buffered formalin, processed, and embedded in paraffin in 2M sucrose before being frozen in cutting medium. Tissues were cut in 5 microns by using a microtome (The Leica RM1255, Austria). For both pAkt S473 and pAkt T308 immunostainings, tissues were deparaffinized and hydrated through graded alcohols to water. Antigen retrieval was performed by using citrate buffer, pH 6.0 in a streamer for 1 hour. Samples were blocked in hydrogen peroxide for 10 minutes. To remove non-specific protein staining, samples were blocked with Background buster (Innovex Biosciences, Richmond, CA) for 10 minutes and rinsed with TBS-Tween solution, pH 7.6. Then, samples were incubated with pAkt S473 primary antibody (#4060, Cell signaling) overnight at 4° C and with pAkt T308 primary antibody (#13038, Cell Signaling) for 1 hour at room temperature at 1:100 dilution. After rinsing with TBS-Tween solution, pH 7.6., samples were stained with secondary anti-rabbit and anti-mouse HRP-Polymer (Biocare Medical, Concord, CA) for 30 minutes. Finally, samples were incubated with DAB (Innovex, Richmond, CA) for 5 minutes and counterstained with hematoxylin, dehydrated and mounted on slides. Results are from at least four tumors per treatment. Visualization of the samples were performed with Nanozoomer Slide Scanner (Hamamatsu, Japan) at 80X magnification and positive staining quantification was performed by NDP.view software. Five fields per tumor were imaged and quantified. Representative images are presented.

For the verification of XPO-1 protein levels in patient tumor samples, BRC1021 tissue microarray from Pantomics, Inc containing 95 cases with known ER, PR, AR, Her2, p53, EGFR, and Ki67 IHC results was used. Subtype of tumors were based on following criteria: Lum A: ER (+), Her2 (-), Ki67<15; Lum B: ER (+), Her2 (+) or ER (+), Her2 (-), Ki67>15; Her2 (+): ER (-),

Her2 (+); TNBC: ER (-), PR (-), Her2 (-)[39; 40]. XPO1 antibody was from Bethyl laboratories (A300-469A). BRC1021 TMA included 43 Her2 3 LumA 31 Lum B 17 TNBC tumor samples. UTSW cohort included 50 Luminal A, 45 Luminal B, 48 TNBC and 35 Her2 positive/amplified breast tumors validated by a pathologist. The samples were collected under the STU 102010-051 IRB protocol number and were collected at UTSW and affiliated hospitals. For each core, a score for XPO1 was assigned based on the signal intensity (0=none, 1=low, 2=moderate, and 3=high).

6.3.4. Western Blot Analysis in Cell Lines

Cells were seeded on 30 mm cell culture plates at 1×10^5 cells/well concentration in corresponding treatment media without phenol red which contains 5% FBS. Cells were treated with 4-OHT (10^{-6} M) (Sigma) and SEL (10^{-7} M) (Selleckchem)-containing treatment media overnight. Next day, cells were collected in Lysis buffer (0.5 M EDTA, 1 M TrisHCl pH 8.1, 10% SDS, 10% Empigen, ddH₂O) with 1x Complete Protease Inhibitor (Roche) and 1x Phosphatase Inhibitor (Thermo Scientific). Samples were further processed with sonication and protein concentrations were determined by BCA assay (Thermo Scientific). Then, samples were boiled in SDS-containing loading buffer and were run in 10% precast gels (BioRad) and transferred to nitrocellulose membrane. The membrane was blocked in Blocking Buffer (Odyssey®, Li-Cor) and target proteins were probed with XPO1 (sc-74454, Santa Cruz Biotechnology), p-Akt S473 (#4060, Cell Signaling), pAkt T308 (#13038, Cell Signaling), Akt (#9272, Cell Signaling), PARP (#9542, Cell Signaling), ATG9A (STJ98598, St. John's Laboratory), AMBRA-1 (STJ98593, St. John's Laboratory) and Beclin-1 (STJ 98594, St. John's Laboratory), antibodies in 1:1000 dilution and β -actin (Sigma SAB1305546) antibody in 1:10000 dilution. The secondary antibodies were obtained from Odyssey were used at 1:10000 dilution. The membranes were visualized by using Licor Odyssey CLx infrared imaging device and software. All results were repeated at least three times and the results were normalized according to signal, which was received from β -actin loading control. Representative blots are presented.

6.3.5. Seahorse Metabolic Profiling Assays

MCF-7, MCF-7 Tam^R and BT474 cells were seeded at a density of 5×10^4 and MDA-MB-134 and HCC 1500 cells were seeded at a density of 1×10^5 in corresponding treatment media without phenol red in each well of the XFp Cell Culture miniplates, respectively (Seahorse

Bioscience Inc., Billerica, MA). The FCCP concentration was 0.5 μ M for MCF-7, BT474, MDA-MB-134 and HCC 1500 cells. Next day, cells were treated with 4-OHT (10^{-6} M) (Sigma) and/or SEL (10^{-7} M) (Selleckchem) -containing treatment media overnight and the cartridges were hydrated with the calibration solution and kept in a non-CO₂ incubator at 37°C overnight. In parallel, a duplicate of each plate was used for cell counting to monitor cell number changes after 24 hours of treatments and Seahorse data was normalized to total cell number. On the assay day, cells were washed with XF Base Media without phenol red (Seahorse Bioscience Inc.) supplemented with 10 mM L-glucose, 2mM L-glutamine (Gibco) and 1 mM sodium pyruvate (Gibco). The ECAR (mpH/min) and OCR (pmol/min) values were obtained by using Seahorse XF_p Cell Energy Phenotype Test Kit (Seahorse Bioscience Inc.), Seahorse XF_p Mito Stress Test Kit (Seahorse Bioscience Inc.), Seahorse XF_p Glycolytic Stress Kit (Seahorse Bioscience Inc.), and Seahorse XF_p Mito Fuel Flex Test Kit (Seahorse Bioscience Inc.), which were run with Seahorse XFp Analyzer (Seahorse Bioscience Inc.). Experiments were performed in triplicate and repeated at least three times.

6.3.6. Caspase Colorimetric Protease Assay

MCF-7 and BT474 cells were seeded at a density of 2×10^3 cells to a 96-well plate and in the next day, they were treated with 4-OHT (10^{-6} M) (Sigma) and SEL (10^{-7} M) (Selleckchem) alone and in combination for 24 hours. The colorimetric caspase activities of Caspase 2, Caspase 3, Caspase 6, Caspase 8 and Caspase 9 were determined according to the manufacturer recommendations by using ApoTarget Caspase Colorimetric Protease Assay Sampler Kit (Invitrogen). Changes in colorimetric density were detected by Cytation™ 5 Cell Imaging Multi-Mode Plate Reader (Biotek Instruments, Inc.) at 400 nm wavelength. Experiments were performed in duplicates and repeated three times.

6.3.7. Autophagy Assay

MCF-7 and BT474 cells were seeded at a density of 2×10^3 cells to 96-well plate and in the next day, they were treated with 4-OHT (10^{-6} M) (Sigma) and SEL (10^{-7} M) (Selleckchem) alone and in combination for either 24 or 48 hours. Autophagosome formation was detected through GFP-labelled LC3-II protein in live cells using the Cyto-ID Autophagy Detection Kit (Enzo Life Sciences, Farmingdale, NY) according to manufacturer recommendations by Cytation™5 Cell

Imaging Multi-Mode Plate Reader (Biotek Instruments Inc., VT) at ~480 nm excitation and ~530 nm emission. In addition, Hoechst 33342 Nuclear Stain was also recorded by means of DAPI filter set at ~340 nm excitation and ~480 nm emission. For flow cytometry analysis, BT474 cells were seeded at a density of 1×10^6 cells/plate, and they were grown for 48 hours in IMEM media without phenol red. Next day, cells were treated with 4-OHT (10^{-6} M) (Sigma) and SEL (10^{-7} M) (Selleckchem) alone and in combination for 24 hours. Negative control plates were treated with EtOH and positive control plates were also treated with Rapamycin (5×10^{-6} M) (CytoID) for 24 hours. Following the treatment process, cells were collected and centrifuged at 1000 rpm for 5 minutes in 1x Assay buffer provided in the CytoID® Autophagy Detection Kit (Enzo Biosciences Inc, Ann Arbor, MI). Cells were resuspended again and incubated with CYTO-ID® Green Detection Reagent for 30 minutes at dark. After this incubation time, cells were washed with 1x Assay buffer 3 times and results were analyzed with results were analyzed by BD™ LSR II Flow cytometry analyzer (BD Biosciences Inc., San Jose, CA). Following analysis, results were analyzed by FCS Express 6 Flow Cytometry Software (DeNovo Software) (<https://www.denovosoftware.com/site/Flow-RUO-Overview.shtml>) and all experiments were repeated with 3 technical replicates at least three times. Experiments were performed in duplicates and repeated three times.

For the inhibition of autophagy BT474 cells were seeded in 96-well plates at a confluency of 2000 cells/well in corresponding treatment media. At day 2, they were treated in a media with with 10^{-6} M 4-OHT and 10^{-7} M SEL alone or combined in the presence or absence of 10^{-5} Chloroquine (Sigma). This treatment was repeated at day 5. Cellular viability was measured with WST1 reagent (Roche) at 450 nm wavelength and results were quantified by using Cytation5 cell imaging Multi-Mode reader (Biotek Instruments Inc., VT). Statistical analyses were done by using GraphPad Prism8© software.

6.3.8. Cell Cycle Analysis

Flow cytometry analysis was performed as previously described [12, 41, 42]. Briefly, MCF-7 and BT474 cells were seeded at a density of 1×10^6 cells/ plate and their media was changed two times in every two days. Following an overnight treatment with 4-OHT (10^{-6} M) (Sigma) and SEL (10^{-7} M) (Selleckchem) alone and in combination, cells were collected with 0.1% FBS containing 1x PBS, washed, resuspended at $1-2 \times 10^6$ cells/ml and fixed with ice cold ethanol for 24

hours. In the next day, cells were washed with 1x PBS and incubated in 10ng/ml RNAase for one hour at room temperature. Cells were stained with 0.25% Propidium Iodide (#10008351, Cayman Chemicals, Ann Arbor, MI) for one hour and results were analyzed by BD™ LSR II Flow cytometry analyzer (BD Biosciences Inc., San Jose, CA). Following analysis, results were analyzed by FCS Express 6 Flow Cytometry Software (DeNovo Software) (<https://www.denovosoftware.com/site/Flow-RUO-Overview.shtml>) and all experiments were repeated three times.

6.3.9. Statistical Analyses

Data from all studies were analyzed using a one-way analysis of variance (ANOVA) model to compare different ligand effects, a two-way-ANOVA model to compare time-dependent changes. All data was tested for normal distribution using pairwise t tests with a Bonferroni correction. All data was normally distributed unless otherwise noted. Normally distributed data was analyzed using pairwise t tests with a Bonferroni correction to identify treatments that were significantly different from each other (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). For every main effect that was statistically significant at $\alpha=0.05$, pairwise t-tests were conducted to determine which ligand treatment levels were significantly different from each other. For these t-tests, the Bonferroni correction was employed to control experiment wise type I error rate at $\alpha=0.05$ followed by Bonferroni post hoc test. Data that were not normally distributed were analyzed using Mann Whitney test for nonparametric data (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). Statistical significance was calculated using GraphPad Prism for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com).

6.3.10. Availability of Data and Materials:

Gene expression data is submitted to GEO database under the accession number GSE112883 and going to be available from the day of the acceptance of the manuscript.

6.4. Results

6.4.1. 4-OHT+SEL treatment synergistically sustains tumor regression in endocrine resistant Luminal B subtype breast cancer cells

Our previous study showed that XPO1 mRNA was overexpressed in the Luminal B subtype of tumors, which are more likely to recur on endocrine treatments relative to the Luminal A subtype [12]. To validate our results, we performed IHC analysis for XPO1 protein expression in two independent breast tumor sets with samples from various breast cancer subtypes. This analysis showed that Luminal B subtype tumors have higher overall XPO1 signal intensity and % XPO1 positive cells in two independent cohorts of breast tumors (Figure 6.1.A and 6.1.B). We then treated endocrine resistant BT474 cells with 4-OHT, SEL or both in combination to assess whether these treatments synergized to reduce cell viability. The isobologram analysis showed that the 4-OHT and SEL combination synergized to achieve a greater decrease in cell viability than with either agent alone (Figure 6.1.D). These results are consistent with our BT474 xenograft studies in nude mice, which showed that the tamoxifen (TAM) and SEL combination provided complete tumor regression, which was sustained several weeks after cessation of drug administration (Figure 6.1.E) [18].

6.4.2. 4-OHT+SEL combination treatment causes gene expression changes distinct from 4-OHT and SEL treatments alone in TAM-resistant cell lines

To understand how the TAM+SEL combination provide a sustained tumor regression, we performed a global gene expression analysis in BT474 cells that were treated with Veh, 4-OHT, SEL or 4-OHT+SEL. Hierarchical clustering of the differentially-expressed genes revealed 10 clusters with different gene regulation patterns. Genes in Clusters 1, 2, 3, 6, 9, and 10 were regulated similarly with all treatments. The 4-OHT+SEL combination and 4-OHT alone resulted in similar patterns of regulation for genes in Cluster 1. The 4-OHT+SEL combination and SEL alone caused similar gene regulation patterns for genes in Clusters 2,3,6,9 and 10. On the other hand, Clusters 4 and 5 include genes that were upregulated more with the combination treatment than either treatment alone, and genes in Clusters 7 and 8 were downregulated more with the combination treatment than either treatment alone (Figure 6.2.A and 6.2.B). Principle component analysis (PCA) showed that treatment groups differed significantly (Figure 6.2.C). Venn-diagram analysis of up- and downregulated genes revealed that the combination treatment caused gene

expression changes similar to as well as different from single agent treatments. The combination treatment increased the expression of 101 genes and decreased the expression of 132 genes that were not affected by either treatment alone (Fig. 6.2.D and 6.2.E).

Next, we used Gene Set Enrichment Analysis (GSEA) analysis to identify functional gene groups that were associated with different treatments (Figure 6.2a.A, B and C). The 4-OHT treatment caused upregulation in genes for cell cycle-, endocrine therapy resistance-, and breast cancer invasiveness-related pathways. On the other hand, genes involved in apoptosis and decreased tumor invasiveness were downregulated by this treatment. Although SEL was effective in decreasing endocrine therapy resistance, breast cancer invasiveness, and metastasis related genes, genes that would regulate apoptosis were also downregulated by this treatment. The 4-OHT+SEL combination was the most effective treatment for down regulating therapy resistance and tumor invasiveness. Specifically, the combination treatment was more efficient than either treatment alone to upregulate gene sets associated with genes downregulated in Luminal B tumors, metastasis, and in bone metastasis. However, the combination treatment was very effective in downregulating ER α target genes that were upregulated by AKT1 overexpression and genes that were upregulated in Luminal B tumors, endocrine therapy resistance and metastasis that were identified in various studies. These findings are consistent with our data showing that differential Akt signaling was modulated by the ER α and XPO1 crosstalk. Of note, 4-OHT+SEL reduced targets of FGFR1 signaling, recently shown to be associated with Palbociclib+Fulvestrant-resistant tumors [43, 44]. Overall, our gene expression analysis showed that the 4-OHT+SEL treatment was very effective in downregulating genes associated with endocrine resistance and metastasis. Data on other gene sets associated with metabolic regulation and autophagy will be presented in the upcoming sections.

6.4.3. XPO1 inhibition modulates differential Akt phosphorylation in TAM-resistant cells and tumor xenografts.

We next focused on the Akt signaling associated gene sets which were identified as differentially downregulated by the 4-OHT+SEL combination (Figure 6.3.A) because they play an important role in cell survival and metabolism. We validated differential Akt phosphorylation with individual or combined drug treatments in BT474 cells (Figure 6.3.B) and MCF-7 TAM^R cells (Figure 6.3a.A, B and C). Of note, 4-OHT treatment induced an increase in the cytoplasmic pAkt

Ser473 signal, whereas SEL treatment increased the pAkt Thr305 signal in dividing cells (arrows) (Figure 6.3.C). As we previously showed, TAM treatment stimulated tumor growth in BT474 tumor xenografts, whereas SEL treatment inhibited tumor growth [12]. However, tumor growth resumed within 3 weeks after SEL treatment was stopped. By contrast, the combination of SEL and TAM not only caused a faster and more complete regression of tumors, but the regression was also sustained [12]. To validate our pathway analysis *in vivo*, we utilized tumor xenograft samples from this experiment (Figure 6.3.D). Our IHC analysis showed that Ser 473 phosphorylation of Akt protein increased only in tumors from TAM treated animals, whereas Thr 308 phosphorylation of Akt was more prevalent in tumors from SEL treated animals. Both of the phosphorylation events were dampened in tumors from animals that were treated with the SEL+TAM combination, suggesting that the combination treatment prevented activation of survival pathways that were otherwise prominent when tumors were treated with single agents (Figure 6.3.D). These results suggest that ER α -XPO1 crosstalk might contribute to drug resistance by regulating differential Akt signaling, which is important for survival and metabolic control.

6.4.4. ER α -XPO1 targeting changes the metabolic phenotype of breast cancer cells from an energetic to a quiescent profile

Our gene expression analysis also pinpointed two of the pathways associated with metabolic regulation: glycolysis and mitochondrial respiration (Figure 6.4.A and B). Since Akt is a major regulator of cell metabolism and TAM responsiveness, we hypothesized that XPO1 modulates Akt activity to rewire metabolism and provide new survival/escape routes to breast cancer cells. We performed a mitochondrial stress test to monitor different parameters of mitochondrial respiration, including basal respiration, proton leak, maximal respiration, spare respiratory capacity, non-mitochondrial respiration, ATP production, and coupling efficiency. The 4-OHT+SEL combination did as well as or slightly better than the individual treatments in reducing different mitochondrial respiration parameters (Figure 6.4.C). We also validated gene expression data related to metabolism using a glycolytic stress test which monitors glycolysis, glycolytic capacity and glycolytic reserve. The 4-OHT+SEL combination was as good as or better than individual treatments in reducing all three parameters (Figure 6.4.D). Next, we tested the individual and combinational effects of 4-OHT and SEL on the metabolic cell phenotypes of TAM-sensitive and TAM-resistant cell lines. Our results showed that although treatment with 4-

OHT alone or SEL alone made all the cell lines less energetic, the cells became even more quiescent with the 4-OHT+SEL treatment (Figure 6.4.E, Figure 6.3.E). Of note, combining SEL with 4-OHT was as effective as combining a PI3K inhibitor (MK2206) with 4-OHT in reducing 4-OHT induced cell viability and increase in mitochondrial respiration (Supplementary Figure 3).

6.4.5. ER α -XPO1 targeting induces autophagic cell death

Our gene expression analyses indicated that gene sets associated with cell cycle and regulation of epithelial cell proliferation were downregulated, whereas gene sets associated with autophagy and cell death were upregulated (Figure 6.5.A). To validate these results, we performed cell cycle analysis in MCF-7 cells and BT474 cells treated with Veh, 4-OHT, SEL or the 4-OHT+SEL combination. The combination treatment was very effective, particularly in BT474 cells, in reducing ratio of S-phase cells (Figure 6.5.B). We also validated autophagic vacuole formation (Figure 6.5.C, D) and an increase in autophagy related proteins in this cell line (Fig. 6.5.E). In addition, we examined different apoptotic caspase activities, PARP cleavage and changes in apoptotic cells in both cell lines. We did not observe any indicator of apoptosis in either of the cell lines at up to 120 hours of treatment (Figure 6.5.G). Finally, treatment of BT474 cells with the autophagy inhibitor chloroquine blocked the 4-OHT+SEL mediated reduction in cell viability (Fig. 6.5.F). Together, these results reveal that co-inhibition of ER α -XPO1 leads to autophagic cell death.

6.5. Discussion

This study is the first of its kind to report the molecular basis of the effectiveness of the novel combination therapy of 4-OHT and SEL in providing sustained tumor regression for ER (+), Tam-resistant tumors. We showed that individual 4-OHT and SEL treatments increased differential Akt signaling in endocrine-resistant cells, but that the combination prevented this activation. We also reported transcriptional and functional consequences of combined ER α and XPO1 targeting. The combination therapy was the most efficient treatment at reversing the expression of endocrine resistance and metastasis-related gene expression programs. In addition, glycolytic and mitochondrial pathways were targeted by this combination. As a result, the 4-OHT+SEL combination effectively blocked glycolysis and mitochondrial respiration and caused cell death by activating autophagy. Our study is the first documented attempt to define the causal

role of metabolic programming in therapy resistant ER (+) tumors and to explore the feasibility of the combined targeting of these pathways to improve the response to endocrine agents and decrease the risk of recurrence.

Targeting XPO1 together with ER α is clinically relevant for several reasons. First, in our published analysis from publicly available tumor datasets [12] and data from patient tumor samples (Fig 6.1.A) mRNA and protein levels of XPO1 were higher in Luminal B subtype tumors which are more likely to recur on endocrine treatments relative to Luminal A subtype. Second, high XPO1 mRNA expression was associated with a poor outcome in women who were treated with TAM [12]. And last, XPO1 was already being targeted in phase 2 and 3 clinical trials (clinicaltrials.gov) by SEL- a specific, covalent, small molecule orally bioavailable inhibitor of XPO1, to treat patients with hematological and solid cancers [24, 25, 26, 45, 46]. SEL is well tolerated with manageable side effects including nausea, fatigue and anorexia that improve over time on treatment. Even in patients that remained on therapy for more than 8 months, no significant cumulative drug toxicities have been identified [16]. Since cancer cells of different tumor types have been shown to be more sensitive to XPO1 inhibition than normal cells [47, 48], combining TAM with SEL potentially offers higher efficacy, specificity and lower toxicity for treatment of endocrine resistant, recurrent ER (+) breast cancer.

We propose that co-targeting ER and XPO1 is an improved therapeutic strategy because inhibiting the proteins in combination caused both a metabolic shift and induced autophagy which together led to prolonged tumor regression. The combined targeting of ER α and XPO1 overcame TAM resistance, modulated cellular signaling to prevent rewiring of tumor cell metabolism and induced cell death by autophagy (Fig. 6.6). We showed that single or combined targeting of ER α and XPO1 caused differential regulation of Akt phosphorylation. The Akt pathway is a master regulator of cancer cell metabolism [49], components of PI3K/AKT/mTOR pathway are mutated in nearly 25% of breast tumors and are associated with drug responses in ER (+) and ER (-) tumors [50, 51, 52]. Estrogens stimulate this pathway to regulate migration and invasion of cancer cells characteristic of ER (+) tumors [53, 54]. In return, mTOR signaling regulates the expression level and activity of ER α and mTOR acts as a coregulator for ER α [55, 56]. Inhibition of PI3K increases ER expression and activity [51, 57], and PI3K inhibitor-endocrine agent combinations were tested with minor success in clinical trials to treat women with endocrine resistant disease (NCT01339442, NCT02273973). Akt signaling regulates both aerobic glycolysis and oxidative

phosphorylation and impact phenotypic features of tumor cells [58]. Our pathway activation assay showed that two established Akt targets, ENOS [59, 60] and PLC γ -1 [61], which are associated with angiogenesis and metastasis [62], had increased phosphorylation levels when cells were treated with SEL only but not with the 4-OHT and SEL combination. A similar pattern of regulation was observed for p53 phosphorylation at Ser15 by SEL and Ser392 by 4-OHT. Interestingly, loss of Ser392 phosphorylation by p70S6K was shown to inhibit autophagy by decreasing expression of ULK1 in the context of oxidative stress [63] whereas the same modification by p38 MAPK activated autophagy [64]. Of note, p38 MAPK phosphorylation was increased only in the 4-OHT+SEL treated cells, which might explain why we observe autophagy only in this treatment condition.

The majority of current research efforts in the therapy resistance field focuses on a delineation of the underlying mechanisms that lead to increased activity of selective signaling pathways. Undoubtedly, interrogating and targeting the end-point kinases in tumors are highly relevant and these studies lead to the development of combination therapies involving PI3K inhibitors or mTOR pathway inhibitors together with endocrine agents. However, resistance to these combination therapies also occurs, and in such cases, the cancer that develops is considerably more aggressive due to hyperactivation of compensatory mitogenic signaling pathways [65]. Moreover, these kinase inhibitors have many adverse side effects. More recently, ER α mutations that decrease sensitivity of the receptor to selective estrogen receptor modulators (SERMs) and selective estrogen receptor degraders (SERDs) were identified in about 15-40% of the metastatic, but not primary, tumors after AI treatment [66, 67, 68, 69, 70]. However, in ER (+) metastatic tumors that occur after TAM treatment, such mutations were not identified [71, 72, 73]. Our proposed treatment approach might prevent/delay the use of AIs and emergence of ESR1 mutations in tumors that recur after endocrine therapies, which is currently a significant clinical challenge.

Our studies described here provide a vast array of novel and important new information that will significantly advance our understanding of adaptive metabolic pathways associated with therapy resistance and cancer cell survival. The nuclear export pathways have not previously been implicated in TAM resistance and given the need for better strategies for selecting patients to receive TAM and improving therapeutic response of relapsed ER α (+) tumors, our results have great potential for uncovering the role of these pathways and their combined targeting in reducing

recurrences and deaths due to metastatic ER (+) tumors. We expect our studies to establish a novel and innovative concept of combined targeting of ER α and XPO1 in TAM resistance that has not been previously explored in the field and will advance a new therapeutic strategy to the clinic. Successful translation of our findings to the clinic will change the treatment regimens for already metastasized patients while on TAM by replacing them with ones that are more effective, less toxic, and will positively impact the patient's survival.

6.6. Figures

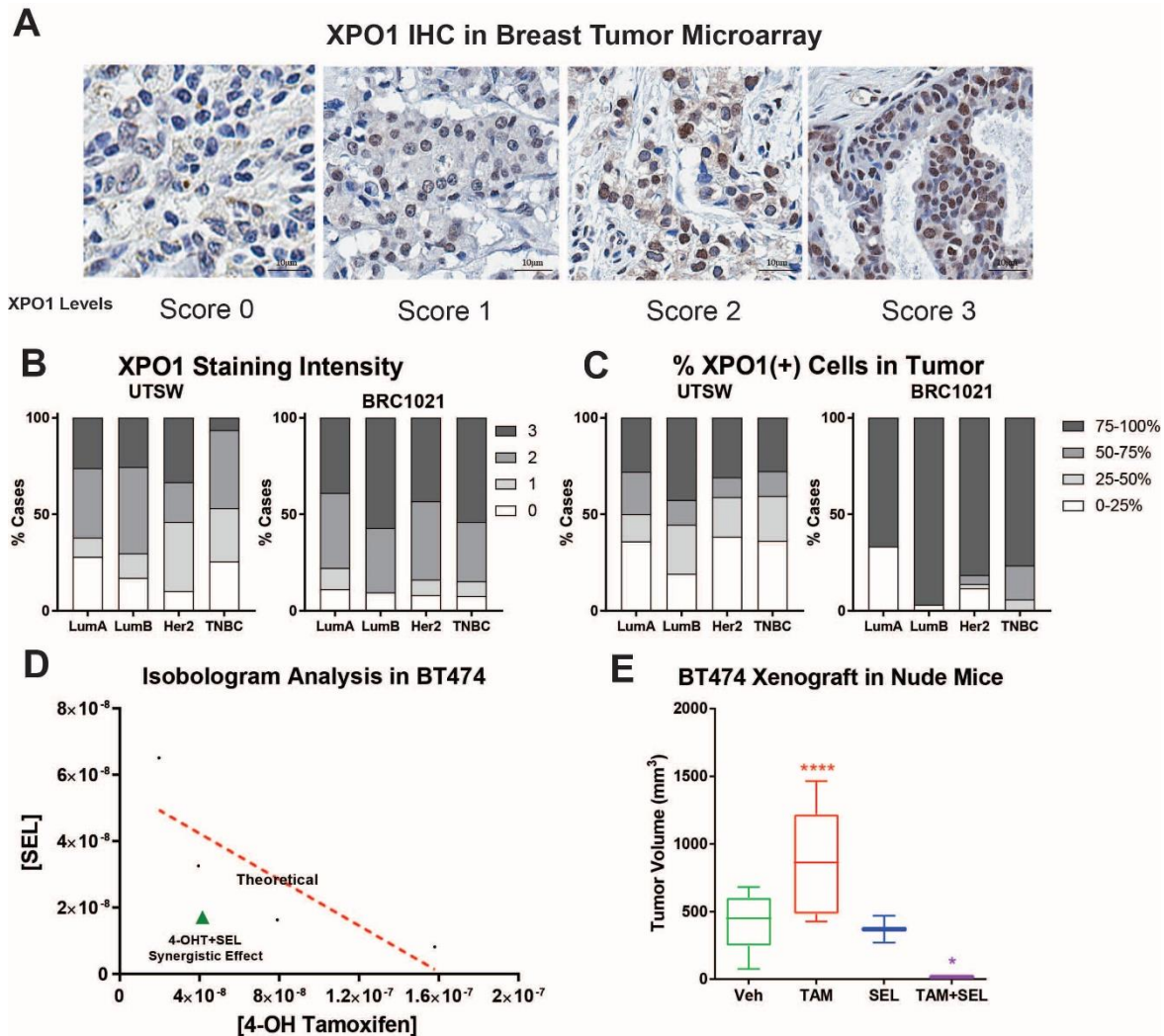


Figure 6.1. 4-OHT+SEL treatment synergistically sustains tumor regression in endocrine resistant Luminal B subtype breast cancer cells. **A)** Verification of XPO-1 protein levels in patient tumor samples (BRC1021 from Pantomics, Inc containing 95 cases with known ER, PR, AR, Her2, p53, EGFR, and Ki67 IHC results). For each core, a score for XPO1 was assigned based on the signal intensity (0=none, 1=low, 2=moderate, and 3=high). **B)** Isobologram analysis of synergy between 4-OHT and SEL in BT474 cells. **C)** BT474 xenograft experiment showing that combined targeting of ER α and XPO1 provided sustained tumor regression. (Adapted from [18]).

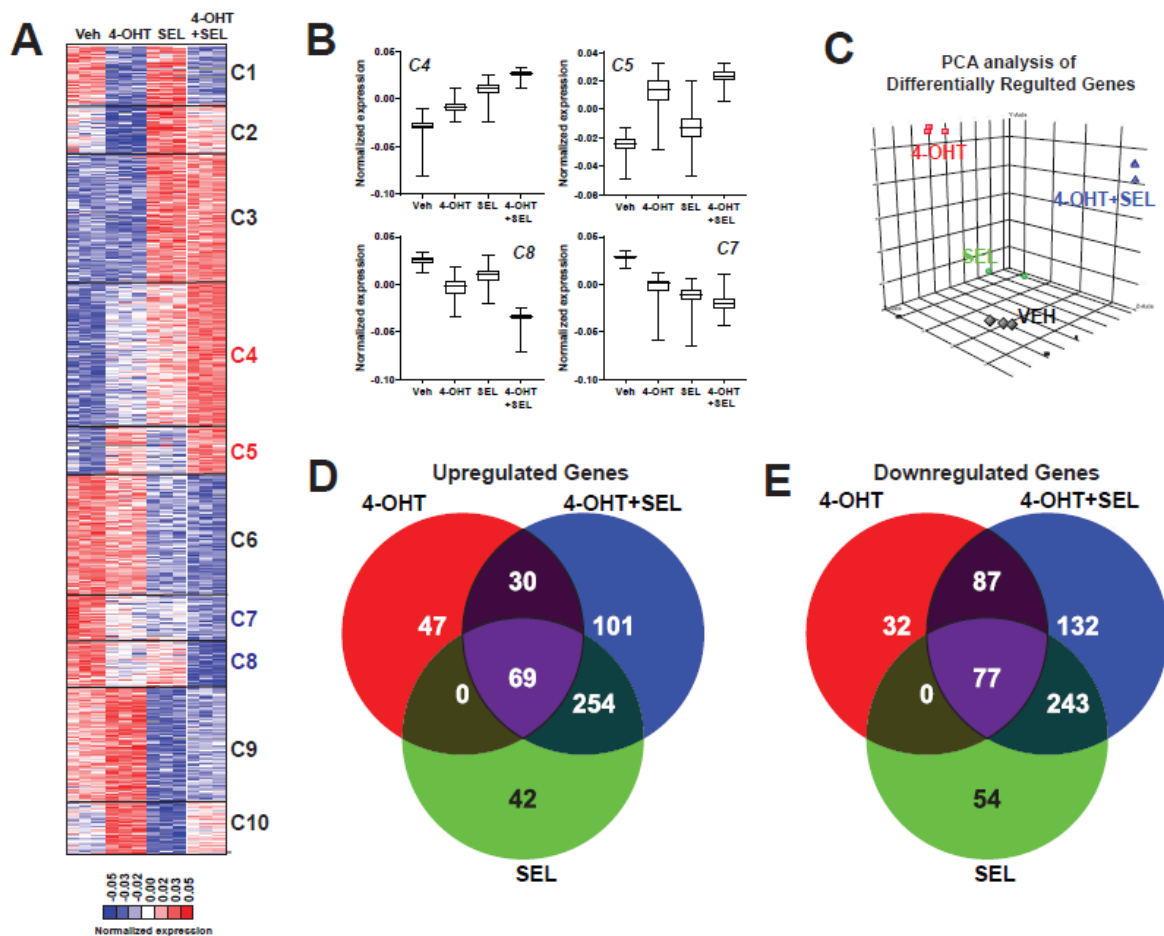


Figure 6.2. The 4-OHT+SEL combination treatment causes gene expression changes distinct from 4-OHT or SEL treatments alone in TAM resistant cell lines. **A)** Hierarchical clustering of differentially expressed genes. **B)** Average expression profiles of clusters that showed differential regulation by the 4-OHT+SEL combination. **C)** PCA analysis Venn diagram analysis of Upregulated **D)** and Downregulated **E)** genes by different ligands.

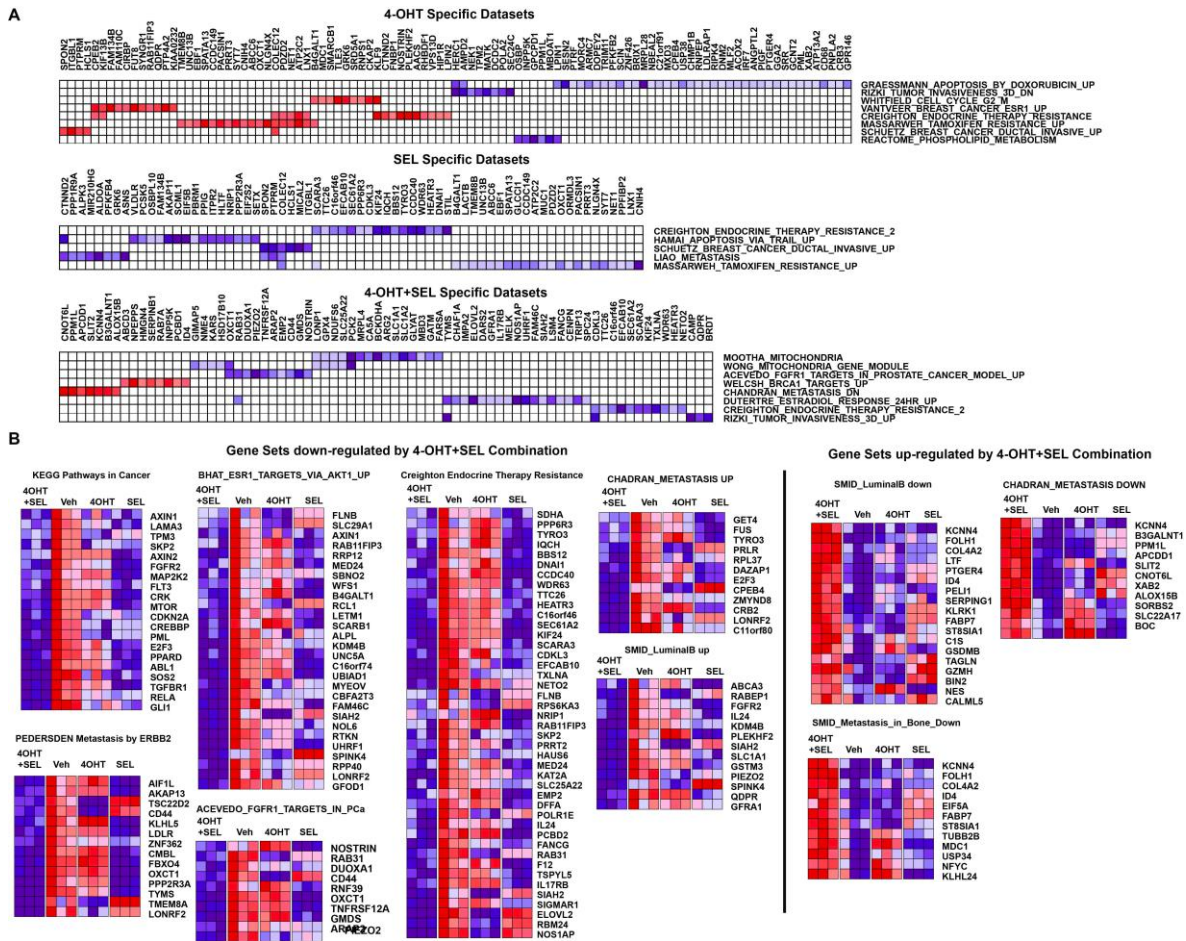


Figure 6.2a. Gene Set Enrichment Analysis (GSEA) analysis to identify functional gene groups that were associated with different treatments. A) Representation of the most activated/inactivated genes in specific pathways in BT474 cells. B) The effect of drug treatments in BT474 cells. Groups were created based on different pathways.

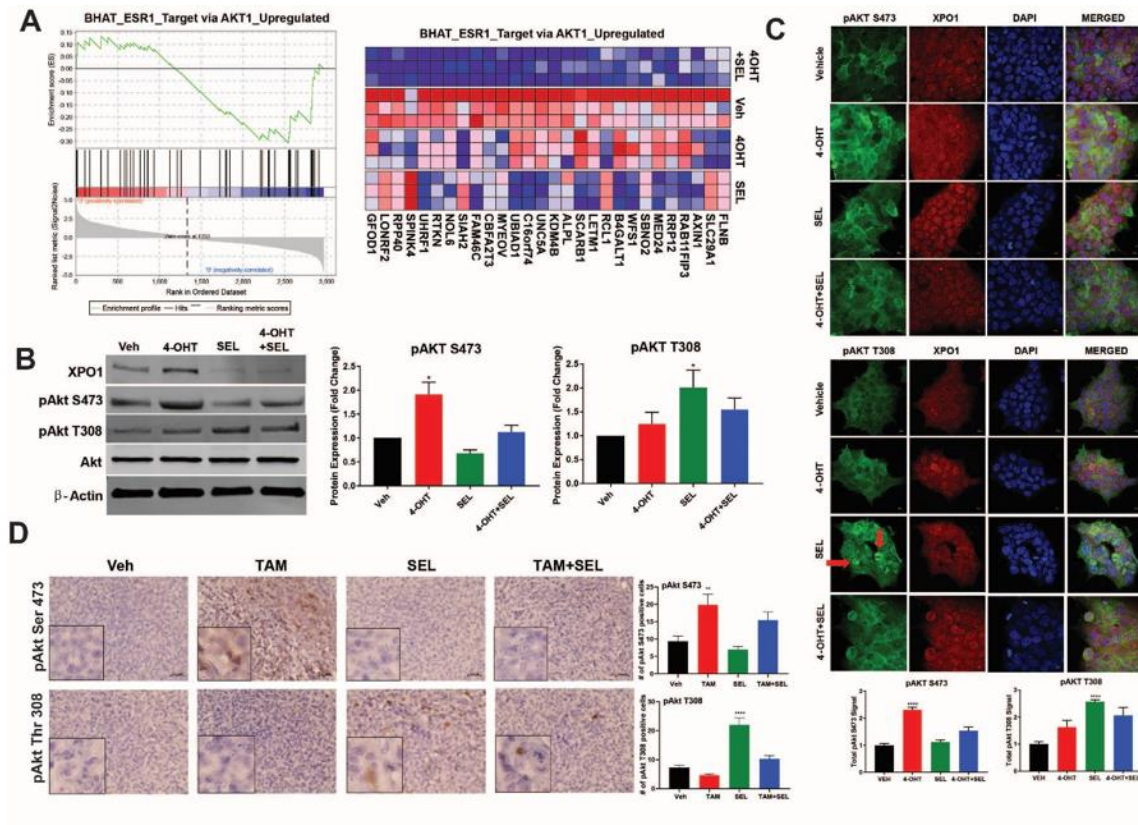


Figure 6.3. XPO1 inhibition modulates differential Akt phosphorylation in TAM-resistant cells and tumor xenografts. **A)** GSEA analysis of RNA-Seq data depicting regulation of Akt signaling by the 4-OHT+SEL combination. ER α -XPO1 targeting changed XPO1 and pAkt protein expressions in both TAM-sensitive and TAM-resistant breast cancer cell lines. Western blot analysis **B)** and immunofluorescence analysis **C)** of BT474 cells treated with 4-OHT (10⁻⁶ M) and SEL (10⁻⁷ M) for 24 hours alone and in combination. Protein expression was quantified and is shown in bar graphs. A one-way analysis of variance (ANOVA) model was used for statistical significance of treatment effect and values were presented as mean $\pm$ SEM from three independent experiments (* p < 0.05, ** p < 0.01). **D)** IHC staining of pAkt T308 and pAkt S473 in tumor samples obtained from BT474 xenografts received individual and combined TAM and SEL treatments (from Fig. 6.1.C). In the figure, red-brownish staining represents pAkt T308 and pAkt S473 proteins. This figure showed that individual 4-OHT and SEL treatments activated two different phosphorylation sites of Akt protein. The top panel indicated that SEL treatment activated pAkt T308 phosphorylation in BT474 tumor xenografts. The bottom panel showed that TAM treatment activated pAkt S473 phosphorylation in BT474 tumor xenografts. Quantification of positive staining was performed digitally by selecting four independent regions on the sample slides and the results were given in the corresponding graphs. Statistical significance was presented as mean $\pm$ SEM by using one-way analysis of variance (ANOVA) model (* p < 0.05, *** p < 0.001).

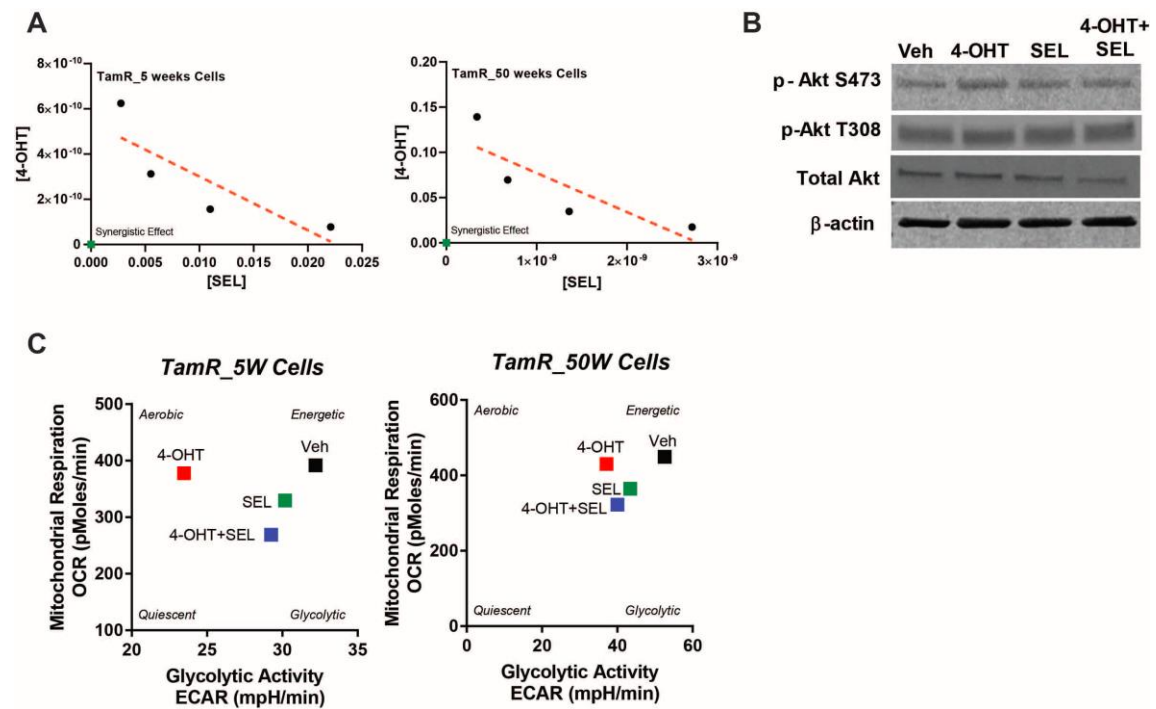


Figure 6.3a. Differential Akt phosphorylation with individual or combined drug treatments in MCF-7 TAM^R cells. A) Isobologram dose-response graphs of TamR_5 weeks and TamR_50 weeks cells. **B)** Western blot analysis of phosphorylated and total Akt protein in TamR_5 week's cells. **C)** Seahorse metabolic cell phenotype analysis of TamR_5 weeks and TamR_50 week's cells.

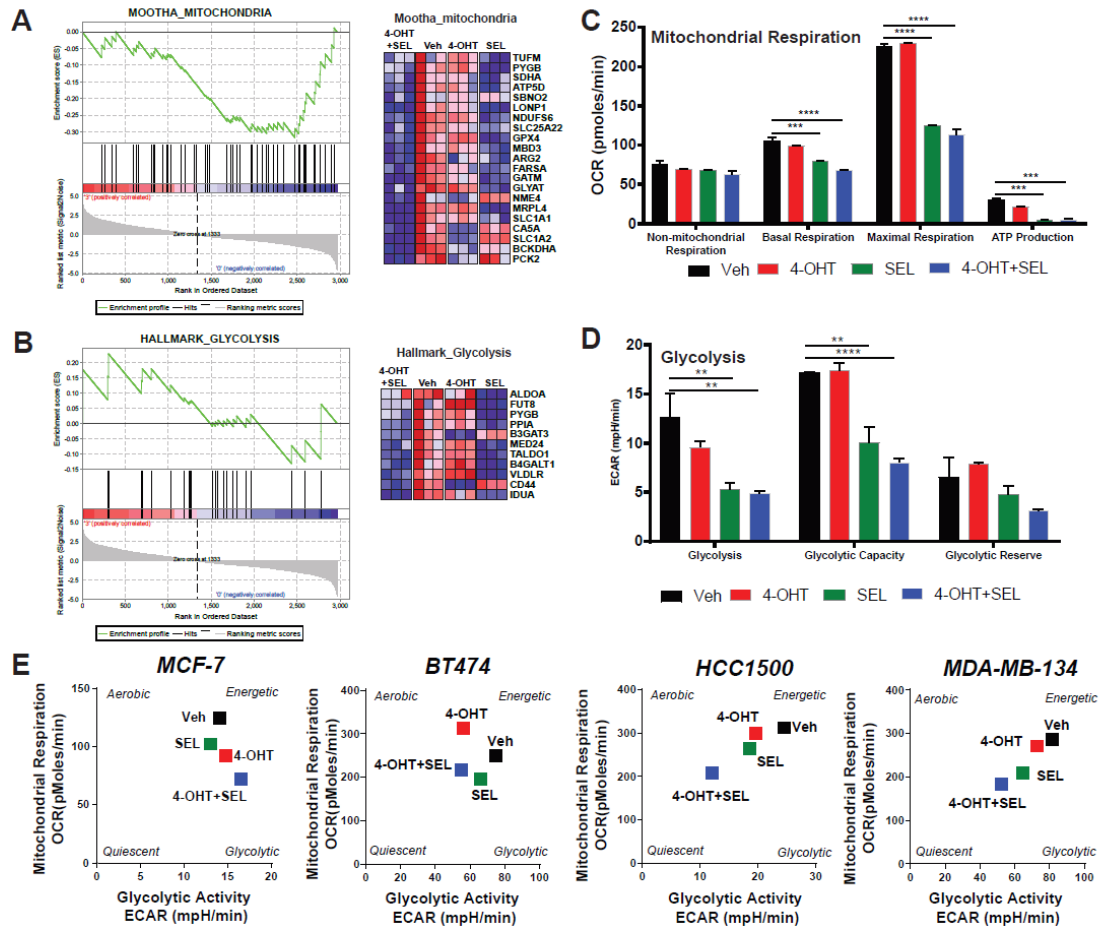


Figure 6.4. ER α -XPO1 targeting changes the metabolic phenotype of breast cancer cells from an energetic to a quiescent profile. GSEA analysis of RNA-Seq data depicting the regulation of glycolytic **A**) and mitochondrial respiration **B**) pathways by the 4-OHT+SEL combination. ER α -XPO1 targeting decreased the glycolytic activity of BT474 cells. Glycolytic functions were determined by the glycolysis stress test. A two-way analysis of variance (ANOVA) model was used for statistical significance of drug treatments alone or in combination and values were presented as mean $\pm$ SEM from two independent experiments and all experiments were performed in triplicates. (* p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001). **C**) ER α -XPO1 targeting decreased the mitochondrial activity of BT474 cells. Mitochondrial activity parameters were measured by the mitochondrial stress test. A two-way analysis of variance (ANOVA) model was used for statistical significance calculations and values were presented as mean $\pm$ SEM from three independent experiments (* p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001). Some key parameters of mitochondrial function (Basal respiration, proton leak, maximal respiration, spare respiratory capacity, non-mitochondrial respiration, ATP production, coupling efficiency and spare respiratory capacity) were calculated and presented for each treatment conditions. **E**) Cell phenotype assay resulted in MCF-7, BT474, MDA-MB-134 and HCC1500 cells which were treated with 4-OHT (10^{-6} M) and SEL (10^{-7} M) for 24 hours alone and in combination. Changes in metabolic profile was determined by measuring oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) under basal and stressed conditions with the cell phenotype assay. Values are presented as $\pm$ SEM from 3 independent experiments.

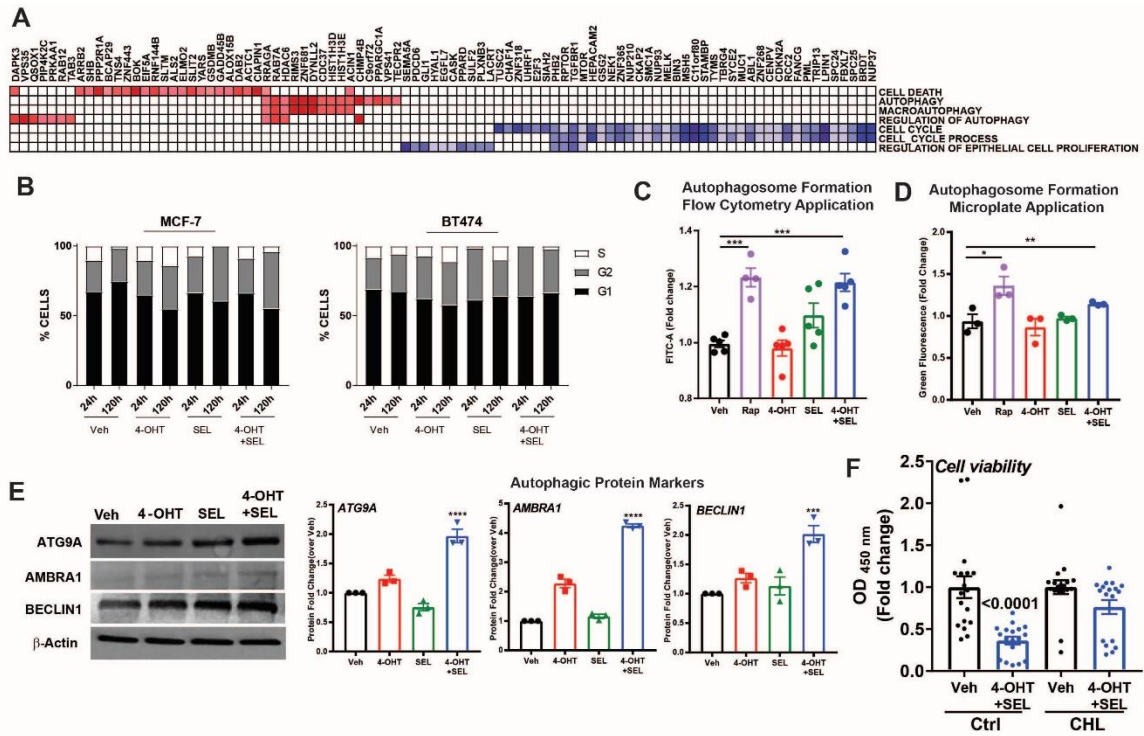


Figure 6.5. ER α -XPO1 targeting induces autophagic cell death. **A)** GSEA analysis of cell proliferation and death pathways regulated by the 4-OHT+SEL combination treatment. **B)** Cell cycle analysis after Veh, 4-OHT, SEL or 4-OHT+SEL treatments in MCF-7 and BT474 cells. LC3-II specific protein expression increased in TAM-resistant BT474 cells in a 24-h period. Comparison of the autophagic markers in BT474 cells treated with 4-OHT (10^{-6} M) and SEL (10^{-7} M) alone and in combination for 24 hours. Autophagosome formation was detected with a colorimetric method **C)** which measures the level of green fluorescent labeled LC3-II specific signal or by FACS analysis of the cells after staining with antibodies specific for LC3-II. **D)** Rapamycin (50 nM) was used as a positive control for autophagy detection. A one-way analysis of variance (ANOVA) model was used for statistical significance of treatments. The experiment was performed in triplicates and values were presented as mean $\pm$ SEM from two independent experiments ($***p < 0.001$). **E)** The western blot analysis showed that the level of autophagic protein markers are higher in BT474 cells treated with both 4-OHT (10^{-6} M) and SEL (10^{-7} M) compared to those treated with individual 4-OHT+SEL treatment. BT474 cells were seeded at a density of 1×10^5 cells/well and treated with 4-OHT (10^{-6} M) and SEL (10^{-7} M) alone and in combination. Protein expressions of autophagic proteins (ATG9A, AMBRA, ULK1 and Beclin-1) were detected with target specific antibodies at 1:1000 dilution. Protein signal was quantitated using Licor Odyssey. A one-way analysis of variance (ANOVA) model was used for statistical significance of treatment and values were presented as mean $\pm$ SEM from three independent experimental repeats. **F)** Autophagy inhibitor chloroquine inhibited 4OHT+SEL induced decrease in cell viability. one-way analysis of variance (ANOVA) model was used for statistical significance of treatments. ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$)

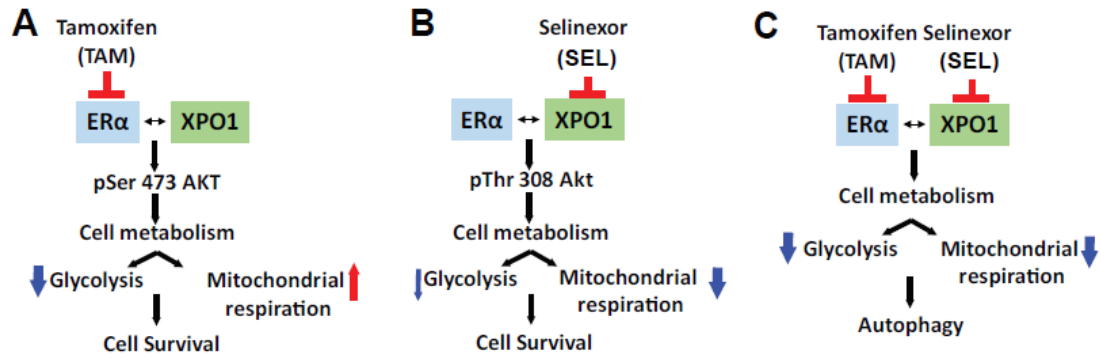


Figure 6.6. Molecular mechanism of sustained tumor regression by combined ER α -XPO1 targeting. **A)** Individual tamoxifen treatment in tamoxifen-resistant breast cancer cells targets cell metabolism through Akt signaling. This effect decreases glycolytic rate and increases mitochondrial respiration and eventually lead cells to cell survival. **B)** Individual selinexor treatment targets XPO1 protein, and changes cellular metabolism through decreasing glycolysis and mitochondrial respiration rate and eventually makes breast cancer cells to survive. **C)** Combined ER α and XPO1 targeting directly affects cellular metabolism and reduces overall cellular metabolism and activates autophagic pathways.

6.7. References

1. B.S. Katzenellenbogen, and J. Frasor, Therapeutic targeting in the estrogen receptor hormonal pathway. *Seminars in oncology* 31 (2004) 28-38.
2. H.S. Rugo, R.B. Rumble, E. Macrae, D.L. Barton, H.K. Connolly, M.N. Dickler, L. Fallowfield, B. Fowble, J.N. Ingle, M. Jahanzeb, S.R.D. Johnston, L.A. Korde, J.L. Khatcheressian, R.S. Mehta, H.B. Muss, and H.J. Burstein, Endocrine Therapy for Hormone Receptor–Positive Metastatic Breast Cancer: *American Society of Clinical Oncology Guideline. Journal of Clinical Oncology* 34 (2016) 3069-3103.
3. K.-D. Yu, J. Wu, Z.-Z. Shen, and Z.-M. Shao, Hazard of Breast Cancer-Specific Mortality among Women with Estrogen Receptor-Positive Breast Cancer after Five Years from Diagnosis: Implication for Extended Endocrine Therapy. *The Journal of Clinical Endocrinology & Metabolism* 97 (2012) E2201-E2209.
4. I. Jatoi, W.F. Anderson, J.-H. Jeong, and C.K. Redmond, Breast Cancer Adjuvant Therapy: Time to Consider Its Time-Dependent Effects. *Journal of Clinical Oncology* 29 (2011) 2301-2304.
5. H.J. Burstein, S. Temin, H. Anderson, T.A. Buchholz, N.E. Davidson, K.E. Gelmon, S.H. Giordano, C.A. Hudis, D. Rowden, A.J. Solky, V. Stearns, E.P. Winer, and J.J. Griggs, Adjuvant Endocrine Therapy for Women With Hormone Receptor–Positive Breast Cancer: American Society of Clinical Oncology Clinical Practice Guideline Focused Update. *Journal of Clinical Oncology* 32 (2014) 2255-2269.
6. M. Colleoni, Z. Sun, K.N. Price, P. Karlsson, J.F. Forbes, B. Thurlimann, L. Gianni, M. Castiglione, R.D. Gelber, A.S. Coates, and A. Goldhirsch, Annual Hazard Rates of Recurrence for Breast Cancer During 24 Years of Follow-Up: Results From the International Breast Cancer Study Group Trials I to V. *J Clin Oncol* 34 (2016) 927-35.
7. H. Pan, R. Gray, J. Braybrooke, C. Davies, C. Taylor, P. McGale, R. Peto, K.I. Pritchard, J. Bergh, M. Dowsett, and D.F. Hayes, 20-Year Risks of Breast-Cancer Recurrence after Stopping Endocrine Therapy at 5 Years. *New England Journal of Medicine* 377 (2017) 1836-1846.
8. I. Jatoi, B.E. Chen, W.F. Anderson, and P.S. Rosenberg, Breast cancer mortality trends in the United States according to estrogen receptor status and age at diagnosis. *J Clin Oncol* 25 (2007) 1683-90.

9. K.D. Yu, J. Wu, Z.Z. Shen, and Z.M. Shao, Hazard of breast cancer-specific mortality among women with estrogen receptor-positive breast cancer after five years from diagnosis: implication for extended endocrine therapy. *J Clin Endocrinol Metab* 97 (2012) E2201-9.
10. E. Kulkoyluoglu, and Z. Madak-Erdogan, Nuclear and extranuclear-initiated estrogen receptor signaling crosstalk and endocrine resistance in breast cancer. *Steroids*.
11. A. Howell, J. Cuzick, M. Baum, A. Buzdar, M. Dowsett, J.F. Forbes, G. Hocht-Boes, J. Houghton, G.Y. Locker, and J.S. Tobias, Results of the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial after completion of 5 years' adjuvant treatment for breast cancer. *Lancet* 365 (2005) 60-2.
12. K. Wrobel, Y.C. Zhao, E. Kulkoyluoglu, K.L. Chen, K. Hieronymi, J. Holloway, S. Li, T. Ray, P.S. Ray, Y. Landesman, A.E. Lipka, R.L. Smith, and Z. Madak-Erdogan, ERalpha-XPO1 Cross Talk Controls Tamoxifen Sensitivity in Tumors by Altering ERK5 Cellular Localization. *Mol Endocrinol* 30 (2016) 1029-1045.
13. J.G. Turner, J.L. Dawson, S. Grant, K.H. Shain, W.S. Dalton, Y. Dai, M. Meads, R. Baz, M. Kauffman, S. Shacham, and D.M. Sullivan, Treatment of acquired drug resistance in multiple myeloma by combination therapy with XPO1 and topoisomerase II inhibitors. *Journal of Hematology & Oncology* 9 (2016) 73.
14. G.L. Gravina, M. Tortoreto, A. Mancini, A. Addis, E. Di Cesare, A. Lenzi, Y. Landesman, D. McCauley, M. Kauffman, S. Shacham, N. Zaffaroni, and C. Festuccia, XPO1/CRM1-selective inhibitors of nuclear export (SINE) reduce tumor spreading and improve overall survival in preclinical models of prostate cancer (PCa). *J Hematol Oncol* 7 (2014) 46.
15. J.E. Neggers, T. Vercruysse, M. Jacquemyn, E. Vanstreels, E. Baloglu, S. Shacham, M. Crochiere, Y. Landesman, and D. Daelemans, Identifying drug-target selectivity of small-molecule CRM1/XPO1 inhibitors by CRISPR/Cas9 genome editing. *Chem Biol* 22 (2015) 107-16.
16. C.I. Chen, M. Gutierrez, P.N. Brown, N. Gabrail, R. Baz, D.E. Reece, M. Savona, S. Trudel, D.S. Siegel, M. Mau-Sorensen, J. Kuruvilla, J.C. Byrd, S. Shacham, T. Rashal, C.Y. Yau, D. McCauley, J.R. Saint-Martin, J. McCartney, Y. Landesman, B. Klebanov, G. Pond, A.M. Oza, M. Kauffman, and M.R. Mirza, Anti Tumor Activity Of Selinexor (KPT-330), A First-In-Class Oral Selective Inhibitor Of Nuclear Export (SINE) XPO1/CRM1

- Antagonist In Patients (pts) With Relapsed / Refractory Multiple Myeloma (MM) Or Waldenstrom's Macroglobulinemia (WM). (2013).
17. A. Bergamaschi, Z. Madak-Erdogan, Y.J. Kim, Y.L. Choi, H. Lu, and B.S. Katzenellenbogen, The forkhead transcription factor FOXM1 promotes endocrine resistance and invasiveness in estrogen receptor-positive breast cancer by expansion of stem-like cancer cells. *Breast Cancer Res* 16 (2014) 436.
 18. K. Wrobel, Y.C. Zhao, E. Kulkoyluoglu, K.L.A. Chen, K. Hieronymi, J. Holloway, S. Li, T. Ray, P.S. Ray, Y. Landesman, A.E. Lipka, R.L. Smith, and Z. Madak-Erdogan, ER α -XPO1 crosstalk controls tamoxifen sensitivity in tumors by altering ERK5 cellular localization. *Molecular Endocrinology* 30 (2016) 1029-1045
 19. D.L. Holliday, and V. Speirs, Choosing the right cell line for breast cancer research. *Breast Cancer Res* 13 (2011) 215.
 20. X. Zhang, X. Mu, O. Huang, Z. Xie, M. Jiang, M. Geng, and K. Shen, Luminal breast cancer cell lines overexpressing ZNF703 are resistant to tamoxifen through activation of Akt/mTOR signaling. *PLoS One* 8 (2013) e72053.
 21. K.L.A. Chen, Y.C. Zhao, K. Hieronymi, B.P. Smith, and Z. Madak-Erdogan, Bazedoxifene and conjugated estrogen combination maintains metabolic homeostasis and benefits liver health. *PLoS One* 12 (2017) e0189911.
 22. Z. Madak-Erdogan, S.H. Kim, P. Gong, Y.C. Zhao, H. Zhang, K.L. Chambliss, K.E. Carlson, C.G. Mayne, P.W. Shaul, K.S. Korach, J.A. Katzenellenbogen, and B.S. Katzenellenbogen, Design of pathway preferential estrogens that provide beneficial metabolic and vascular effects without stimulating reproductive tissues. *Sci Signal* 9 (2016) ra53.
 23. Y.C. Zhao, and Z. Madak Erdogan, Systems Biology of Metabolic Regulation by Estrogen Receptor Signaling in Breast Cancer. *J Vis Exp* (2016).
 24. T.B. Alexander, N.J. Lacayo, J.K. Choi, R.C. Ribeiro, C.-H. Pui, and J.E. Rubnitz, Phase I Study of Selinexor, a Selective Inhibitor of Nuclear Export, in Combination With Fludarabine and Cytarabine, in Pediatric Relapsed or Refractory Acute Leukemia. *Journal of Clinical Oncology* 34 (2016) 4094-4101.
 25. M.M. Gounder, A. Zer, W.D. Tap, S. Salah, M.A. Dickson, A.A. Gupta, M.L. Keohan, H.H. Loong, S.P. D'Angelo, S. Baker, M. Condry, K. Nyquist-Schultz, L. Tanner, J.P.

- Erinjeri, F.H. Jasmine, S. Friedlander, R. Carlson, T.J. Unger, J.-R. Saint-Martin, T. Rashal, J. Ellis, M. Kauffman, S. Shacham, G.K. Schwartz, and A.R. Abdul Razak, Phase IB Study of Selinexor, a First-in-Class Inhibitor of Nuclear Export, in Patients With Advanced Refractory Bone or Soft Tissue Sarcoma. *Journal of Clinical Oncology* 34 (2016) 3166-3174.
26. A.R.A. Razak, M. Mau-Soerensen, N.Y. Gabrail, J.F. Gerecitano, A.F. Shields, T.J. Unger, J.R. Saint-Martin, R. Carlson, Y. Landesman, D. McCauley, T. Rashal, U. Lassen, R. Kim, L.-A. Stayner, M.R. Mirza, M. Kauffman, S. Shacham, and A. Mahipal, First-in-Class, First-in-Human Phase I Study of Selinexor, a Selective Inhibitor of Nuclear Export, in Patients With Advanced Solid Tumors. *Journal of Clinical Oncology* 34 (2016) 4142-4150.
 27. A.M. Bolger, M. Lohse, and B. Usadel, Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30 (2014) 2114-2120.
 28. D.R. Zerbino, P. Achuthan, W. Akanni, M.R. Amode, D. Barrell, J. Bhai, K. Billis, C. Cummins, A. Gall, C.G. Girón, L. Gil, L. Gordon, L. Haggerty, E. Haskell, T. Hourlier, O.G. Izuogu, S.H. Janacek, T. Juettemann, J.K. To, M.R. Laird, I. Lavidas, Z. Liu, J.E. Loveland, T. Maurel, W. McLaren, B. Moore, J. Mudge, D.N. Murphy, V. Newman, M. Nuhn, D. Ogeh, C.K. Ong, A. Parker, M. Patricio, H.S. Riat, H. Schuilenburg, D. Sheppard, H. Sparrow, K. Taylor, A. Thormann, A. Vullo, B. Walts, A. Zadissa, A. Frankish, S.E. Hunt, M. Kostadima, N. Langridge, F.J. Martin, M. Muffato, E. Perry, M. Ruffier, D.M. Staines, S.J. Trevanion, B.L. Aken, F. Cunningham, A. Yates, and P. Flicek, Ensembl 2018. *Nucleic Acids Research* 46 (2018) D754-D761.
 29. A. Dobin, C.A. Davis, F. Schlesinger, J. Drenkow, C. Zaleski, S. Jha, P. Batut, M. Chaisson, and T.R. Gingeras, STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* 29 (2013) 15-21.
 30. Y. Liao, G.K. Smyth, and W. Shi, The Subread aligner: fast, accurate and scalable read mapping by seed-and-vote. *Nucleic Acids Research* 41 (2013) e108-e108.
 31. M.D. Robinson, D.J. McCarthy, and G.K. Smyth, edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26 (2010) 139-140.

32. M.E. Ritchie, B. Phipson, D. Wu, Y. Hu, C.W. Law, W. Shi, and G.K. Smyth, limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Research* 43 (2015) e47-e47.
33. B. Phipson, S. Lee, I.J. Majewski, W.S. Alexander, and G.K. Smyth, Robust hyperparameter estimation protects against hypervariable genes and improves power to detect differential expression. *Ann. Appl. Stat.* 10 (2016) 946-963.
34. Y. Benjamini, and Y. Hochberg, Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society. Series B (Methodological)* 57 (1995) 289-300.
35. A. Subramanian, P. Tamayo, V.K. Mootha, S. Mukherjee, B.L. Ebert, M.A. Gillette, A. Paulovich, S.L. Pomeroy, T.R. Golub, E.S. Lander, and J.P. Mesirov, Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. *Proceedings of the National Academy of Sciences* 102 (2005) 15545.
36. V.K. Mootha, C.M. Lindgren, K.-F. Eriksson, A. Subramanian, S. Sihag, J. Lehar, P. Puigserver, E. Carlsson, M. Ridderstråle, E. Laurila, N. Houstis, M.J. Daly, N. Patterson, J.P. Mesirov, T.R. Golub, P. Tamayo, B. Spiegelman, E.S. Lander, J.N. Hirschhorn, D. Altshuler, and L.C. Groop, PGC-1 α -responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nature Genetics* 34 (2003) 267.
37. G. Arpino, C. Gutierrez, H. Weiss, M. Rimawi, S. Massarweh, L. Bharwani, S. De Placido, C.K. Osborne, and R. Schiff, Treatment of human epidermal growth factor receptor 2-overexpressing breast cancer xenografts with multiagent HER-targeted therapy. *J Natl Cancer Inst* 99 (2007) 694-705.
38. C.X. Wang, D.C. Koay, A. Edwards, Z. Lu, G. Mor, I.T. Ocal, and M.P. Digiovanna, In vitro and in vivo effects of combination of Trastuzumab (Herceptin) and Tamoxifen in breast cancer. *Breast Cancer Res Treat* 92 (2005) 251-63.
39. B. Kondov, Z. Milenkovic, G. Kondov, G. Petrushevska, N. Basheska, M. Bogdanovska-Todorovska, N. Tolevska, and L. Ivkovski, Presentation of the Molecular Subtypes of Breast Cancer Detected By Immunohistochemistry in Surgically Treated Patients. *Open Access Macedonian Journal of Medical Sciences* 6 (2018) 961-967.

40. Z. Inic, M. Zegarac, M. Inic, I. Markovic, Z. Kozomara, I. Djuriscic, I. Inic, G. Pupic, and S. Jancic, Difference between Luminal A and Luminal B Subtypes According to Ki-67, Tumor Size, and Progesterone Receptor Negativity Providing Prognostic Information. *Clinical Medicine Insights. Oncology* 8 (2014) 107-111.
41. A. Bergamaschi, Z. Madak-Erdogan, Y.J. Kim, Y.L. Choi, H. Lu, and B.S. Katzenellenbogen, The forkhead transcription factor FOXM1 promotes endocrine resistance and invasiveness in estrogen receptor-positive breast cancer by expansion of stem-like cancer cells. *Breast Cancer Res* 16 (2014) 436.
42. Z. Madak-Erdogan, T.H. Charn, Y. Jiang, E.T. Liu, J.A. Katzenellenbogen, and B.S. Katzenellenbogen, Integrative genomics of gene and metabolic regulation by estrogen receptors alpha and beta, and their coregulators. *Mol Syst Biol* 9 (2013) 676.
43. J.M. Giltane, K.E. Hutchinson, T.P. Stricker, L. Formisano, C.D. Young, M.V. Estrada, M.J. Nixon, L. Du, V. Sanchez, P.G. Ericsson, M.G. Kuba, M.E. Sanders, X.J. Mu, E.M. Van Allen, N. Wagle, I. Mayer, V. Abramson, H. Gómez, M. Rizzo, W. Toy, S. Chandarlapaty, E.L. Mayer, J. Christiansen, D. Murphy, K. Fitzgerald, K. Wang, J.S. Ross, V.A. Miller, P.J. Stephens, R. Yelensky, L. Garraway, Y. Shyr, I. Meszoely, J.M. Balko, and C.L. Arteaga, Genomic profiling of ER+ breast cancers after short-term estrogen suppression reveals alterations associated with endocrine resistance. *Science translational medicine* 9 (2017) eaai7993.
44. L. Formisano, Y. Lu, V.M. Jansen, J.A. Bauer, A.B. Hanker, M.E. Sanders, P. González-Ericsson, S. Kim, M. Arnedos, F. André, and C.L. Arteaga, Abstract 1008: Gain-of-function kinase library screen identifies FGFR1 amplification as a mechanism of resistance to antiestrogens and CDK4/6 inhibitors in ER+ breast cancer. *Cancer Research* 77 (2017) 1008-1008.
45. P. Ranganathan, T. Kashyap, X. Yu, X. Meng, T.-H. Lai, B. McNeil, B. Bhatnagar, S. Shacham, M. Kauffman, A.M. Dorrance, W. Blum, D. Sampath, Y. Landesman, and R. Garzon, XPO1 Inhibition using Selinexor Synergizes with Chemotherapy in Acute Myeloid Leukemia by Targeting DNA Repair and Restoring Topoisomerase II α to the Nucleus. *Clinical Cancer Research* 22 (2016) 6142-6152.
46. J. Kuruvilla, M. Savona, R. Baz, P.M. Mau-Sorensen, N. Gabrail, R. Garzon, R. Stone, M. Wang, L. Savoie, P. Martin, I. Flinn, M. Jacoby, T.J. Unger, J.-R. Saint-Martin, T. Rashal,

- S. Friedlander, R. Carlson, M. Kauffman, S. Shacham, and M. Gutierrez, Selective inhibition of nuclear export with selinexor in patients with non-Hodgkin lymphoma. *Blood* 129 (2017) 3175-3183.
47. R.A. Salas Fragomeni, H.W. Chung, Y. Landesman, W. Senapedis, J.R. Saint-Martin, H. Tsao, K.T. Flaherty, S. Shacham, M. Kauffman, and J.C. Cusack, CRM1 and BRAF inhibition synergize and induce tumor regression in BRAF-mutant melanoma. *Mol Cancer Ther* 12 (2013) 1171-9.
 48. J.G. Turner, D.C. Marchion, J.L. Dawson, M.F. Emmons, L.A. Hazlehurst, P. Washausen, and D.M. Sullivan, Human multiple myeloma cells are sensitized to topoisomerase II inhibitors by CRM1 inhibition. *Cancer Res* 69 (2009) 6899-905.
 49. D. Hanahan, and Robert A. Weinberg, Hallmarks of Cancer: The Next Generation. *Cell* 144 (2011) 646-674.
 50. S. Yndestad, E. Austreid, I.R. Svanberg, S. Knappskog, P.E. Lønning, and H.P. Eikesdal, Activation of Akt characterizes estrogen receptor positive human breast cancers which respond to anthracyclines. *Oncotarget* 8 (2017) 41227-41241.
 51. A. Bosch, Z. Li, A. Bergamaschi, H. Ellis, E. Toska, A. Prat, J.J. Tao, D.E. Spratt, N.T. Viola-Villegas, P. Castel, G. Minuesa, N. Morse, J. Rodón, Y. Ibrahim, J. Cortes, J. Perez-Garcia, P. Galvan, J. Grueso, M. Guzman, J.A. Katzenellenbogen, M. Kharas, J.S. Lewis, M. Dickler, V. Serra, N. Rosen, S. Chandarlapaty, M. Scaltriti, and J. Baselga, PI3K inhibition results in enhanced estrogen receptor function and dependence in hormone receptor-positive breast cancer. *Science Translational Medicine* 7 (2015).
 52. J. Bostner, E. Karlsson, M.J. Pandiyan, H. Westman, L. Skoog, T. Fornander, B. Nordenskjöld, and O. Stål, Activation of Akt, mTOR, and the estrogen receptor as a signature to predict tamoxifen treatment benefit. *Breast cancer research and treatment* 137 (2013) 397-406.
 53. K. Zhou, P. Sun, Y. Zhang, X. You, P. Li, and T. Wang, Estrogen stimulated migration and invasion of estrogen receptor-negative breast cancer cells involves an ezrin-dependent crosstalk between G protein-coupled receptor 30 and estrogen receptor beta signaling. *Steroids* 111 (2016) 113-120.

54. G. Zhang, Upregulation of estrogen receptor mediates migration, invasion and proliferation of endometrial carcinoma cells by regulating the PI3K/AKT/mTOR pathway. *Oncology Reports* (2013) 1175-1182.
55. A. Alayev, R.S. Salamon, S.M. Berger, N.S. Schwartz, R. Cuesta, R.B. Snyder, and M.K. Holz, mTORC1 directly phosphorylates and activates ERalpha upon estrogen stimulation. *Oncogene* 35 (2016) 3535-43.
56. A. Shrivastav, M. Bruce, D. Jaksic, T. Bader, S. Seekallu, C. Penner, Z. Nugent, P. Watson, and L. Murphy, The mechanistic target for rapamycin pathway is related to the phosphorylation score for estrogen receptor-alpha in human breast tumors in vivo. *Breast Cancer Res* 16 (2014) R49.
57. E. Toska, H.U. Osmanbeyoglu, P. Castel, C. Chan, R.C. Hendrickson, M. Elkabets, M.N. Dickler, M. Scaltriti, C.S. Leslie, S.A. Armstrong, and J. Baselga, PI3K pathway regulates ER-dependent transcription in breast cancer through the epigenetic regulator KMT2D. *Science* 355 (2017) 1324-1330.
58. R.B. Robey, and N. Hay, Is Akt the “Warburg kinase”?—Akt-energy metabolism interactions and oncogenesis. *Seminars in cancer biology* 19 (2009) 25.
59. B.J. Michell, J.E. Griffiths, K.I. Mitchelhill, I. Rodriguez-Crespo, T. Tiganis, S. Bozinovski, P.R.O. de Montellano, B.E. Kemp, and R.B. Pearson, The Akt kinase signals directly to endothelial nitric oxide synthase. *Current Biology* 9 (1999) 845-S1.
60. D. Fulton, J.-P. Gratton, T.J. McCabe, J. Fontana, Y. Fujio, K. Walsh, T.F. Franke, A. Papapetropoulos, and W.C. Sessa, Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature* 399 (1999) 597-601.
61. Y. Wang, J. Wu, and Z. Wang, Akt Binds to and Phosphorylates Phospholipase C-γ1 in Response to Epidermal Growth Factor. *Molecular Biology of the Cell* 17 (2006) 2267-2277.
62. L.M. Balz, K. Bartkowiak, A. Andreas, K. Pantel, B. Niggemann, K.S. Zänker, B.H. Brandt, and T. Dittmar, The interplay of HER2/HER3/PI3K and EGFR/HER2/PLC-γ1 signalling in breast cancer cell migration and dissemination. *The Journal of Pathology* 227 (2012) 234-244.

63. Y. Ci, K. Shi, J. An, Y. Yang, K. Hui, P. Wu, L. Shi, and C. Xu, ROS inhibit autophagy by downregulating ULK1 mediated by the phosphorylation of p53 in selenite-treated NB4 cells. *Cell Death Dis* 5 (2014) e1542.
64. B. Liu, Y. Cheng, B. Zhang, H.-j. Bian, and J.-k. Bao, Polygonatum cyrtonema lectin induces apoptosis and autophagy in human melanoma A375 cells through a mitochondria-mediated ROS–p38–p53 pathway. *Cancer Letters* 275 (2009) 54-60.
65. E.C. Dees, and L.A. Carey, Improving endocrine therapy for breast cancer: it's not that simple. *J Clin Oncol* 31 (2013) 171-3.
66. P.G. Alluri, C. Speers, and A.M. Chinnaiyan, Estrogen receptor mutations and their role in breast cancer progression. *Breast Cancer Res* 16 (2014) 494.
67. D. Chu, C. Paoletti, C. Gersch, D. VanDenBerg, D. Zabransky, R. Cochran, H.Y. Wong, P. Valda Toro, J. Cidado, S. Croessmann, B. Erlanger, K. Cravero, K. Kyker-Snowman, B. Button, H. Parsons, W.B. Dalton, R. Gillani, A. Medford, K. Aung, N. Tokudome, A. Chinnaiyan, A.F. Schott, D.R. Robinson, K. Jacks, J. Lauring, P.J. Hurley, D.F. Hayes, J.M. Rae, and B.H. Park, ESR1 mutations in circulating plasma tumor DNA from metastatic breast cancer patients. *Clin Cancer Res* (2015).
68. S. Li, D. Shen, J. Shao, R. Crowder, W. Liu, A. Prat, X. He, S. Liu, J. Hoog, C. Lu, L. Ding, O.L. Griffith, C. Miller, D. Larson, R.S. Fulton, M. Harrison, T. Mooney, J.F. McMichael, J. Luo, Y. Tao, R. Goncalves, C. Schlosberg, J.F. Hiken, L. Saied, C. Sanchez, T. Giuntoli, C. Bumb, C. Cooper, R.T. Kitchens, A. Lin, C. Phommaly, S.R. Davies, J. Zhang, M.S. Kavuri, D. McEachern, Y.Y. Dong, C. Ma, T. Pluard, M. Naughton, R. Bose, R. Suresh, R. McDowell, L. Michel, R. Aft, W. Gillanders, K. DeSchryver, R.K. Wilson, S. Wang, G.B. Mills, A. Gonzalez-Angulo, J.R. Edwards, C. Maher, C.M. Perou, E.R. Mardis, and M.J. Ellis, Endocrine-therapy-resistant ESR1 variants revealed by genomic characterization of breast-cancer-derived xenografts. *Cell Rep* 4 (2013) 1116-30.
69. D.R. Robinson, Y.M. Wu, P. Vats, F. Su, R.J. Lonigro, X. Cao, S. Kalyana-Sundaram, R. Wang, Y. Ning, L. Hodges, A. Gursky, J. Siddiqui, S.A. Tomlins, S. Roychowdhury, K.J. Pienta, S.Y. Kim, J.S. Roberts, J.M. Rae, C.H. Van Poznak, D.F. Hayes, R. Chugh, L.P. Kunju, M. Talpaz, A.F. Schott, and A.M. Chinnaiyan, Activating ESR1 mutations in hormone-resistant metastatic breast cancer. *Nat Genet* 45 (2013) 1446-51.

70. W. Toy, Y. Shen, H. Won, B. Green, R.A. Sakr, M. Will, Z. Li, K. Gala, S. Fanning, T.A. King, C. Hudis, D. Chen, T. Taran, G. Hortobagyi, G. Greene, M. Berger, J. Baselga, and S. Chandarlapaty, ESR1 ligand-binding domain mutations in hormone-resistant breast cancer. *Nat Genet* 45 (2013) 1439-45.
71. R. Jeselsohn, R. Yelensky, G. Buchwalter, G. Frampton, F. Meric-Bernstam, A.M. Gonzalez-Angulo, J. Ferrer-Lozano, J.A. Perez-Fidalgo, M. Cristofanilli, H. Gómez, C.L. Arteaga, J. Giltane, J.M. Balko, M.T. Cronin, M. Jarosz, J. Sun, M. Hawryluk, D. Lipson, G. Otto, J.S. Ross, A. Dvir, L. Soussan-Gutman, I. Wolf, T. Rubinek, L. Gilmore, S. Schnitt, S.E. Come, L. Pusztai, P. Stephens, M. Brown, and V.A. Miller, Emergence of Constitutively Active Estrogen Receptor- α Mutations in Pretreated Advanced Estrogen Receptor-Positive Breast Cancer. *Clinical Cancer Research* 20 (2014) 1757-1767.
72. C. Fribbens, B. O'Leary, L. Kilburn, S. Hrebien, I. Garcia-Murillas, M. Beaney, M. Cristofanilli, F. Andre, S. Loi, S. Loibl, J. Jiang, C.H. Bartlett, M. Koehler, M. Dowsett, J.M. Bliss, S.R.D. Johnston, and N.C. Turner, Plasma ESR1 Mutations and the Treatment of Estrogen Receptor-Positive Advanced Breast Cancer. *Journal of Clinical Oncology* 34 (2016) 2961-2968.
73. G. Schiavon, S. Hrebien, I. Garcia-Murillas, R.J. Cutts, A. Pearson, N. Tarazona, K. Fenwick, I. Kozarewa, E. Lopez-Knowles, R. Ribas, A. Nerurkar, P. Osin, S. Chandarlapaty, L.-A. Martin, M. Dowsett, I.E. Smith, and N.C. Turner, Analysis of *ESR1* mutation in circulating tumor DNA demonstrates evolution during therapy for metastatic breast cancer. *Science Translational Medicine* 7 (2015) 313ra182-313ra182.

CHAPTER 7: EXPERIMENTAL PART III:
ROLE OF MITOCHONDRIA AND GLUTAMINE METABOLISM IN THERAPY
RESISTANCE: COMBINED TARGETING OF ESTROGEN RECEPTOR ALPHA AND
EXPORTIN 1 IN ENDOCRINE RESISTANT BREAST CANCERS

7.1. Abstract

Majority of breast cancer specific deaths in women with ER α (+) tumor occur due to metastases that are resistant to endocrine therapy. There is a critical need for novel therapeutic approaches to prevent or delay recurrence of ER α (+) tumors. The objective of this study was to elucidate the role of mitochondrial pathways that are activated in the presence of single therapies and undermine therapy effectiveness in ER (+) breast cancers. In our previous studies, we identified Exportin 1 (XPO1), a nuclear export protein, as an important player in endocrine resistance progression. Selinexor (SEL), an XPO1 antagonist, has been evaluated in multiple later stage clinical trials in patients with relapsed and /or refractory hematological and solid tumor malignancies. Using a combination of transcriptomics, kinase arrays, metabolomics and metabolic flux experiments, we identified glutamine metabolism pathways to be rewired during endocrine resistance. In limited media conditions mimicking nutrient deprived tumor microenvironment, endocrine resistant cells were more dependent on mitochondria for energy production. Their glucose and fatty acid dependency decreased in the presence of SEL and cells were more dependent on glutamine. The effect of glutamine was dependent on conversion of the glutamine to glutamate and mitochondrial complex 1 activity. In order to examine metabolites that might result in the observed phenotype we performed GC/MS whole metabolite profiling and identified amino acid metabolism pathways to be upregulated when cells were treated with SXR. We demonstrated that combined targeting of XPO1 and ER α rewires metabolic pathways and shuts down both glycolytic and mitochondrial pathways that would eventually lead to autophagy. Remodelling metabolic pathways to regenerate new vulnerabilities in endocrine resistant breast tumors is novel, our findings show great promise for uncovering the role ER α -XPO1 crosstalk plays in reducing cancer recurrences.

Keywords: Breast cancer; Combined drug targeting; Metabolic rewiring; Glutamine

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7.2. Introduction

The nuclear hormone estrogen receptor alpha (ER α) is present in approximately 70% of both early and late stage human breast cancers (BCas) [1, 2]. ER α is targeted by endocrine therapies, which are well tolerated and provide long term disease free survival if patients have localized disease [1]. Unfortunately, 10-40% patients with ER+ disease experience recurrence and metastasis within 20 years [3-7]. The recurrence of cancer in ER- patients is higher in the first five years after the diagnosis, yet for ER+ patients there is a substantial long-term risk of death due to recurrent BCa even 20 years after the initial diagnosis [8]. 5-year relative survival of patients with ER+ metastatic disease is 24%, almost none are cured and each year nearly 28,000 women with recurrent ER+ metastatic tumors die [2, 9, 10].

Extended endocrine therapy combined with agents, such as CDK inhibitors or mTOR inhibitors is the most current treatment option for ER+ metastatic cancer [2]. Yet therapy-resistance develops during the course of initial and sequential treatment in almost all of patients due to alterations of sequence or copy number for critical genes such as ESR1, PIK3CA or CCND1, signaling pathways such as Her2, PI3K or FGF signaling, and drug uptake and metabolism [5, 11]. Thus, acquired therapy resistance remains a significant clinical problem, a problem that indicates a critical need for therapeutic approaches that include novel, targetable mechanism-based strategies by which a sustained regression of ER+ metastatic tumors can be achieved or tumors can be kept therapy responsive. Identification of pathways that promote therapy resistance and metastasis provide an opportunity for developing more effective therapy regimens.

In our previously published studies we identified a group of nuclear transport proteins, including XPO1, which are upregulated in tamoxifen resistant cell lines and tumors. XPO1 up-regulation caused nuclear export of factors that are required in the nucleus for ER α -dependent transcriptional responses [12, 13]. We previously showed that XPO1 mRNA and protein expression are higher in Luminal B subtype of tumors that are more refractory to endocrine treatments [12] [14]. High XPO1 mRNA expression is associated with a poor outcome in women who are treated with TAM [12]. In TAM-resistant cells, combined targeting of ER α and XPO1 prevented 4-OH-Tam induced cell proliferation and anchorage independent growth [12] and induced autophagy [14]. In BT474 tumor xenografts, TAM stimulated tumor growth whereas SEL inhibited growth. However, resistance to SEL developed within 3 weeks after therapy was stopped

[12]. The combination of SEL and TAM not only caused a faster and more complete regression of tumors, but also the regression was sustained. In our follow-up studies, we showed that XPO1 induce endocrine resistance by modulating PI3K/Akt signaling and increasing survival of BCa cells by inducing metabolic rewiring. Combined ER α and XPO1 targeting effectively shut-down cell survival mechanisms to provide sustained tumor regression [14]. However, we don't know if we can combine XPO1 inhibitors with other therapies currently used in the clinics for treatment of metastatic BCas, which is the focus of this study.

In this study, we elucidated the causes and mechanisms of therapy resistance by focusing on the role of metabolic changes in tumor cells to adapt to and survive in premetastatic niches (PMNs) and test therapeutic potential of combining XPO1 inhibitors with currently utilized clinical drugs to prevent therapy resistance during metastatic tumor treatment. Our studies established XPO1 as a target, whose inhibition would enhance the effectiveness of current therapies, by preventing metabolic adaptation and improved survival in metastatic organ sites during sequential therapies.

7.3. Materials and Methods

7.3.1. Cell Culture, Ligand Treatments

All cell lines were obtained from American Type Culture Collection (Waltham, MA, USA). MCF-7 parental cells were cultured in Roswell Park Memorial Institute (RPMI-1640) with NEAA salts (Sigma, St Louis, MO, USA), supplemented with 5% fetal bovine serum (FBS) (HyClone, Logan, UT, USA), 100 μ g/mL penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) and 50 mg/ml Gentamicin (Gibco, Gaitersburg, MD). MCF7-ESR1^{D537S} and MCF7-ESR1^{Y537S} cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with NEAA salts (Sigma, St Louis, MO, USA), supplemented with 5% FBS, 100 μ g/mL penicillin/streptomycin and 50 mg/ml Gentamicin (Gibco, Gaitersburg, MD). Human breast cancer cell lines BT474, HCC-1500 and MDA-MB-134, which are 4-OH-Tam-resistant, were used as a model of de novo resistance [12, 15, 16]. BT474 were cultured in ATCC recommended Hybri-care medium with 10% inactivated FBS, sodium bicarbonate and antibiotics. MDA-MB-134 cells were cultured in Leibovitz's medium with 20% FBS (HyClone), and 100 μ g/mL penicillin/streptomycin (Invitrogen). HCC-1500 cells were cultured in ATCC-formulated RPMI-1640 media with 10% FBS, sodium bicarbonate and antibiotics. Acquired resistance was studied from resistance

progression cell lines previously derived and characterized by long-term exposure of parental MCF-7 cells to 4-OHT (MCF-7 TamR) [12, 15]. These cells retain ER α expression, do not require E2 for growth, are not growth inhibited by SERMs and 4-OHT stimulates their growth [12, 15]. For drug treatments, cells were treated with media including 10⁻⁶ M 4-hydroxytamoxifen (4-OHT) (Sigma), 10⁻⁶ M Fulvestrant (Fulv) (Sigma), 10⁻⁶ M Palbociclib (Palb) (Sigma) alone or in combination with and 10⁻⁷ M Selinexor (SEL) (Selleckchem, Houston, TX, USA) for 24 hours. For other drug treatments, SR-18292 (*PPARGC1a* inhibitor) (Selleckchem) and ZLN005 (*PPARGC1a* activator) (Selleckchem) were used at concentrations between 1x10⁻⁵ M, 5x10⁻⁶ M and 1x10⁻⁶ M. MK2206 (*PI3K* inhibitor) was used at a concentration of 5x10⁻⁵ M [17-19].

7.3.2. Cell Viability Assays and siRNA Knock Down

Cells were seeded at a density of 2x10³ cells/well in a 96-well plate, and were grown overnight in corresponding media with/without phenol red. On day 1, cells were treated with drugs alone and in combination. Treatment was repeated again on day 4. On day 7, WST1 assay (Roche Molecular Systems, Inc., Pleasanton, CA, USA) was used to quantify cell proliferation ratio. Absorbance was measured at 450 nm using a Cytation5 plate reader (BioTek, Winooski, VT, USA). Statistical analyses were done by using Graphpad© Prism8 software (GraphPad Software Inc., La Jolla, CA, USA, www.graphpad.com). All experiment conditions had six technical repeats and experiments were repeated at least for three times.

For siRNA knock down experiments, cells were seeded at a concentration of 2x10³ cells/well in corresponding treatment media without phenol red and antibiotics. Next day, cells were incubated with 25 nM siRNAs targeting *PPARGC1a* (Dharmacon, ON-TARGET plus SMARTpool Human *PPARGC1a* (10891)) and *MDH1* (Dharmacon, ON-TARGET plus SMARTpool Human *MDH1* (4190)) genes. Target sequences of both siRNAs were given as below (J-005111-05, *PPARGC1a*: GAGAAUUCAUGGAGCAAUA), (J-005111-06, *PPARGC1a*: GAAGAGCGCCGUGUGAUUU), (J-005111-07, *PPARGC1a*: ACACUCAGCUAAGUUAUAA), (J-005111-08, *PPARGC1a*: GCAGGUAACAUGUUCCCUA), (J-009264-09, *MDH1*: CCUUAGAUAAAUACGCCAA), (J-009264-10, *MDH1*: GGGAGAAUUGUCACGACU), (J-009264-11, *MDH1*: CAACAGAUAAAGAAGACGU), (J-009264-12, *MDH1*: AGGUUAUUGUUGUGGGUAA). Cells were incubated with the media containing siRNAs for 48 hours. Cells were treated with

different endocrine agents alone or in combination with SEL two times in corresponding in antibiotic-free and phenol red-free media on day 3 and day 6. On day 9, cell viability was measured and analyzed as explained above.

For limited media condition mimicking experiments, MCF-7, MCF7-ESR1^{Y537S}, MCF7-ESR1^{D538G}, and BT474 cells were seeded at a density of 2×10^3 cells/well in 96 well plates. Next day, cells were treated with 10^{-6} M 4-OHT and 10^{-7} M SEL alone and on combination in different limited media conditions (Plain media: containing only 7.5% sodium bicarbonate, Low glucose media: 1 g/L D-Glucose, High glucose media: 4.5 g/L D-Glucose, Glutamine-only media: 2 mM L-Glutamine. Treatments were repeated on day 4. On day 7, cell viability was measured with WST-1 reagent.

For individual amino acid treatments, parental MCF-7, MCF7-ESR1^{Y537S}, MCF7-ESR1^{D538G}, and BT474 cells were seeded at a density of 2×10^3 cells/well in 96 well plates. On day 1, cells were treated with corresponding media containing 2 mM individual amino acids. Amino acid treatments were repeated on day 4. Absorbance was measured at 450 nm using a Cytation5 plate reader (BioTek, Winooski, VT, USA).

7.3.3. 3-D Cell Culture Models

To culture cells on different surface of matrix hydrogels (XYLYX TissueSpec® Bone, Liver and Lung) in a 6mg/ml solution, 2×10^3 cells/well were mixed with corresponding tissue culture matrix in growth media and seeded on 96-well plates. Then, they were incubated at 37°C in a humidified environment with 5% CO₂ for at least 45 minutes to achieve gelation. Cells were treated with media containing different endocrine agents alone or in combination with SEL every Monday and Friday in line with clinical practice of endocrine treatments for 40 days. Oncosphere formations were visualized and captured by Invitrogen™ EVOS™ XL Core Light Microscope (Waltham, MA, USA). Number of oncospheres were counted automatically after each drug treatments with OpenCFU colony counting software (<http://opencfu.sourceforge.net/>) and all statistical analyses were completed by using GraphPad® Prism8 software.

7.3.4. Mutation Correlation Analysis

cBioPortal for Cancer Genomics (<https://www.cbioportal.org/>) website was used to see the correlation between XPO1 and other most common mutation patterns. To identify co-expression

profiles of *XPO1* and *PIK3CA*, *XPO1* and *ESR1*, and *XPO1* and *CCND1* in different breast tumors, METABRIC (Nature 2012 & Nature Commun. 2015) and TCGA (Nature, 2012) databases were selected. Selection criteria was narrowed down by selecting ER⁺ tumors. Then, ER⁺ tumors with *PIK3CA*, *ESR1* and *CCND1* mutations were selected individually, and tumors carrying *XPO1* and these common mutations were specifically selected [20, 21].

7.3.5. Western Blot Analysis in Cell Lines

Cells were seeded on 30 mm cell culture plates at 1×10^5 cells/well concentration in corresponding treatment media without phenol red which contains 5% FBS. Cells were treated with media containing endocrine agent alone and in combination overnight. Next day, cells were collected in Lysis buffer (0.5 M EDTA, 1 M TrisHCl pH 8.1, 10% SDS, 10% Empigen, ddH₂O) with $1 \times$ Complete Protease Inhibitor (Roche) and $1 \times$ Phosphatase Inhibitor (Thermo Scientific, Waltham, MA, USA). Samples were further processed with sonication and protein concentrations were determined by BCA assay (Thermo Scientific). Then, samples were boiled in SDS-containing loading buffer and were run in 10% precast gels (BioRad) and transferred to nitrocellulose membrane. The membrane was blocked in Blocking Buffer (Odyssey®, Li-Cor, Lincoln, NE, USA) and target proteins were probed with *XPO1* (sc-74454, Santa Cruz Biotechnology, Dallas, TX, USA), p-Akt S473 (#4060, Cell Signaling), pAkt T308 (#13038, Cell Signaling), Akt (#9272, Cell Signaling), PGC1a (#SAB2500781, Sigma-Aldrich) antibodies in 1:1000 dilution and β -actin (Sigma SAB1305546) antibody in 1:10000 dilution. The secondary antibodies were obtained from Odyssey were used at 1:10000 dilution. The membranes were visualized by using Licor Odyssey CLx infrared imaging device and software. All results were repeated two times and the results were normalized according to signal, which was received from β -actin loading control. Representative blots are presented.

7.3.6. Gene Expression Analysis

To identify metabolism related gene sets regulated by SEL only treatment we used our previously published RNA-Seq dataset (GSE112883). RNASeq analysis was performed as previously described [19–21]. Briefly, BT474 cells were treated with Vehicle (Veh, 0.5% EtOH), 10^{-6} M 4-OHT (Sigma), 10^{-6} M Fulvestrant (Sigma), 10^{-6} M Palbociclib (Sigma) alone or in combination with 10^{-7} M Selinexor (SEL) (Selleckchem) for 24 h. Concentrations of ligands are

based on our previously published study [12] and clinical data [22-24]. Total RNA was extracted with TRIzol reagent (Life Technologies, Cancers 2019, 11, 479 4 of 20 Carlsbad, CA, USA) according to the manufacturer's protocol and cleaned using a clean-up kit (QIAGEN, Hilden, Germany). RNA quality was assessed using bioanalyzer. Total RNA from each sample (three per treatment group) were sequenced at the UIUC sequencing center, and data was generated in Fastqc file format to compare the expressions between the four treatment groups. Preprocessing and Quality Control: Fastqc files containing raw RNA sequencing data were trimmed using Trimmomatic (Version 0.38) [25]. Next, the reads were mapped to the Homo sapiens reference genome (GRCh37) from the Ensembl [26] database and aligned using the STAR alignment tool (Version 2.7.0f) [27]. After this, the read counts were generated from SUBREAD (Version 1.6.3) [28] and featured counts were exported for statistical analysis in R. Quality control and normalization was conducted in R using edgeR (Version 3.24.3) [29].

Statistical Analysis and DEGs: Statistical analysis was conducted in R using limma (Version 3.38.3) [30, 31]. Empirical Bayesian statistics were conducted on the fitted model of the contrast matrix. Differentially expressed genes were then determined by fold-change and p-value with Benjamini and Hochberg multiple test correction for each gene, for each treatment relative to the vehicle control. We considered genes with fold-change >1.5 and p-value < 0.05 as statistically significant, differentially expressed. Cluster3 software was used for clustering the differentially expressed genes. Data was visualized using Treeview Java. PCA analysis was performed using StrandNGS (Version 3.1.1). GSEA [32, 33] analysis was used to identify GO terms associated with different treatments.

7.3.7. Metabolomics analysis

BT-474 cells were seeded at a density of 2×10^5 cells/plate in treatment media. Next day, they were treated with 10^{-6} M 4-OHT and 10^{-7} M SEL alone or in combination for 24 hours. After this incubation period, samples were submitted to the Metabolomics Center at UIUC. GC/MS whole metabolite profiling was performed to detect and quantify the metabolites by using Gas chromatography-mass spectrometry (GC/MS) analysis. Metabolites were extracted from samples according to Agilent Inc. application notes. The hentriacontanoic acid was added to each sample as the internal standard prior to derivatization. Metabolite profiles were acquired using an Agilent

GC-MS system (Agilent 7890 gas chromatograph, an Agilent 5975 MSD, and an HP 7683B autosampler). The spectra of all chromatogram peaks were evaluated using the AMDIS 2.71 and a custom-built database with 460 unique metabolites. All known artificial peaks were identified and removed prior data mining. To allow comparison between samples, all data were normalized to the internal standard in each chromatogram. Metabolomics data with sample class annotations (healthy and susceptible) were uploaded to the Statistical Analysis tool of MetaboAnalyst software version 4.0 [34]. Features with more than 50% missing values were removed. Data were normalized based on values from “Healthy” samples. Data were log transformed and scaled using Auto scaling feature. VIP scores for top 25 metabolites that discriminate between healthy and susceptible individuals were calculated and displayed using the Partial Least Squares-Discriminant analysis tool. Heatmap of class averages of 25 metabolites was generated using Heatmap feature using default options for clustering and restricting the data to top 25 metabolites ranked by t-test. Heatmap for abundance of each metabolite and association with class, menopausal status and BMI were generated using Cluster3 and visualized using Java Treeview. We calculated the correlation between all identified circulating factors (metabolite and protein) using the Pearson correlation coefficient using R software (R Core Team, 2015). R Code to calculate correlation coefficients and p-values are available upon request. Correlation coefficients and p-values were clustered using Cluster3 software and visualized using Treeview Java tool.

7.3.8. Seahorse Metabolic Profiling Assays

MCF-7, MCF-7 TamR and BT474 cells were seeded at a density of 5×10^4 and MDA-MB-134 and HCC 1500 cells were seeded at a density of 1×10^5 , and MCF-7 *ESR1*^{D537S} and MCF-7 *ESR1*^{Y537S} cells were seeded at a density of 3×10^4 in corresponding treatment media without phenol red in each well of the XFp Cell Culture miniplates, respectively (Seahorse Bioscience Inc., Billerica, MA, USA). The FCCP concentration was 0.5 μ M for MCF-7, BT474, MDA-MB-134 and HCC 1500 cells. Next day, cells were treated with treatments media containing endocrine agents alone or in combination with SEL overnight and the cartridges were hydrated with the calibration solution and kept in a non-CO₂ incubator at 37 °C overnight. In parallel, a duplicate of each plate was used for cell counting to monitor cell number changes after 24 h of treatments and Seahorse data was normalized to total cell number. On the assay day, cells were washed with XF Base Media without phenol red (Seahorse Bioscience Inc.) supplemented with 10 mM L-glucose,

2 mM L-glutamine (Gibco) and 1 mM sodium pyruvate (Gibco, Waltham, MA, USA). The ECAR (mpH/min) and OCR (pmol/min) values were obtained by using Seahorse XFp Cell Energy Phenotype Test Kit (Seahorse Bioscience Inc.), which were run with Seahorse XFp Analyzer (Seahorse Bioscience Inc.). Experiments were performed in triplicate and repeated at least three times.

For the Seahorse experiments conducted in different extracellular matrix conditions, Seahorse XFp plates were coated with different Native Coat™ ECMs for bone, lung and liver (XYLYX, NY, USA). Then, these 2D coats were incubated at 37°C in a humidified environment with 5% CO² for at least for 1 hour. Cells were seeded in these coated Seahorse plates after removing native coat mixtures. The rest of the treatment and assay processes were applied as explained above.

7.3.9. Luminescence-Based Assays

NAD⁺/NADH-Glo™ Assay (Promega, Madison, WI), NADP⁺/NADPH-Glo™ Assay (Promega, Madison, WI), Reactive oxygen species (ROS)-Glo™ (Promega, Madison, WI), Glutathione (GSH)-Glo™ Assay (Promega, Madison, WI) were run based on same procedure. BT-474 cells were seeded at a concentration of 2x10³ cells/well in 96-well plates in corresponding phenol red-free media. Next day, cells were treated with endocrine agents alone or in combination with SEL in corresponding concentrations, and they were incubated with drugs for 24 hours. All assays were run according to the supplier's instructions for use of products. After 24 hours incubation with drugs, luminescence values were measured using a Cytation5 plate reader (BioTek, Winooski, VT, USA). Standard curve and sample values were analyzed after subtraction of values from blank sample, and all statistical analyses were done by using Graphpad© Prism8 software (GraphPad Software Inc., La Jolla, CA, USA, www.graphpad.com). All experiment conditions had six technical repeats and experiments were repeated in six technical repeats at least for two times.

7.3.10. Statistical Analyses

Data from all studies were analyzed using a one-way analysis of variance (ANOVA) model to compare different ligand effects, a two-way-ANOVA model to compare time-dependent changes. All data was tested for normal distribution using pairwise t tests with a Bonferroni

correction. All data was normally distributed unless otherwise noted. Normally distributed data was analyzed using pairwise t tests with a Bonferroni correction to identify treatments that were significantly different from each other (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). For every main effect that was statistically significant at $\alpha = 0.05$, pairwise t-tests were conducted to determine which ligand treatment levels were significantly different from each other. For these t-tests, the Bonferroni correction was employed to control experiment wise type I error rate at $\alpha = 0.05$ followed by Bonferroni post hoc test. Data that were not normally distributed were analyzed using Mann Whitney test for nonparametric data (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$). Statistical significance was calculated using GraphPad Prism for Windows.

7.3.11. Availability of Data and Materials

Gene expression data is submitted to GEO database under the accession number GSE112883 and going to be available from the day of the acceptance of the manuscript.

7.4. Results

7.4.1. Clinical relevance of combining XPO1 inhibitors with current therapies in metastatic ER (+) tumors

To assess viability of XPO1 as a therapeutic target we utilized TCGA and METABRIC datasets and studied XPO1 mRNA expression with other biomarkers that help clinicians make decisions in the clinic. In both datasets, XPO1 mRNA level correlated with ESR1, PIK3CA and CCND1 mRNAs in ER(+) tumors, as well as in ER(+) tumors with PIK3CA mutations (most common mutation found in breast tumors) or CCND1 amplification, rendering this protein a viable target in tumors with various genomic mutations or amplifications (Figure 7.1.A and 7.1a). To examine pathways that might be affected by XPO1 and ER crosstalk in MCF7 cells with ESR1 mutations, we performed a RNASeq experiment. We treated MCF7-ESR1^{Y537S} cells with 4-OHT, Fulv and Palb in the presence or absence of SEL (Figure 7.1.B). For all treatments, cotreatment with SEL decreased genes associated with cell cycle, and increased genes associated with apoptosis and p53 signaling (Figure 7.1.C). Validating our results from gene expression analysis, in endocrine resistant MCF7-ESR1^{Y537S}, MCF7-ESR1^{D538G}, HCC1500 and BT474 cells combining SEL with Fulv, 4-OHT Tam or Palb reduced agonist activities of these drugs to increase cell viability (Figure 7.1.D).

7.4.2. Combination of SEL with other treatments decrease colony formation of BCa cells in hydrogels from major metastatic tissues

Next, we cultured various endocrine-responsive and -resistant BCa cell lines in hydrogels mimicking tissue extracellular matrices from liver, bone and lung. We have observed tissue extracellular matrix-specific responses to drugs. For example, for MCF-7 *ESR1*^{Y537S} treatment with 4-OHT blocked growth in lung but increased it in bone and liver hydrogels. Fulv and SEL were agonists in all three-tissue matrices. Palb increased colony size and number in liver but not in bone or lung. Finally, combining each agent with SEL prevented agonistic activities of these drugs in different tissue hydrogels (Figure 7.2.A and B). These results showed feasibility of combining SEL with other therapies to treat already metastasized ER⁺ tumors.

7.4.3. ER α -XPO1 targeting changes metabolic phenotype of BCa cells from an energetic to quiescent profile

Previously, to understand how TAM+SEL combination provides a sustained tumor regression, previously we performed a global gene expression analysis in BT474 cells that were treated with Veh, 4-OHT, SEL or 4-OHT+SEL. This analysis identified Akt signaling associated gene circuits as well as two additional pathways associated with metabolic regulation: glycolysis and mitochondrial respiration. We hypothesized that XPO1 modulates Akt activity to rewire metabolism and provide new survival/escape routes to BCa cells. We observed that different endocrine resistant models (HCC-1500, BT474, MCF7/Tam^R, MCF7-ESR1D^{538G} and ESR1^{Y537S}) had higher mitochondrial and glycolytic respiration (Energetic phenotype) compared to endocrine sensitive cell lines (Figure 7.3.A). We tested the individual and combinational effects of 4-OHT and SEL on metabolic cell phenotypes of TAM-resistant cell lines, which showed that combining 4-OHT with SEL induced a quiescent phenotype, where both glycolysis and mitochondrial respiration is reduced, in all models tested [14]. We next cataloged the metabolic phenotype of therapy-resistant BCa cell lines using Seahorse metabolic profiler. Growing endocrine-sensitive or -resistant BCa cells on an extracellular matrix from the bone, liver or lung tissue (major sites for BCa metastasis) resulted in an increase in both glycolytic and mitochondrial activity, suggesting a metabolic rewiring taking place when cells are adapting to new microenvironments (Figure 7.3.B).

7.4.4. Single agent treatments rewire metabolic pathways to enhance survival of BCa cells

In our *in vivo*, tumors that initially regressed while on SEL came back within 3 weeks after the treatment was stopped [12]. To identify any survival pathways that might be activated with SEL only treatment, we compared gene sets that were differentially activated or repressed with SEL treatment, but not with 4-OHT or 4-OHT+SEL treatments. We found that SEL treatment of BT474 cells activated a glutamine (Gln) deprivation gene program (Figure 7.4.A). We then monitored mitochondrial fuel dependency of BT474 cells when we treated them with SEL only (Figure 7.4.B). This analysis showed that Gln, but not glucose or free fatty acids, was not used for ATP production, but it was necessary for maintenance of mitochondrial respiration (Figure 7.4.B). Metabolomics analysis also showed that Gln levels in the cells treated with SEL were lower (Figure 7.4.C).

SEL is an inhibitor of cell viability in our cell culture models, but *in vivo* fails to provide sustained tumor regression. Under limiting nutrient conditions, tumors can activate different stress mechanisms to acquire vital nutrients [35]. We next tested, in a scenario where limited nutrient availability activates glutamine acquisition from tumor microenvironment, if Gln supplementation would reverse cell inhibitory effect of SEL. In limiting media conditions with no glucose or serum supplementation, glutamine addition converted SEL to an activator for cell viability in BT474 (Figure 7.4.D). This effect was blocked by BPTES, a glutamine uptake inhibitor (Figure 7.4.E)

To identify if Gln supplementation with SEL increased production of metabolites, which were previously shown to be dependent on Gln [36], we monitored NAD^+/NADH ratio, $\text{NADP}^+/\text{NADPH}$ ratio, reactive oxygen species and glutathione production and found that the increase in NAD^+/NADH ratio was highest in cells that are grown in limited media conditions supplemented with Gln and SEL (Figure 7.5.A, B, C and D). NAD^+ is a critical metabolite that links cellular metabolism, signaling and transcription [37]. In addition to providing ATP, TCA cycle and glycolysis regenerates NAD^+ levels. When glycolysis is inhibited (low pyruvate levels, Figure 7.5.E), Gln, through conversion to glutamate and α -Ketoglutarate, is utilized by the TCA cycle to produce ATP and regenerate NAD^+ . When glycolysis and TCA cycle is blocked, Gln is converted to aspartate through a series of steps that regenerates NAD^+ by cytosolic MDH1 [38, 39]. Our metabolomics analysis showed that SEL treatment reduced cellular pyruvate level, whereas aspartate level was increased (Figure 7.5.E). In limited media supplemented with Gln, SEL-dependent cell viability was blocked by knock-down of MDH1 (Figure 7.5.F), suggesting

that in limited conditions, which might mimic tumor microenvironment, or when glycolysis and TCA cycle is inhibited, Gln is shunted to replenish NAD^+ in a MDH1-dependent manner and increased cell survival (Figure 7.5.G).

7.4.5. PGC1 α level determines the metabolic plasticity and response to therapies

Our RNASeq analysis showed that PPARGC1A (PGC1 α) to be induced by combination treatments with SEL (Figure 7.6.A and B). PGC-1 α is a major regulator of mitochondrial biogenesis, and overexpression of this protein was implicated in regulation of Gln metabolism [40] and resistance to metabolic drugs [41]. However, PGC1 α was also indicated as an autophagy regulator [42]. Therefore, we hypothesized that this protein might be involved in the metabolic rewiring and different outcomes we observed with different treatments. In our models of endocrine resistant BCa cells had higher PGC1 α expression (Figure 7.6.C), consistent with higher mitochondrial and glycolytic respiration in these cell lines compared to endocrine sensitive cell lines (Figure 7.3.A). Importantly, knockdown of PGC-1 α prevented survival of BCa cells under limiting media conditions with Gln, underscoring the importance of this protein in cell survival in these conditions (Figure 7.6.D). Interestingly, small molecule activator of PGC1 α , ZLN005 increased therapy resistant cell line viability with 4-OHT (T), Fulv (F) or Palb (P) at low dose, which was blocked by SEL (S) (Figure 7.6.E). Interestingly, inhibition of PI3K/Akt signaling or PGC1 α activity using a small molecule inhibitor reduced energetic phenotype of endocrine resistant cells (Figure 7.6.F). Our results showed that extracellular matrix composition affected metabolic phenotype of BCa cells and inhibiting PI3K pathway, PGC1 α or XPO1 reduced the effect of extracellular matrix. However, the effect of the combined therapies with SEL was even more effective in inducing quiescence in different hydrogels (Figure 7.7.A), consistent with our colony formation assays (Figure 7.2). In addition, SEL combinations were very effective in preventing SEL induced cell viability in limited media conditions with various amino acids (Figure 7.7.B), emphasizing the therapeutic potential of these combinations in reducing metastatic tumor growth and adaptation to stress conditions and preventing therapy resistance in already metastasized tumors.

7.5. Discussion

In this study, we elucidated the causes and mechanisms of therapy resistance by focusing on the role of metabolic changes in tumor cells to adapt to and survive in PMNs and test therapeutic potential of combining XPO1 inhibitors with currently utilized clinical drugs to prevent therapy resistance during metastatic tumor treatment. In this study, we started understanding the clinical relevance of combining XPO1 inhibitors with current therapies in metastatic ER (+) tumors, and we found out that XPO1 mutations in these tumors are highly correlated with other most common mutations (e.g. *PIK3CA*, *ESR1* and *CCDN1*) in these tumors. Then, we looked at the effect of this combined treatment in different premetastatic tumor environments in breast cancer, and we identified that combined endocrine and SEL treatment works better than individual treatments to reduce colony area, but SEL only treatment decreases number of colonies. After that, we looked at the effect of this combined treatment on cellular metabolic phenotype of endocrine-resistant breast cancer cells, and we found that endocrine-resistant cells have a higher metabolic phenotype. Furthermore, our results showed that endocrine-resistant cells growing in metastatic environments have a higher cellular metabolic profile. Then, we investigated the reason behind poor outcome of individual SEL treatment, and our results showed that SEL treatment increases Gln dependency in endocrine-resistant breast cancer cells through a mechanism, which converts glutamine to other TCA cycle intermediates to sustain NAD^+ levels in endocrine-resistant breast cancer cells. Then, we elucidated the mechanism behind combined endocrine agent and SEL treatment, and we found out that *PGC1 α* levels determines the metabolic plasticity and response to therapies.

Our studies established XPO1 as a target, whose inhibition would enhance the effectiveness of current therapies, by preventing metabolic adaptation and improved survival in metastatic organ sites during sequential therapies. Resistance to therapies is a significant concern for metastatic breast cancer patients. The majority of current research efforts in the therapy resistance field focused on a delineation of the underlying mechanisms that lead to increased activity of selective signaling pathways. Undoubtedly, interrogating and targeting the end-point kinases in tumors is highly relevant and these studies led to the development of combination therapies involving PI3K inhibitors or mTOR pathway inhibitors together with endocrine agents. However, resistance to these combination therapies also occurs, and in such cases, the cancer that develops is considerably more aggressive due to hyperactivation of compensatory mitogenic signaling pathways. Moreover, these kinase inhibitors have many adverse side effects. Additionally, *ESR1* mutations that decrease

sensitivity of the receptor to endocrine therapies were identified in about 15-40% of the metastatic, but not primary tumors. Our proposed approach might offer an alternative treatment strategy to prevent emergence of these adaptive strategies for cancer cells and therapy resistance, which is a significant clinical challenge currently. Further, what is learned may serve to help us understand therapy responsiveness of other cancers, including therapy-resistant TNBC for which XPO1 inhibitors are already in line for phase two clinical trials. The most obvious benefit of successful completion of our aims to the breast cancer community is reduced mortality from breast cancer, due to improved therapies.

Tumor cells can create favorable environments in target metastatic tissues by secreting various factors even before they metastasize [43]. These PMNs are characterized by altered local deposition of extracellular matrices, a rudimentary vascular network, recruitment of bone marrow derived cells (BMDCs) and presence of proinflammatory molecules and cells [44]. There is a current shortfall of relevant in vitro models that accurately reflect the PMNs for ER+ BCa cells, as well as novel analysis tools to understand the molecular mechanisms driving adaptation and survival of metastatic ER+ BCa cells in these environments. Therefore, adaptation of previously published PMN models [43, 45] using tissue-specific extracellular matrices to mimic metastatic tissue sites for ER+ BCa cells enabled us to study the impact of PMN microenvironment on how ER+ BCa cells behave, adapt, and respond to therapy agents in metastatic tissue environments.

Our research represents a new and substantive departure from the status quo by shifting focus to inhibiting dynamic mechanisms enabling adaptation to metastatic tissue environments and therapies. PGC1 α was previously implicated in metabolic rewiring and drug resistance of triple negative BCa, yet the role of PGC1 α has been controversial [41, 42, 46, 47]. Our hypothesis that lenient activation of PGC1 α , but not the chronic overexpression, promote survival and adaptation mechanisms is novel. In addition, none of the publications addressed the impact of different PGC1 α isoforms on ER+ BCa cell metabolism and metastasis. Our data supports our hypothesis that the expression level of PGC1 α might determine whether the cancer cells will find escape routes (lower PGC1 α) or regress due to autophagy or other cell death mechanisms (very high level of PGC1 α) (Figure 7.7.C).

Through our study, we evaluated the combining SEL with current therapies to overcome therapy resistance in ER⁺ metastatic tumors. Using our molecular characterization methods in clinically relevant cell lines we analyzed how combined XPO1 targeting affects PGC1 α level,

therapy responsiveness, ER α signaling and tumor outcome, thereby evaluating the potential of its inhibition as a means to prevent metastatic tumor adaptation and therapy resistance. Overall, results of our studies validated XPO1 as a target whose co-inhibition should enhance the effectiveness of therapies in metastatic ER⁺ breast tumors. XPO1 and acute regulation of PGC1 α have not been implicated in adaptation to metastatic environments and therapy resistance of ER⁺ metastatic tumors before. Given the need for better strategies for improving therapy response of relapsed ER⁺ tumors and avoid increasingly toxic therapy applied in this setting, our proposed research has great significance and potential for uncovering the role of this protein and its targeting in reducing therapy resistance in recurrent ER⁺ tumors. In summary, the nuclear export pathways have not previously been implicated or examined in the metastatic tissue adaptation and therapy resistance, and given the need for better strategies for improving or maintaining therapy response of metastatic ER⁺ tumors, our proposed studies carry high potential for uncovering the role of these pathways and their use in reducing deaths due to metastatic BCas.

7.6. Figures

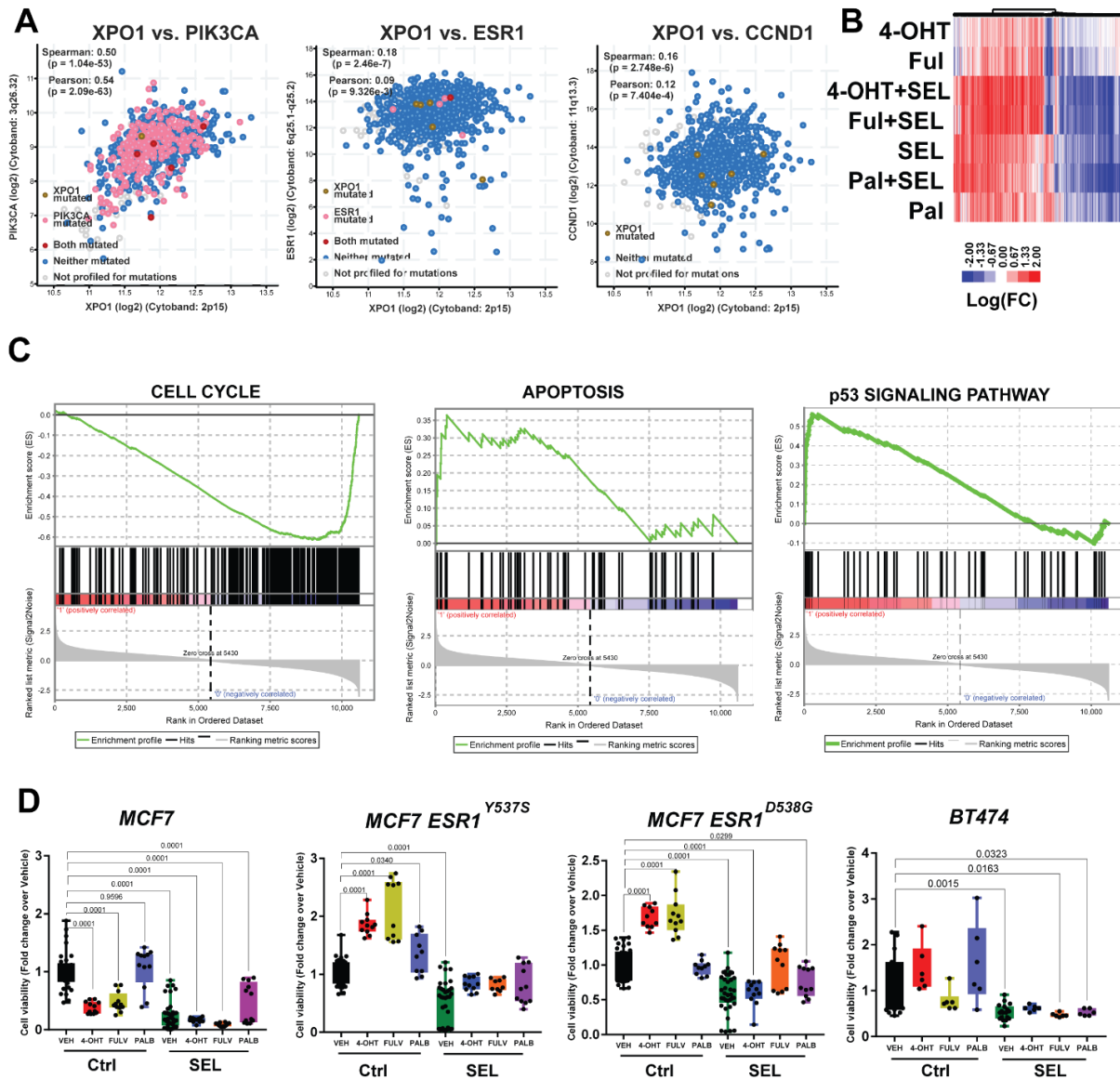


Figure 7.1. Clinical relevance of combining XPO1 inhibitors with current therapies in metastatic ER (+) tumors. **A)** We assessed the correlation between mRNA levels of XPO1 with ESR1, PIK3CA and CCND1 mRNAs in ER(+) tumors by using TCGA and METABRIC tumor databases (cBioPortal for Cancer Genomics (<https://www.cbioportal.org/>)). Correlated mutations were shown in different colors in the figure. Spearman and Pearson correlation analyses were given in each figure. **B)** RNA sequencing experiment was performed in MCF7 ESR1^{Y537S} cells, treated with 4-OHT [10^{-6}], Fulv [10^{-6}] and Palb [10^{-6}] in the presence or absence of SEL [10^{-7}]. **C)** Most important pathways gets activated with combined treatment strategy based on GSEA results of RNA sequencing experiment. **D)** Cell viability assay in different endocrine-resistant breast cancer cell lines (MCF7-ESR1^{Y537S}, MCF7-ESR1^{D538G}, HCC1500 and BT474), cotreated with 4-OHT [10^{-6} M], Fulv [10^{-6} M] and Palb [10^{-6} M] and SEL [10^{-7} M]. A one-way analysis of variance (ANOVA) model was used for statistical significance of treatment and values were presented as mean $\pm$ SEM from three independent experimental repeats. Significances were compared according to Veh condition for each treatment condition.

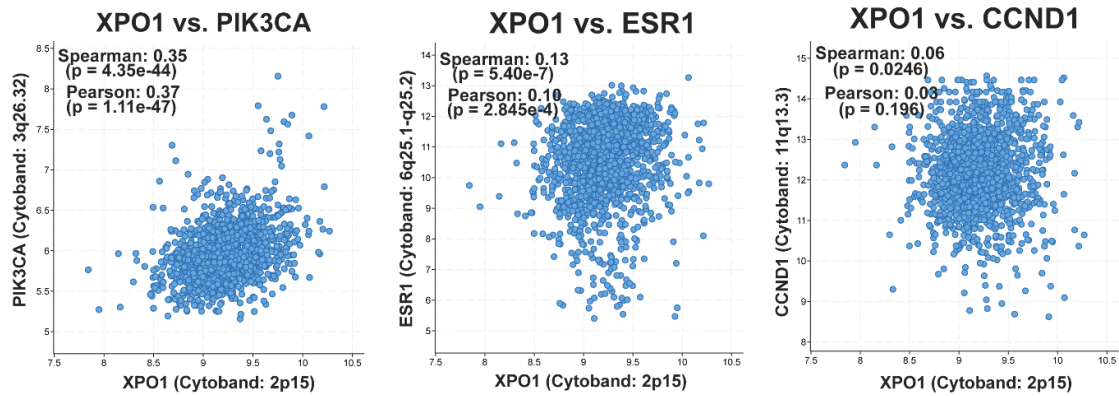


Figure 7.1a. Clinical relevance of combining XPO1 inhibitors with current therapies in metastatic ER (+) tumors. We assessed the correlation between mRNA levels of XPO1 with ESR1, PIK3CA and CCND1 mRNAs in ER(+) tumors by using METABRIC tumor databases (cBioPortal for Cancer Genomics (<https://www.cbioportal.org/>)). Correlated mutations were shown in different colors in the figure. Spearman and Pearson correlation analyses were given in each figure.

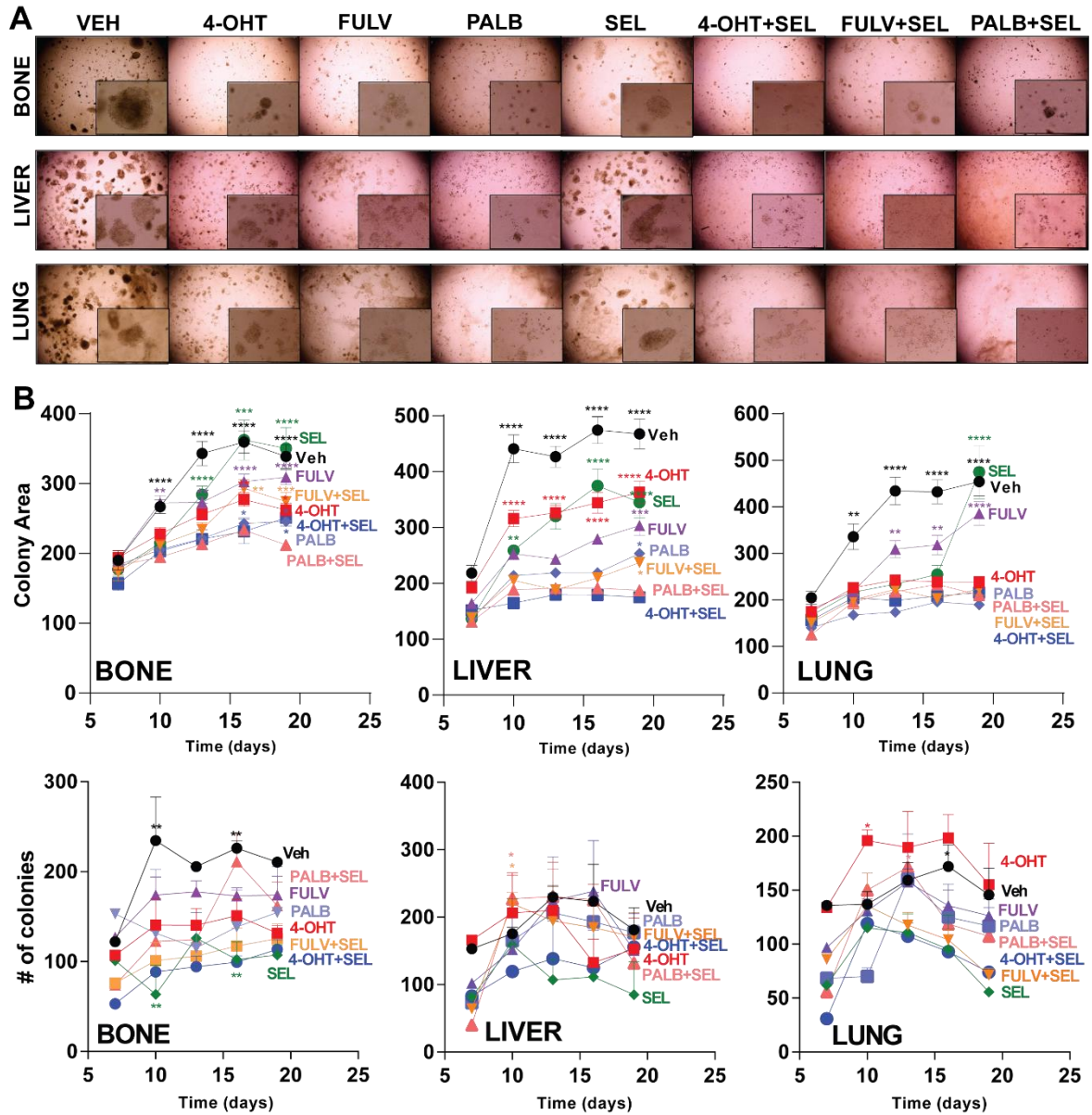


Figure 7.2. Combination of SEL with other treatments decrease colony formation of BCa cells in hydrogels from major metastatic tissues. A) MCF7-ESR1^{Y537S} cells were cultured at a density of 2×10^3 cells/well and embedded in 3-D hydrogel environments to mimic different premetastatic niches (bone, liver, lung) in breast cancer cells, and they were treated with different endocrine agents (4-OHT [10^{-6}], Fulv [10^{-6}] and Palb [10^{-6}]) and in combination with SEL [10^{-7}]. B) Colony area and C) colony number parameters were quantified with OpenCFU colony counting software (<http://opencfu.sourceforge.net/>). Experimental statistics were analyzed by using GraphPad[®] Prism8 software. A one-way analysis of variance (ANOVA) model was used for statistical significance of treatment and values were presented as mean $\pm$ SEM from three independent experimental repeats. Significances were compared according to first measurement values for each treatment condition. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

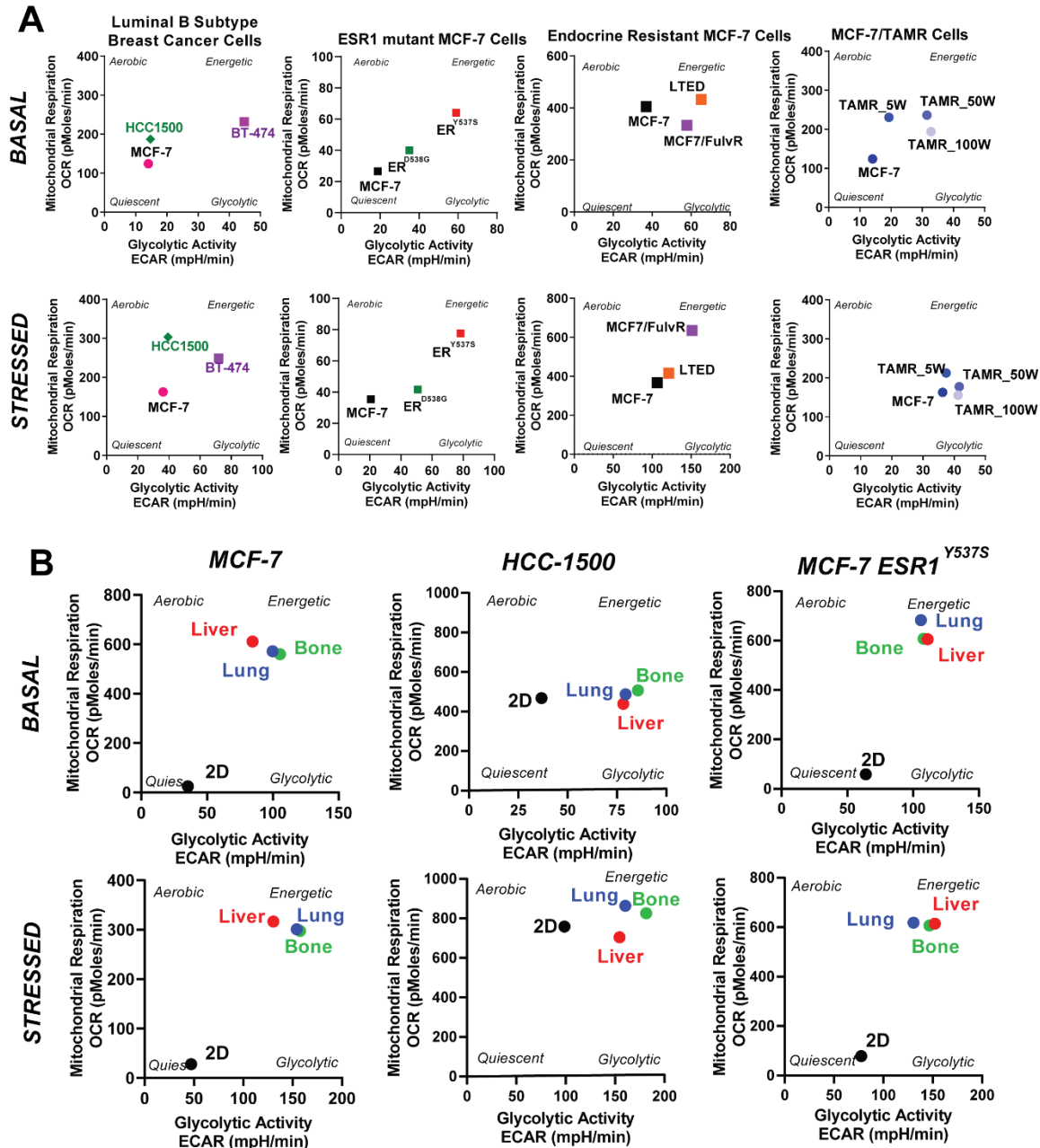


Figure 7.3. ER α -XPO1 targeting changes metabolic phenotype of BCa cells from an energetic to quiescent profile. **A)** Cellular metabolic changes in different endocrine-resistant models were monitored with Seahorse metabolic profiling. Different *de novo* endocrine-resistant cell models (HCC-1500, BT474, MCF7-ESR1^{D538G} and ESR1^{Y537S}) and acquired resistant models (MCF7/Tam^R cells generated with a consistent drug treatment strategy) were cultured at a density of 3×10^4 cells/XFp plate wells, and treated with different endocrine agents (4-OHT [10^{-6}], Fulv [10^{-6}] and Palb [10^{-6}]) and in combination with SEL [10^{-7}]. **B)** Different endocrine-sensitive and -resistant cell models were grown on an extracellular matrix from bone, liver or lung tissue (major sites for BCa metastasis) at a density of 3×10^4 cells/XFp plate wells. All experimental conditions were repeated at least two times and Seahorse metabolic profiling values were normalized according to cell number for each assay.

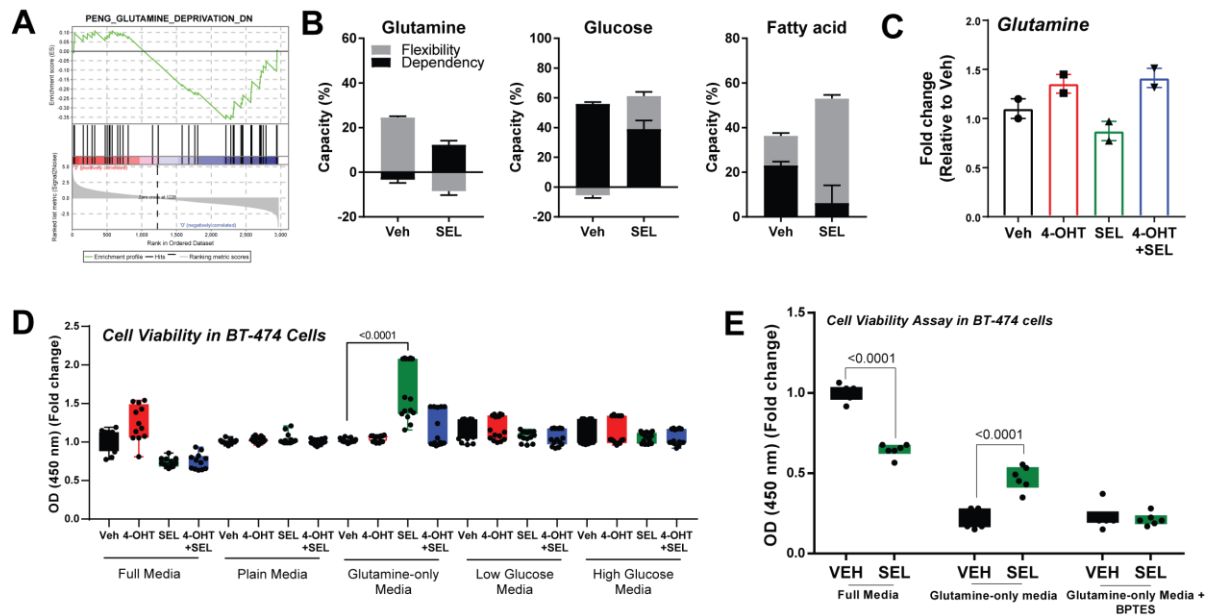


Figure 7.4. Single agent treatments rewire metabolic pathways to enhance survival of BCa cells. **A)** GSEA analysis showing activation of Gln deprivation genes in *de novo* endocrine-resistant BT474 cells. **B)** Mitochondrial Fuel Flex Test results in BT474 cells showing glutamine dependency. BT474 cells were cultured at a density of 3×10^4 /XFp plate wells, and treated with different endocrine agents (4-OHT [10^{-6}], Fulv [10^{-6}] and Palb [10^{-6}]) and in combination with SEL [10^{-7}]. All results were visualized after normalizing OCR and ECAR values according to cell number. **C)** Metabolomics analysis result showing Gln levels in SEL-only treated BT474 cells. For this experiment, BT474 cells were cultured at a density of 2×10^5 cells/10 cm plates in two technical repeats, and treated with different endocrine agents (4-OHT [10^{-6}], Fulv [10^{-6}] and Palb [10^{-6}]) and in combination with SEL [10^{-7}]. **D)** Cell viability assay showing the effect of Gln addition to the media. BT474 cells were cultured at a density of 2×10^3 cells/well in a 96-well plate and treated with 4-OHT [10^{-6}] alone and in combination with SEL [10^{-7}] in different limited media conditions. **E)** Similar experimental conditions were tested in presence of Glutaminase inhibitor, BPTES [10^{-6}]. A one-way analysis of variance (ANOVA) model was used for statistical significance of treatment and values were presented as mean $\pm$ SEM from three independent experimental repeats. Significances were compared according to Veh condition.

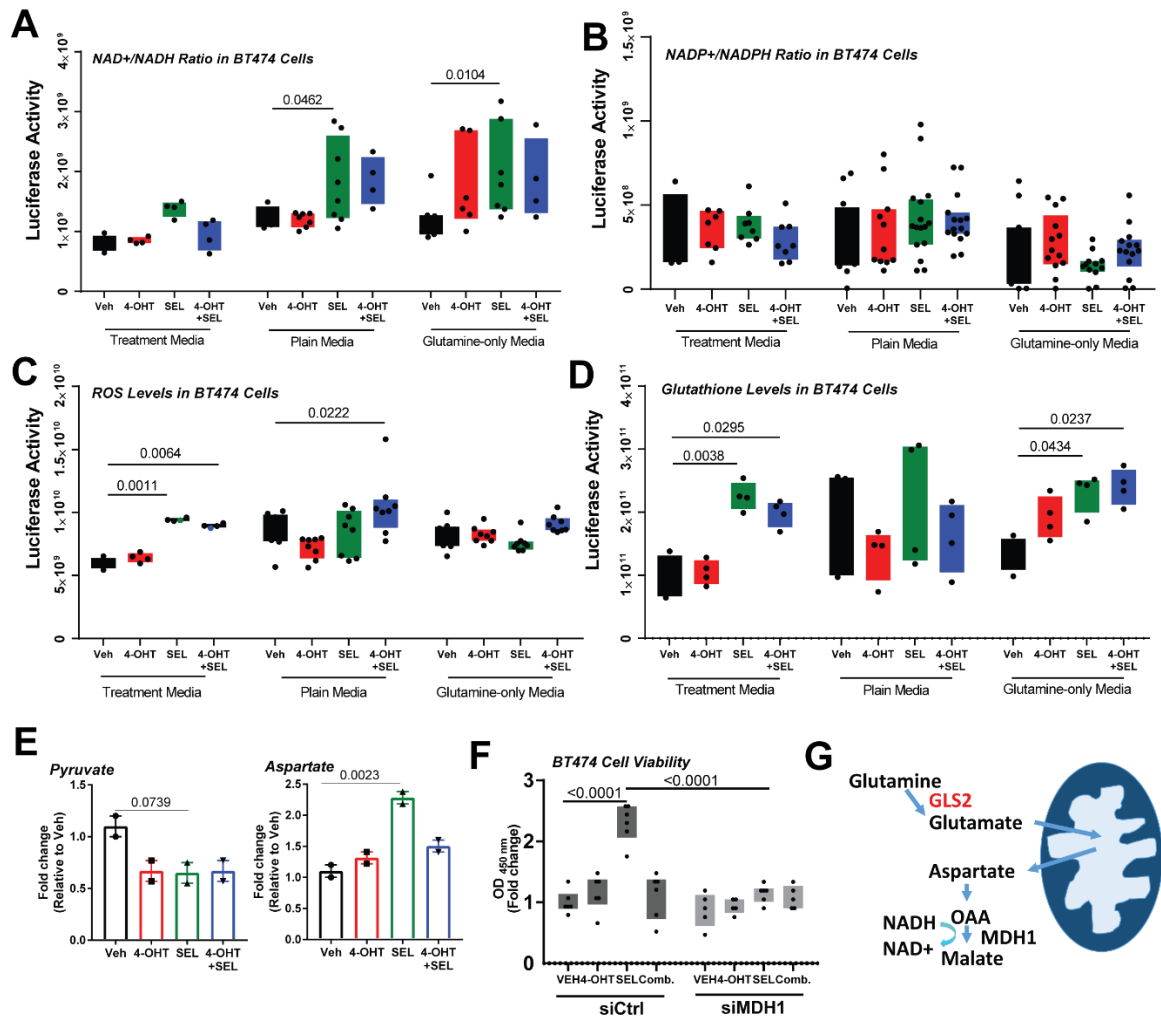


Figure 7.5. Single agent treatments rewire metabolic pathways to enhance survival of BCa cells. **A)** NAD^+/NADH and **B)** $\text{NADP}^+/\text{NADPH}$ ratios and **C)** Reactive oxygen species (ROS) and **D)** GSH levels were quantified by using different luminescence-base assays. For each test kit, BT474 cells were seeded at a density of 2×10^3 in 96-well plates and treated with different endocrine agents (4-OHT [10^{-6}], Fulv [10^{-6}] and Palb [10^{-6}]) and in combination with SEL [10^{-7}]. Experimental statistics were analyzed by using GraphPad[®] Prism8 software. A two-way analysis of variance (ANOVA) model was used for statistical significance of treatment and values were presented as mean $\pm$ SEM from three independent experimental repeats. Significances were compared according to first measurement values for each treatment condition. **E)** Metabolomics analysis result showing pyruvate and aspartate levels in SEL-only treated BT474 cells. For this experiment, BT474 cells were cultured at a density of 2×10^5 cells/10 cm plates in two technical repeats, and treated with different endocrine agents (4-OHT [10^{-6}], Fulv [10^{-6}] and Palb [10^{-6}]) and in combination with SEL [10^{-7}]. A one-way analysis of variance (ANOVA) model was used for statistical significance of treatment and values were presented as mean $\pm$ SEM from three independent experimental repeats. Significances were compared according to first measurement values for each treatment condition. **F)** The function of *MDH1* gene on the cell viability of BT474 cells was tested. BT474 cells were seeded in 96-well plates, and treated with 4-OHT [10^{-6}] alone and in combination with SEL [10^{-7}]. A one-way analysis of variance (ANOVA) model was used for statistical significance of treatment and values were presented as mean $\pm$ SEM from three independent experimental repeats. Significances were compared according to first measurement values for each treatment condition. **G)** A model explaining how Gln is used in mitochondria, and shunt into malate-aspartate shuttle to sustain NAD^+/NADH ratio in cancer cells.

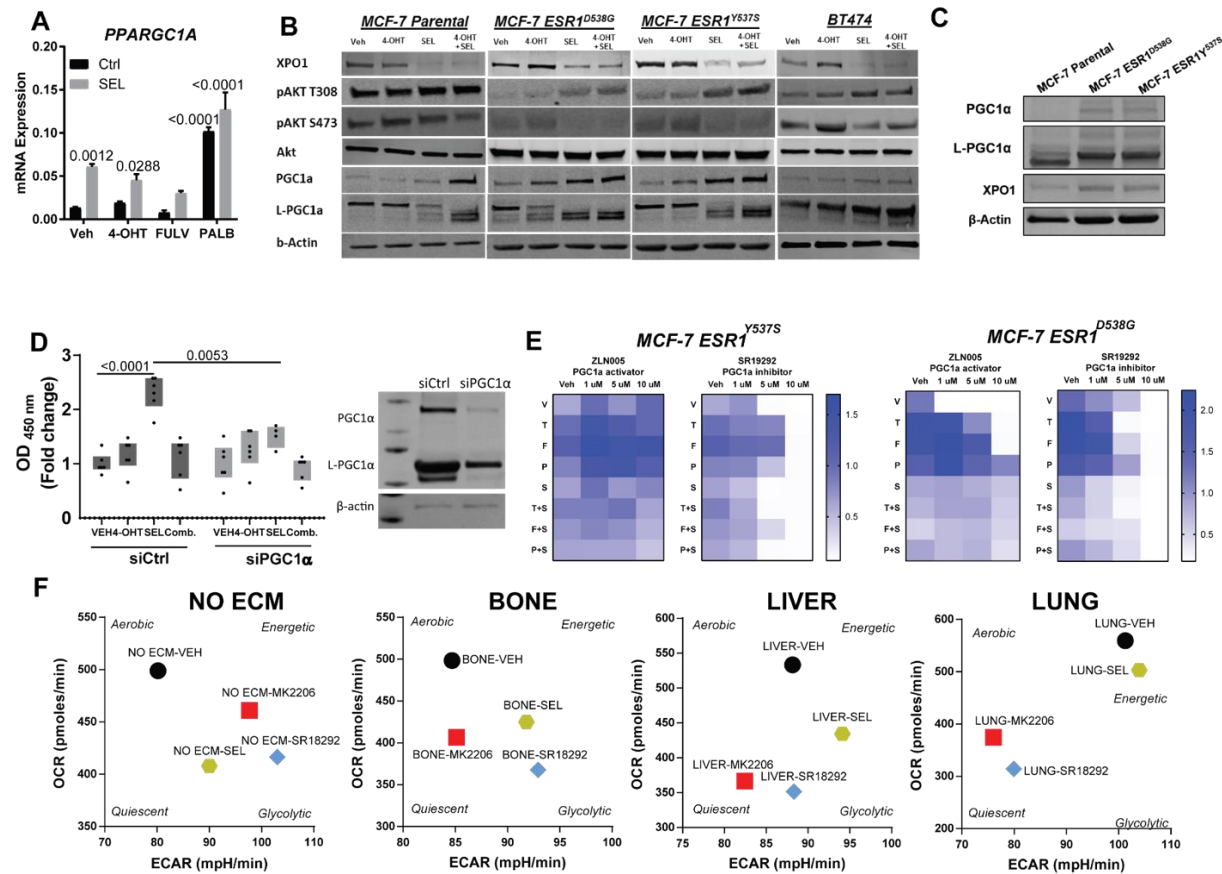


Figure 7.6. PGC1 α level determines the metabolic plasticity and response to therapies. **A)** RNA sequencing results showing SEL treatment induces PPARGC1A (PGC1 α) mRNA expression in combination with other endocrine therapy agents. Statistical analyses were analyzed according to one-way ANOVA test. **B** and **C)** Western blot analyses showing that PGC1 α protein expression were relatively higher in endocrine-resistant breast cancer cells. All cell lines were seeded at a density of 2×10^5 cells/30 mm plates and levels of each target proteins were detected after protein extraction. **D)** The function of *PGC1 α* gene on the cell viability of BT474 cells was tested. BT474 cells were seeded in 96-well plates, and treated with 4-OHT [10^{-6}] alone and in combination with SEL [10^{-7}]. A one-way analysis of variance (ANOVA) model was used for statistical significance of treatment and values were presented as mean $\pm$ SEM from three independent experimental repeats. Significances were compared according to first measurement values for each treatment condition. **E)** MCF7-ESR1^{D538G} and ESR1^{Y537S} cells were treated with different doses of *PGC1 α* activator (ZLN005) and *PGC1 α* inhibitor (SR-18292) in combination with different endocrine agents (4-OHT [10^{-6}], Fulv [10^{-6}] and Palb [10^{-6}]) and in combination with SEL [10^{-7}]. Data visualization was done by GraphPad Prism 8[®]. **F)** Cellular metabolic phenotypes of MCF7- ESR1^{Y537S} cells were determined by seeding 3×10^4 cells/XFp plate well in different extracellular matrix environments, and the effect of small molecule inhibitors for PGC1 α [10^{-6}] (SR-18292), XPO1 [10^{-7}] (SEL) and PI3K/Akt [5×10^{-6}] (MK2206) targets. All Seahorse assays were repeated two times in three technical replicates and OCR and ECAR values were normalized according to cell number.

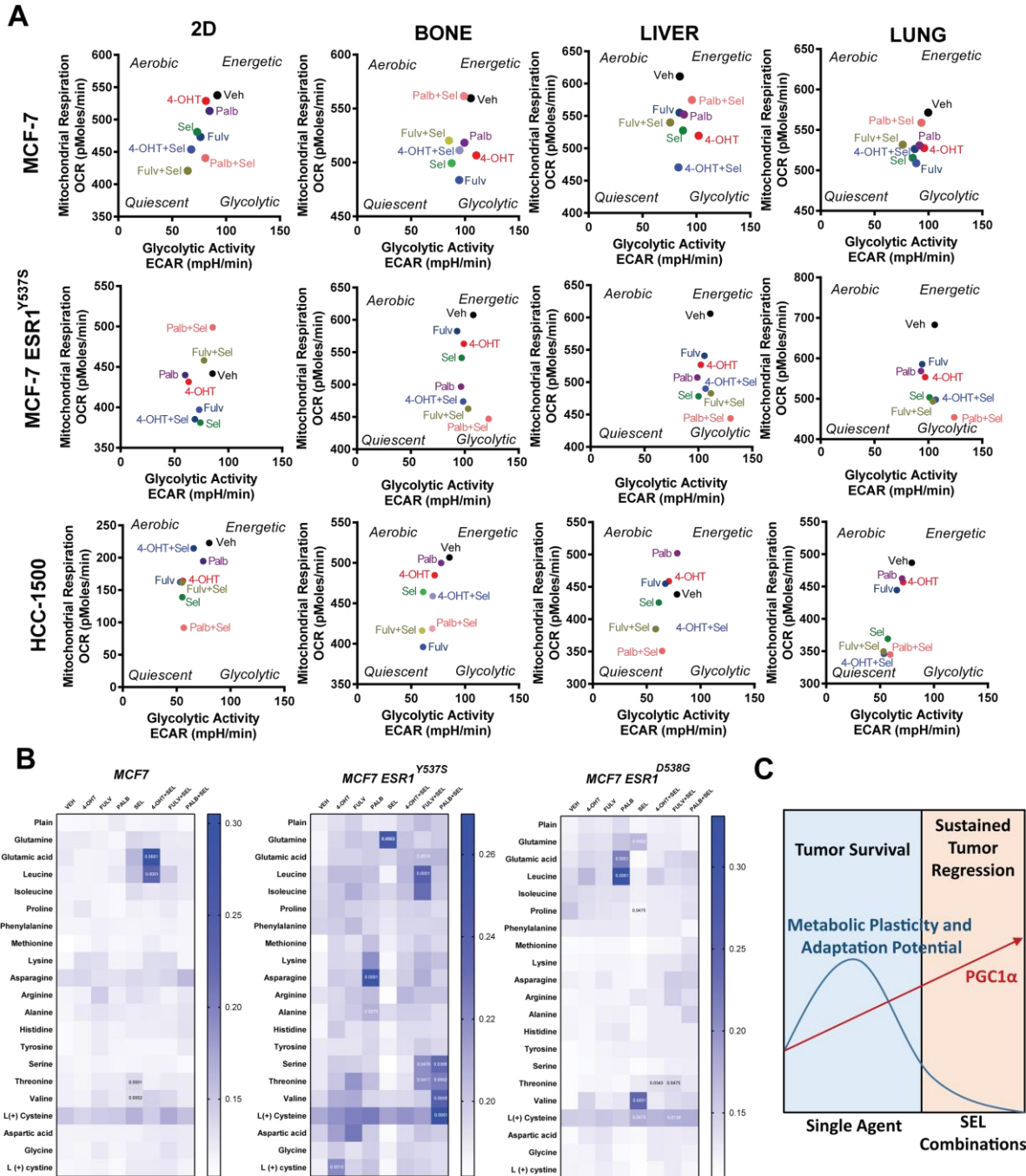


Figure 7.7. PGC1 α level determines the metabolic plasticity and response to therapies. **A)** Cellular metabolic phenotypes of endocrine-sensitive MCF7 Parental cells and endocrine-resistant MCF7- ESR1^{Y537S} and HCC1500 cells were determined by seeding 3×10^4 cells/XFp plate well in different extracellular matrix environments, and they were treated with different endocrine agents (4-OHT [10^{-6}], Fulv [10^{-6}] and Palb [10^{-6}]) and in combination with SEL [10^{-7}]. **B)** Endocrine-sensitive MCF7 Parental cells and endocrine-resistant MCF7- ESR1^{Y537S} and ESR1^{D538G} cells were grown in limited media conditions containing only 2 mM individual amino acids. Cells were treated with different endocrine agents (4-OHT [10^{-6}], Fulv [10^{-6}] and Palb [10^{-6}]) and in combination with SEL [10^{-7}], and cell viability of cells were quantified with WST-1 assay. All experiment conditions were repeated in six technical replicates and statistical significance values were calculated according to two-way ANOVA test. **C)** A model explaining overall effect of PGC1 α in endocrine resistance in breast cancer cells.

7.7. References

1. B. S. Katzenellenbogen and J. Frasor, "Therapeutic targeting in the estrogen receptor hormonal pathway," *Semin Oncol*, vol. 31, pp. 28-38, Feb 2004.
2. H. S. Rugo, R. B. Rumble, E. Macrae, D. L. Barton, H. K. Connolly, M. N. Dickler, *et al.*, "Endocrine Therapy for Hormone Receptor–Positive Metastatic Breast Cancer: American Society of Clinical Oncology Guideline," *Journal of Clinical Oncology*, vol. 34, pp. 3069-3103, September 1, 2016 2016.
3. K.-D. Yu, J. Wu, Z.-Z. Shen, and Z.-M. Shao, "Hazard of Breast Cancer-Specific Mortality among Women with Estrogen Receptor-Positive Breast Cancer after Five Years from Diagnosis: Implication for Extended Endocrine Therapy," *The Journal of Clinical Endocrinology & Metabolism*, vol. 97, pp. E2201-E2209, 2012.
4. I. Jatoi, W. F. Anderson, J.-H. Jeong, and C. K. Redmond, "Breast Cancer Adjuvant Therapy: Time to Consider Its Time-Dependent Effects," *Journal of Clinical Oncology*, vol. 29, pp. 2301-2304, June 10, 2011 2011.
5. J. Richman and M. Dowsett, "Beyond 5 years: enduring risk of recurrence in oestrogen receptor-positive breast cancer," *Nature Reviews Clinical Oncology*, 2018/12/18 2018.
6. H. Pan, R. Gray, J. Braybrooke, C. Davies, C. Taylor, P. McGale, *et al.*, "20-Year Risks of Breast-Cancer Recurrence after Stopping Endocrine Therapy at 5 Years," *New England Journal of Medicine*, vol. 377, pp. 1836-1846, 2017.
7. O. M. Rueda, S.-J. Sammut, J. A. Seoane, S.-F. Chin, J. L. Caswell-Jin, M. Callari, *et al.*, "Dynamics of breast-cancer relapse reveal late-recurring ER-positive genomic subgroups," *Nature*, vol. 567, pp. 399-404, 2019/03/01 2019.
8. H. J. Burstein, S. Temin, H. Anderson, T. A. Buchholz, N. E. Davidson, K. E. Gelmon, *et al.*, "Adjuvant Endocrine Therapy for Women With Hormone Receptor–Positive Breast Cancer: American Society of Clinical Oncology Clinical Practice Guideline Focused Update," *Journal of Clinical Oncology*, vol. 32, pp. 2255-2269, July 20, 2014 2014.
9. I. Jatoi, B. E. Chen, W. F. Anderson, and P. S. Rosenberg, "Breast cancer mortality trends in the United States according to estrogen receptor status and age at diagnosis," *J Clin Oncol*, vol. 25, pp. 1683-90, May 1 2007.
10. K. D. Yu, J. Wu, Z. Z. Shen, and Z. M. Shao, "Hazard of breast cancer-specific mortality among women with estrogen receptor-positive breast cancer after five years from

- diagnosis: implication for extended endocrine therapy," *J Clin Endocrinol Metab*, vol. 97, pp. E2201-9, Dec 2012.
11. E. Kulkoyluoglu and Z. Madak-Erdogan, "Nuclear and extranuclear-initiated estrogen receptor signaling crosstalk and endocrine resistance in breast cancer," *Steroids*, 2016.
 12. K. Wrobel, Y. C. Zhao, E. Kulkoyluoglu, K. L. A. Chen, K. Hieronymi, J. Holloway, *et al.*, "ER α -XPO1 crosstalk controls tamoxifen sensitivity in tumors by altering ERK5 cellular localization," *Molecular Endocrinology*, vol. 0, pp. me.2016-1101, 2016.
 13. Z. Madak-Erdogan, R. Ventrella, L. Petry, and B. S. Katzenellenbogen, "Novel roles for ERK5 and cofilin as critical mediators linking ER α -driven transcription, actin reorganization, and invasiveness in breast cancer," *Molecular cancer research : MCR*, vol. 12, pp. 714-27, May 2014.
 14. E. Kulkoyluoglu-Cotul, B. Smith, K. U. Wrobel, Y. C. Zhao, K. L. Chen, K. Hieronymi, *et al.*, "Combined targeting of Estrogen Receptor α and XPO1 prevent Akt activation, remodel metabolic pathways and induce autophagy to overcome tamoxifen resistance," *accepted to Cancers*, 2019.
 15. A. Bergamaschi, Z. Madak-Erdogan, Y. J. Kim, Y. L. Choi, H. Lu, and B. S. Katzenellenbogen, "The forkhead transcription factor FOXM1 promotes endocrine resistance and invasiveness in estrogen receptor-positive breast cancer by expansion of stem-like cancer cells," *Breast Cancer Res*, vol. 16, p. 436, Sep 12 2014.
 16. D. L. Holliday and V. Speirs, "Choosing the right cell line for breast cancer research," *Breast Cancer Res*, vol. 13, p. 215, Aug 12 2011.
 17. L. N. Zhang, H. Y. Zhou, Y. Y. Fu, Y. Y. Li, F. Wu, M. Gu, *et al.*, "Novel small-molecule PGC-1 α transcriptional regulator with beneficial effects on diabetic db/db mice," *Diabetes*, vol. 62, pp. 1297-307, Apr 2013.
 18. K. Sharabi, H. Lin, C. D. J. Tavares, J. E. Dominy, J. P. Camporez, R. J. Perry, *et al.*, "Selective Chemical Inhibition of PGC-1 α Gluconeogenic Activity Ameliorates Type 2 Diabetes," *Cell*, vol. 169, pp. 148-160 e15, Mar 23 2017.
 19. C. Costa, H. Ebi, M. Martini, S. A. Beausoleil, A. C. Faber, C. T. Jakubik, *et al.*, "Measurement of PIP3 levels reveals an unexpected role for p110 β in early adaptive responses to p110 α -specific inhibitors in luminal breast cancer," *Cancer Cell*, vol. 27, pp. 97-108, Jan 12 2015.

20. J. Gao, B. A. Aksoy, U. Dogrusoz, G. Dresdner, B. Gross, S. O. Sumer, *et al.*, "Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal," *Sci Signal*, vol. 6, p. pl1, Apr 2 2013.
21. E. Cerami, J. Gao, U. Dogrusoz, B. E. Gross, S. O. Sumer, B. A. Aksoy, *et al.*, "The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data," *Cancer Discov*, vol. 2, pp. 401-4, May 2012.
22. T. B. Alexander, N. J. Lacayo, J. K. Choi, R. C. Ribeiro, C. H. Pui, and J. E. Rubnitz, "Phase I Study of Selinexor, a Selective Inhibitor of Nuclear Export, in Combination With Fludarabine and Cytarabine, in Pediatric Relapsed or Refractory Acute Leukemia," *J Clin Oncol*, vol. 34, pp. 4094-4101, Dec 2016.
23. M. M. Gounder, A. Zer, W. D. Tap, S. Salah, M. A. Dickson, A. A. Gupta, *et al.*, "Phase IB Study of Selinexor, a First-in-Class Inhibitor of Nuclear Export, in Patients With Advanced Refractory Bone or Soft Tissue Sarcoma," *J Clin Oncol*, vol. 34, pp. 3166-74, Sep 10 2016.
24. A. R. Abdul Razak, M. Mau-Soerensen, N. Y. Gabrail, J. F. Gerecitano, A. F. Shields, T. J. Unger, *et al.*, "First-in-Class, First-in-Human Phase I Study of Selinexor, a Selective Inhibitor of Nuclear Export, in Patients With Advanced Solid Tumors," *J Clin Oncol*, vol. 34, pp. 4142-4150, Dec 2016.
25. A. M. Bolger, M. Lohse, and B. Usadel, "Trimmomatic: a flexible trimmer for Illumina sequence data," *Bioinformatics*, vol. 30, pp. 2114-20, Aug 1 2014.
26. D. R. Zerbino, P. Achuthan, W. Akanni, M. R. Amode, D. Barrell, J. Bhai, *et al.*, "Ensembl 2018," *Nucleic Acids Res*, vol. 46, pp. D754-D761, Jan 4 2018.
27. A. Dobin, C. A. Davis, F. Schlesinger, J. Drenkow, C. Zaleski, S. Jha, *et al.*, "STAR: ultrafast universal RNA-seq aligner," *Bioinformatics*, vol. 29, pp. 15-21, Jan 1 2013.
28. Y. Liao, G. K. Smyth, and W. Shi, "The Subread aligner: fast, accurate and scalable read mapping by seed-and-vote," *Nucleic Acids Res*, vol. 41, p. e108, May 1 2013.
29. M. D. Robinson, D. J. McCarthy, and G. K. Smyth, "edgeR: a Bioconductor package for differential expression analysis of digital gene expression data," *Bioinformatics*, vol. 26, pp. 139-40, Jan 1 2010.

30. M. E. Ritchie, B. Phipson, D. Wu, Y. Hu, C. W. Law, W. Shi, *et al.*, "limma powers differential expression analyses for RNA-sequencing and microarray studies," *Nucleic Acids Res*, vol. 43, p. e47, Apr 20 2015.
31. B. Phipson, S. Lee, I. J. Majewski, W. S. Alexander, and G. K. Smyth, "Robust Hyperparameter Estimation Protects against Hypervariable Genes and Improves Power to Detect Differential Expression," *Ann Appl Stat*, vol. 10, pp. 946-963, Jun 2016.
32. M. J. de Hoon, S. Imoto, J. Nolan, and S. Miyano, "Open source clustering software," *Bioinformatics*, vol. 20, pp. 1453-4, Jun 12 2004.
33. D. R. Rhodes, J. Yu, K. Shanker, N. Deshpande, R. Varambally, D. Ghosh, *et al.*, "ONCOMINE: a cancer microarray database and integrated data-mining platform," *Neoplasia*, vol. 6, pp. 1-6, Jan-Feb 2004.
34. J. Chong, O. Soufan, C. Li, I. Caraus, S. Li, G. Bourque, *et al.*, "MetaboAnalyst 4.0: towards more transparent and integrative metabolomics analysis," *Nucleic Acids Res*, vol. 46, pp. W486-W494, Jul 2 2018.
35. Natalya N. Pavlova and Craig B. Thompson, "The Emerging Hallmarks of Cancer Metabolism," *Cell Metabolism*, vol. 23, pp. 27-47, 2016.
36. B. J. Altman, Z. E. Stine, and C. V. Dang, "From Krebs to clinic: glutamine metabolism to cancer therapy," *Nature Reviews Cancer*, vol. 16, p. 619, 07/29/online 2016.
37. C. Cantó, R. H. Houtkooper, J. Auwerx, and R. J. Wanders, "The Secret Life of NAD⁺: An Old Metabolite Controlling New Metabolic Signaling Pathways," *Endocrine Reviews*, vol. 31, pp. 194-223, 2010.
38. K. Birsoy, T. Wang, Walter W. Chen, E. Freinkman, M. Abu-Remaileh, and David M. Sabatini, "An Essential Role of the Mitochondrial Electron Transport Chain in Cell Proliferation Is to Enable Aspartate Synthesis," *Cell*, vol. 162, pp. 540-551, 2015.
39. J. Son, C. A. Lyssiotis, H. Ying, X. Wang, S. Hua, M. Ligorio, *et al.*, "Glutamine supports pancreatic cancer growth through a KRAS-regulated metabolic pathway," *Nature*, vol. 496, p. 101, 03/27/online 2013.
40. S. McGuirk, S.-P. Gravel, G. Deblois, D. J. Papadopoli, B. Faubert, A. Wegner, *et al.*, "PGC-1 α supports glutamine metabolism in breast cancer," *Cancer & Metabolism*, vol. 1, p. 22, December 05 2013.

41. S. Andrzejewski, E. Klimcakova, R. M. Johnson, S. Tabariès, M. G. Annis, S. McGuirk, *et al.*, "PGC-1 α Promotes Breast Cancer Metastasis and Confers Bioenergetic Flexibility against Metabolic Drugs," *Cell Metabolism*, vol. 26, pp. 778-787.e5, 2017/11/07/ 2017.
42. F. Mastropasqua, G. Girolimetti, and M. Shoshan, "PGC1 α : Friend or Foe in Cancer?," *Genes*, vol. 9, p. 48, 2018.
43. R. A. Carpenter, J.-G. Kwak, S. R. Peyton, and J. Lee, "Implantable pre-metastatic niches for the study of the microenvironmental regulation of disseminated human tumour cells," *Nature Biomedical Engineering*, vol. 2, pp. 915-929, 2018/12/01 2018.
44. H. Peinado, H. Zhang, I. R. Matei, B. Costa-Silva, A. Hoshino, G. Rodrigues, *et al.*, "Pre-metastatic niches: organ-specific homes for metastases," *Nature Reviews Cancer*, vol. 17, p. 302, 03/17/online 2017.
45. B. A. Aguado, G. G. Bushnell, S. S. Rao, J. S. Jeruss, and L. D. Shea, "Engineering the pre-metastatic niche," *Nature Biomedical Engineering*, vol. 1, p. 0077, 06/13/online 2017.
46. V. S. LeBleu, J. T. O'Connell, K. N. Gonzalez Herrera, H. Wikman, K. Pantel, M. C. Haigis, *et al.*, "PGC-1 α mediates mitochondrial biogenesis and oxidative phosphorylation in cancer cells to promote metastasis," *Nature cell biology*, vol. 16, pp. 992-15, 2014.
47. S.-P. Gravel, "Deciphering the Dichotomous Effects of PGC-1 α on Tumorigenesis and Metastasis," *Frontiers in Oncology*, vol. 8, 2018-March-23 2018.

CHAPTER 8: CONCLUSIONS

In the first three chapters of this thesis, we mainly investigated molecular mechanism and clinical significance of estrogen receptor regulation. We also reviewed literature for the metabolic aspects of estrogen receptor signaling and the translational efficacy of metabolism-directed treatments in current clinical practice of ER (+) breast cancer treatment. In chapter 4, we built main hypothesis and long-term aim of the project based on the literature review in the first three chapters.

In chapter 5, we focused on the characterization of XPO1 protein in terms of tamoxifen resistance in ER (+) breast cancers, and showed that *XPO1* overexpression is associated with tamoxifen resistance in breast cancer cells. Also, a nuclear export signature can be used as a promising biomarker to predict tamoxifen responsiveness in ER (+) breast cancer patients. *XPO1* overexpression increases cell proliferation, and this effect can be blocked by *XPO1* knock down. Nuclear localization of active ERK5 (pERK5) is lost during tamoxifen resistance progression. In presence of 4-OHT, pERK5 is extranuclear, but combined 4-OHT and SEL treatment relocates pERK5 back to nucleus.

In chapter 6, we concentrated on the molecular mechanism and the clinical significance of combined tamoxifen and selinexor treatment strategy in *de novo* resistant ER (+) breast cancer patients, and we showed that combined ER α -XPO1 targeting modulates Akt signaling to rewire tumor cell metabolism and induce autophagy. Based on this overall conclusion, our results were suggesting that XPO1 protein is upregulated in Luminal B subtype of tumors. Combined 4-OHT+SEL treatment causes gene expression changes distinct from 4-OHT and SEL treatments alone in tamoxifen-resistant cell lines. Different phosphorylation sites of Akt are activated with 4-OHT, SEL and in combined treatments. Combined ER α -XPO1 inhibition reduces Akt phosphorylation that was observed with single treatments in tamoxifen-resistant cells and tumor xenografts. Combined ER α -XPO1 targeting changes metabolic phenotype of tamoxifen-resistant breast cancer cells from an energetic to quiescent profile. 4-OHT and SEL combination reduces mitochondrial activity in tamoxifen-resistant cells. Combined ER α -XPO1 targeting activates autophagy markers, but not result in apoptosis.

In chapter 7, we directed our interest on the efficacy of ER α -XPO1 dual targeting in different potential metastatic sites of breast cancer, and found that single agent treatments are not

as effective as combined treatments due to metabolic rewiring. In addition, Combined 4-OHT+SEL treatment is an effective strategy to target endocrine resistant breast cancer cells. Other results that we gathered from this part of the project indicated that *XPO1* mutations are highly correlated with *ESR1* and *PIK3CA* mutations in breast cancer. Selinexor alone and in combination with other endocrine and chemotherapy agents decreases cell viability of endocrine resistant breast cancers better. Endocrine resistant cells have a more energetic phenotype. Selinexor works better in combination with other endocrine treatment agents to stop oncosphere formation in possible breast cancer metastatic sites (e.g. lung, liver, bone). Endocrine resistant breast cancer have a higher capacity in the lung and liver compared to bone. Energetic phenotype of both endocrine sensitive and resistant breast cancer cells decreases with SEL + endocrine agent combinations compared to individual treatments. *in vivo* and *ex vivo* models indicated that SEL-treated tumors come back after treatment. SEL-treatment makes resistant cells more dependent on glutamine. Glutamine is necessary for cells treated with SEL. Glutamine cannot alone compensate the lack of other fuels for continued mitochondrial respiration (Excess glutamine is not used for ATP production). Combined 4-OHT + SEL treatment reduces the rate of synthesis of glycolysis and mitochondrial respiration. Aspartate production increases with SEL treatment, and this excess aspartate is used for NAD⁺ synthesis via malate-aspartate shuttle. *MDH1* is essential for glutamine usage.

Overall, our results showed that combination of selinexor with current endocrine therapy agents is a promising adjuvant treatment strategy, which can be easily applicable to clinic. This clinical significance of our project support the bench-to-bedside translation efficacy of this project.

CHAPTER 9: FUTURE DIRECTIONS

Studies we conducted in this project indicated that 4-OHT+SEL treatment offer a promising treatment option especially for treatment ER (+) breast cancer patients. An increasing number of clinical trials started in the last decade due to beneficial effects of selinexor treatment in other cancer types (leukemia, lymphoma, prostate cancer). In parallel, our next aim is to validate the impact of this combined drug strategy on *in vitro* drug resistant breast cancer cell models, which were exposed to drug treatments for several weeks. We are going to test the effect of combined and individual drug treatments on the cell viability of these cell lines generated as acquired drug resistance models.

As a next step, we will evaluate the efficacy of these treatment strategies with *in vivo* models, which is the best way to mimic metastatic breast tumor environment. Specifically, we want to analyze how combined XPO1 targeting affects PGC1 α level, therapy responsiveness, ER α signaling and tumor outcome, thereby evaluating the potential of its inhibition as a means to prevent metastatic tumor adaptation and therapy resistance.

Eventually, we want to test the translational impact of this combined treatment with clinical trials with the help of our collaborators at University of Texas MD Anderson Cancer Center.

Overall, results of our studies should validate XPO1 as a target whose co-inhibition should enhance the effectiveness of therapies in metastatic ER (+) breast tumors. XPO1 and acute regulation of PGC1- α have not been implicated in adaptation to metastatic environments and therapy resistance of ER (+) metastatic tumors before. Given the need for better strategies for improving therapy response of relapsed ER (+) tumors and avoid increasingly toxic therapy applied in this setting, our proposed researches have great significance and potential for uncovering the role of this protein and its targeting in reducing therapy resistance in recurrent ER (+) tumors.

In summary, the nuclear export pathways have not previously been implicated or examined in the metastatic tissue adaptation and therapy resistance, and given the need for better strategies for improving or maintaining therapy response of metastatic ER (+) tumors, our proposed studies carry high potential for uncovering the role of these pathways and their use in reducing deaths due to metastatic BCas.

APPENDIX A: Abbreviations

ACC	Acetyl-CoA carboxylase
ACS	Acetyl-CoA synthases
ACLY	ATP citrate lyase
AdCMV	Adenovirus with a cytomegalovirus construct
AdXPO1	Adenovirus with an XPO1 construct
AI	Aromatase inhibitor
AF-1	Activation factor-1
ATP S	ATP synthase
ASL	Argininosuccinate lyase
CD	Charcoal dextran
CDK 4/6	Cyclin-dependent kinase 4/6
CD-36	Cluster of differentiation 36
CDO	Cysteine dioxygenase
CSAD	Cysteine sulfinic acid decarboxylase
DAPI	4',6'-diamino-2-phenylindole
DBD	DNA binding factor
DCIS	Ductal carcinoma <i>in situ</i>
DES	Diethylstilbestrol
E2	17 β -estradiol
EGRF	Epidermal growth factor receptor
ER	Estrogen receptor
ER α [+]	Estrogen receptor positive
ER α [-]	Estrogen receptor negative
ER β	Estrogen receptor beta
ERE	Estrogen receptor element
ERK-5	Extracellular-regulated kinase-5
FASN	Fatty acid synthase
FBS	Fetal bovine serum
FFA	Free fatty acid

FFPE	Formalin fixed, paraffin embedded
FULV	Fulvestrant
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
G6P isomerase	Glucose-6-phosphate isomerase
G6PD	Glucose-6-phosphate dehydrogenase
GPOR	G-protein coupled ER
GLUTs	Glucose transporters
GSL-1	Glutaminase-1
GS	Glutamine synthase
HER	Human epidermal growth factor
HIF-1	Hypoxia-inducible factor-1
HK	Hexokinase
HMG-CoA	3-Hydroxy-3-methylglutaryl-CoA
HMG-CoAR	3-Hydroxy-3-methylglutaryl-CoA reductase
HSP	Heat-shock protein
LBD	Ligand binding domain
LCIS	Lobular carcinoma <i>in situ</i>
LDHA	Lactate dehydrogenase-A,
MAGL	Monoacylglycerol lipase
MAPK	Mitogen-activated kinase pathway
MNAR	Modulator of non-genomic activity of estrogen receptor
NCoR	Nuclear receptor corepressor
NO	Nitric oxide
PALB	Palbociclib
PCAF	p300 coactivator associated factor
PDH	Pyruvate dehydrogenase
pERK-5	Phosphorylated ERK-5
PFKs	Phosphofructokinase
PFKBP	Phosphofructokinase fructose biphosphatases
PGAM	Phosphoglycerate mutase
PGDH	Phosphoglycerate dehydrogenase

PKA	Protein kinase
PKM2	Pyruvate kinase isoenzyme M2
PR	Progesterone Receptor
PSP	Phosphoserine phosphatase
PTM	Post-translational modification
RIP140	Receptor interacting protein 140
RAR α	Retinoic acid receptor alpha
RSK	Ribosomal S6 kinase
qPCR	Quantitative PCR
SCD1	Stearoyl-CoA desaturase-1
SEL	Selinexor
SERM	Selective estrogen receptor modulator
SERD	Selective estrogen receptor degrader
SLC1A5:	Solute carrier family 1 member 5
SMRT	Silencing mediator for retinoid and thyroid receptor
SphKP-1	Sphingosine-1 phosphate kinase
SRC-1	Steroid receptor coactivator-1
TNBC	Triple negative breast cancer
TPR	Tetratricopeptide repeat motif
Veh	Vehicle
WST-1	Water-soluble tetrazolium-1
XPO1	Exportin 1
4-OH-TAM	4-hydroxytamoxifen