Contribution of the unfolded protein response to breast and prostate tissue homeostasis and its significance to cancer endocrine response

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Abbreviations: 4OH-TAM, 4-hydroxytamoxifen; AR, androgen receptor; ATF4, activating transcription factor 4; ATF6, activating transcription factor 6; BC, breast cancer; BiP, sensor-binding immunoglobulin protein; CHOP, C/EBP homologous protein; DHT, dihydrotestosterone; E2, 17β-estradiol; eIF2α, eukaryotic initiation factor 2α; EnR, endoplasmic reticulum; ERα, estrogen receptor alpha; ERG, ETS related gene; IP_3, inositol triphosphate; IRE1α, inositol-requiring enzyme 1α; mTOR, mammalian target of rapamycin; PC, prostate cancer; PERK, protein kinase RNA-like endoplasmic reticulum kinase; PLCγ, phospholipase C gamma; PQC, protein quality control; TAM, hydroxytamoxifen; UPR, unfolded protein response; UPS, ubiquitin-proteasome-system; XBP-1, X-box binding protein 1; XBP-1s, spliced XBP-1; XBP-1u, unspliced XBP-1.

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Abstract

Resistant Breast and Prostate cancer remain a major clinical problem and new therapeutic approaches and better predictors of therapeutic response are clearly needed. Due to the involvement of the Unfolded Protein Response (UPR) in cell proliferation and apoptosis evasion, an increasing number of publications support the hypothesis that impairments in this network trigger and/or exacerbate cancer. Moreover, UPR activation could contribute to the development of drug-resistance phenotypes in both breast and prostate cancers. Therefore, targeting this pathway has recently emerged as a promising strategy in anti-cancer therapy. This review addresses the contribution of UPR to breast and prostate tissues homeostasis and its significance to cancer endocrine response with focus on the current progress on UPR research related to cancer biology, detection, prognosis and treatment are also discussed.

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1. Introduction

Breast cancer (BC) and prostate cancer (PC) are currently the first and fourth cause of death among female and male cancer patients worldwide (1). About 70% of all diagnosed BCs express estrogen receptor alpha (ERα) and 90% of all PCs express androgen receptor (AR). ERα and AR are ligand-activated transcription factors which exert their molecular functions by regulating gene expression through genomic and non-genomic mechanisms. These two proteins are still to date the gold standard for diagnosis and treatment decisions. Despite the favorable improvements in patients' survival associated with endocrine therapies, about 50% initially hormone responsive BCs (2) and almost all PCs (3,4) develop resistance to these treatments. This contributes to the progression of the disease towards an incurable and lethal stage. Therefore, understanding the molecular mechanisms involved in intrinsic or acquired resistance remains a major clinical challenge.

Several studies have pointed out ERα and AR cross-talk with receptor tyrosine-kinase pathways as a key alteration leading to endocrine resistance (5-10). Moreover, receptor activating mutations are also associated to endocrine resistance in about 15-20% BC (11) and 10% PC (12), respectively. Recent evidence also suggests that adaptive stress responses which have evolved to protect cells from proteotoxic stress may play a role in endocrine resistance (13-16).

Adaptive stress responses are activated in response to metabolic and environmental stress and involve the protein quality control (PQC) network. The PQC comprises the translational machinery, protein degradation machinery as well as molecular chaperones and co-chaperones (17). Normal cells respond to acute stress by activating the PQC in order to rapidly restore protein homeostasis (from now on proteostasis) and thus protect from proteotoxic stress. However, if proteostasis is not restored cells commit to apoptosis. Therefore, the PQC network targets survival or

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death pathways, which are selectively activated depending on the restoration capacity of the cells.

An increasing number of publications support the hypothesis that impairments in PQC mechanisms trigger and/or exacerbate cancer, with some components of these network associated to therapy resistance in both BC and PC (15,18-25). In this review, we discuss the current knowledge on the deregulation of one of these PQC mechanisms, the unfolded protein response (UPR), in breast and prostate tissue homeostasis, in BC and PC, as well as its significance to endocrine therapy resistance.

2. The Unfolded Protein Response

This pathway is activated to protect cells from detrimental conditions that activate endoplasmic reticulum (EnR) stress. As such, hypoxia, acidosis, nutrient deprivation and gene mutations that contribute to protein misfolding cause EnR stress leading to UPR activation (26) followed by restoration of EnR functions (protein folding, post-translational modifications, lipid and steroid synthesis and calcium signaling) (27). UPR is initiated when the chaperone *EnR stressor sensor-binding immunoglobulin protein* (BiP) frees itself from the EnR-resident proteins that regulate the UPR cascades and in turn associates with misfolded proteins. This results in activation of the three UPR branches: *inositol-requiring enzyme 1α* (IRE1α) arm, *protein kinase RNA-like endoplasmic reticulum kinase* (PERK) arm and *activating transcription factor 6* (ATF6) arm (Figure 1). UPR activation results in two temporally distinct events to allow EnR recovery: an initial reaction to decrease mRNA transcription, lower protein translation rates and enhance degradation of damaged proteins, followed by a later induction of genes involved in proteostasis and lipid biosynthesis control. Therefore, cells can adapt to different intensity and duration of intrinsic and environmental stress by selectively regulating UPR branch activation (26-28).

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IRE1α branch: Upon dissociation from BiP, IRE1α dimerizes and autophosphorylates activating its RNAse domain, leading to the subsequent splicing of *X-box binding protein 1* (XBP-1) mRNA. Spliced XBP-1 (XBP-1s) encodes a transcription factor that up-regulates genes involved in protein folding, lipid metabolism, quality control and endoplasmic-reticulum-associated degradation (ERAD). XBP-1 heterodimerizes with several other transcription factors, hence its target genes may vary according to the cell context and stimuli. Unspliced XBP-1 (XBP-1u) can function as a negative regulator of XBP-1s activity, therefore XBP-1s/XBP-1u balance can have significant consequences for UPR activation as well as XBP-1 function and transcription of its target genes (29,30). IRE1α also regulates the stability of multiple RNAs through its endonuclease activity in a process known as regulated IRE1-dependent decay (RIDD) which targets glucose metabolism, inflammation and apoptosis. IRE1α cleavage of XBP-1 or activation of RIDD follow different kinetics and may depend on IRE1α oligomeric state (31).

PERK branch: Release of PERK from BiP results in PERK oligomerization and trans-autophosphorylation, which activates its kinase function. p-PERK phosphorylates *eukaryotic initiation factor 2α* (eIF2α) in Ser51 to reduce the rate of formation of the eIF2 ternary complex (eIF2-GTP-tRNA Methionine) which is essential for ribosome binding to the start codon (32). Despite this translational inhibition, some mRNAs, such as *activating transcription factor 4* (ATF4), escape and are translated (33). ATF4 induces the expression of anti-oxidative enzymes, promotes amino acid synthesis, autophagy, protein folding and differentiation, and downregulates genes involved in cellular senescence and inhibitors of angiogenesis (27,34). In response to chronic stress, sustained ATF4 expression induces *C/EBP homologous protein* (CHOP) gene transcription, which encodes a transcription factor that stimulates growth arrest and apoptosis (35). CHOP´s target gene, *growth arrest and DNA damage-inducible protein*

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34 (GADD34) in association with *phosphatase protein 1* (PP1) dephosphorylate eIF2α, which enables the recovery of protein translation (36). However, if CHOP accumulates, due to chronic stress, Bcl-2-like protein 11 (BIM) expression is induced, and cells commit to apoptosis (35). Thus, PERK arm integrates adaptive and chronic EnR stress responses.

ATF6α branch: Following its release from BiP, ATF6α translocates to the Golgi where it is cleaved by the proteases SP1 and SP2, releasing an N-terminal fragment (ATF6f) that acts as a transcription factor of XBP-1u, EnR chaperones including BiP and ERAD-associated proteins (26,27,37). ATF6f also forms heterodimers with XBP-1s which drives specific gene expression programs. Despite the functional overlap between ATF6α and IRE1α branch gene targets, it appears that ATF6αhas evolved to enhance the protective mechanisms of PERK and IRE1α signaling, contributing for cell survival during chronic stress (38). In fact, ATF6α deletion results in impaired function of the secretory pathway during EnR stress thus resulting in impaired long-term EnR function (39).

3. Role of the unfolded protein response in tissue homeostasis: focus on the mammary gland

During lactation, normal breast cells must balance the increased production of milk proteins with the risk that an excessive protein load accumulation could impair basic cell survival functions (28,37,40). Similarly, to observations in other secretory cells such as plasmocytes and pancreatic cells, mammary cells have a well-coordinated and active UPR to adapt to the high EnR activity required during their functional differentiation during pregnancy and lactation (41-45). Notably, to the date, there is no information available regarding UPR involvement in normal prostate physiology and development.

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IRE1-α/XBP1 branch: A thorough characterization of mammary tissue from virgin, early pregnant and lactating mice lacking XBP-1 disclosed that its deletion correlates to poor branching morphogenesis and impaired terminal end bud formation at the virgin stage, possibly due to a stromal effect. XBP-1 deletion was sufficient to impair lobuloalveolar development during early lactation. This is due to reduced epithelial cell proliferation which prevents lobuloalveolar compartment expansion as shown using tissue transplantation techniques (41). The same authors also showed that XBP-1 was detectable only during lactation and was nearly absent in virgin and pregnant mice. However, other have shown that XBP-1mRNA and protein gradually increase from pregnancy, reaching highest levels during lactation. Moreover, in agreement with a role for XBP-1 in functional differentiation, blocking its expression in HC11 mammary epithelial cells reduced lactogenic protein mRNA levels in response to dexamethasone-prolactin-insulin stimulation. The authors explained these as resulting from reduced mRNA of prolactin and insulin receptors in cells with XBP-1 knock-down (42). Therefore, the increased protein and lipid synthesis demands during lactogenic differentiation induce EnR stress to activate the IRE-1α/XBP-1 branch and consequently increase the EnR capacity needed to support alveolar expansion and the secretory phenotype.

PERK/ATF4 branch: PERK activation inhibited MCF10A acini formation under normal growth conditions and its inhibition with dominant-negative PERK mutants resulted in hyperproliferation and *in vivo* tumorigenicity (43). In line with this, overexpression of the PERK downstream effector ATF4 in mice decreased proliferation and differentiation of mammary alveolar epithelium and accelerated involution (44). The effect of this branch on involution appears to result from an interplay with autophagy regulation where elevated BiP and p-eIF2a expression (as well as XBPu and autophagy genes) occurred

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in the reversible involution phase 24–48 h postweaning in mice. Whereas, ATF4 expression was increased in the irreversible involution phase (72–168 h) and stimulated CHOP expression, which coincided with the expression of apoptosis markers such as active caspases and cleaved PARP (46). This study showed a sequential contribution of UPR and autophagy pathways in the involution process, promoting pro-survival or death signaling during the reversible and irreversible involution phases, respectively (46). During mid-lactation, PERK physiological activation appears to be necessary for the lipogenic maturation of mammary epithelial cells, as demonstrated by the increased levels of p-eIF2αbetween lactation days 7 and 12 (45), as well as activation of the lipogenic phenotype which characterizes lactogenic differentiation (47). Tissue-specific PERK deletion in the mouse mammary epithelium reduced levels of the lipogenic genes (45). Regarding ATF4, its expression was found differentially regulated during the development of the mammary gland although results are not consistent. One study found that in total tissue lysates ATF4 expression was highest during virgin and pregnancy stages and lowest during lactation (44). The second study reported that ATF4 protein levels gradually increased during pregnancy and reached a significantly higher level on lactation days 4-7 (42). Supporting the latter study, in the HC11 cell line, ATF4 knock-down reduced insulin and glucocorticoid receptor mRNA levels which suggests that ATF4 is necessary to allow lactogenic protein synthesis. Moreover, in the same cell line insulin and prolactin increased CHOP mRNA and transcriptional activity on the STAT5a-driven beta-casein gene (48). However, CHOP´s function in lactogenic differentiation remains to be clarified since in whole mouse mammary tissue CHOP mRNA significantly decreased from day 15 of pregnancy to day 4 of lactation (42), while in another study it transiently peaked at lactation day 5 (48).

In summary, PERK activation may induce a positive feedback loop where ATF4 reduces proliferation and increases the response to lactogenic hormones, which in turn

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enhance CHOP´s transcriptional activation. Reduced proliferation in ATF4 overexpressing mammary glands could be related to CHOP-induced G0/G1 arrest (49), thus supporting a role of PERK/ATF4 branch in inhibition of alveolar cell proliferation and stimulation of their functional differentiation.

ATF6 branch: there was no significant change in the expression level of ATF6 during the mammary gland developmental stages (42). However, since ATF6 branch enhances the response to IRE-1α and PERK activation, more studies are needed to asses ATF6 activation.

4.The Unfolded Protein Response in Breast and Prostate Cancer

Cancer cells have increased metabolic demands to sustain biomolecule biosynthesis, survive chronic hypoxia, acidosis and nutrient depletion (50). In addition, accumulation of gene mutations alters protein folding which could increase formation of toxic protein aggregates (51). Cancer cells have evolved PQC mechanisms that allow their survival in normally deleterious conditions, with the overall result of chronically disturbed proteostasis along with enhanced survival (52). One explanation for this phenomenon is that cancer cells display sub-functional death pathways, thus chronic proteostasis loss does not result in cell death, but may be a selective advantage contributing to cancer cell survival (27).

Due to the importance of UPR in morphogenesis and differentiation of the secretory epithelium, the exploitation of UPR by neoplastic breast and prostate cells in order to deal with metabolic and oxidative stress seems predictable. This evolutionary strategy could induce cancer cell dormancy (34) and may be involved in the acquisition of a therapy resistant cancer phenotype. Amongst PQC effectors the ubiquitin-proteasome-system (UPS) is frequently up-regulated in cancer cells but its activity *per se*is not sufficient to maintain cancer cell proteostasis (53). Curiously, BC and PC

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cancer stem cells have lower proteasome activity, which is related with higher metastatic capacity, worse prognosis and therapeutic resistance (54-57). Thus, in breast and prostate cancer stem cells protein folding mechanisms must be overactivated to compensate for the low proteasomal activity. In fact, it was recently demonstrated that disseminated BC with mesenchymal characteristics have higher level of UPR proteins, namely the chaperones BiP and GRP94, thanMDA-MB-468 and MCF7 cells (58).

A number of studies have shown alterations in BC and PC that support the hypothesis that impairments in UPR appear to trigger and/or exacerbate the disease as well as influence response to anti-cancer therapy (15,18,24,28,59-67). However, the current knowledge on UPR function in PC is still limited and controversial: So, *et al* found that all the 3 UPR branches are selectively down-regulated in mouse models of prostate tumorigenesis (68); on the other hand, Liu et al found that IRE1α, PERK and ATF6 were increased and significantly associated with Gleason grade, T and M stages, PSA level ad shorter survival (62). The following sections focus on receptor-positive BC and PC and the significance of UPR on endocrine response.

5. Regulation of UPR branch activation by ER and AR agonists and antagonists

Estrogens and androgens exert their effects through distinct molecular mechanisms (Figure 2). In the classical nuclear receptor (NR) pathway, ER and AR bind their agonist, dimerize and bind to DNA consensus sequences which results in direct gene regulation. ER and AR may also interact with other transcription factors and regulate gene expression by indirect DNA binding (69-71). In the absence of agonist, these NRs can be activated by phosphorylation through cross-talk with receptor tyrosine kinase signaling which results in ligand-independent regulation of gene expression. On the other hand, a ligand activates a membrane-associated receptor or a receptor located in the cytoplasm, such as phospholipase C (PLC)/protein kinase C

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(PKCs), Ras/Raf/MAPK, phosphatidyl inositol 3 kinase (PI3K)/Akt and cAMP/ protein kinase A (PKA), to induce a rapid physiological response without direct gene regulation by ER or AR (69-71). Increasing evidence shows that ER or AR activate or inhibit the UPR branches in a cell context-dependent manner and that this could be associated to endocrine therapy resistance.

BiP: BC and PC cells usually overexpress molecular chaperones such as HSP27, HSP60, HSPA1A, GRP94 and BiP (24,72,73) which aid to restore proteostasis by facilitating protein folding and the pro-survival and cytoprotective response of cancer cells to environmental stress (74).

- BiP in breast cancer: Non-genomic activation of *Phospholipase C Gamma* (PLCγ) by ERα is followed by rapid Ca^{2+} efflux into the cytosol, causing BiP release from UPR effectors and anticipatory UPR activation of all three arms of the UPR (66,75) which prepares the cells for increased protein and lipid synthesis needed for proliferation. A small ERα biomodulator molecule, BHPI, was recently shown to induce a massive and sustained UPR activation involving non-genomic activation of PLCγ by ERα, which initiates a fruitless cycle of EnR Ca^{2+} depletion and ATP consumption and converts UPR from cytoprotective to cytotoxic (76). ERα abundantly interacts with BiP in the EnR, which probably reflects the need of this chaperone for ERα mediated non-genomic signaling (77). In T47-D cells, pre-exposure to E2 for 8 days elicits a 10-fold increase in the concentration of tunicamycin necessary to induce apoptosis (66). Therefore, cells can exploit the UPR anticipatory response produced by ERα activation as a protective mechanism against chemotherapy

E2 induces BiP transcription in MCF-7 and T47-D cells (48,66,78). In E2-dependent MCF-7/WS8 cells and in E2-refractory MCF7-2A cells, E2 also upregulates BiP mRNA. On the contrary, in E2-sensitive/anti-estrogen resistant MCF7-5C cells BiP expression was not sufficiently induced and as a result IRE1α/XBP-1 branch was

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activated leading to up-regulation of pro-apoptotic caspase-4 and BIM (79). Thus, in order to support cell survival under estrogen stimulation, cells must up-regulate BiP levels. In agreement with this, in MCF-7/BUS cells which undergo apoptosis in response to E2 starvation, BiP over-expression protected from mitochondria permeabilization and apoptosis, while its knock-down sensitized the cells to E2-starvation (80). Therefore, BiP induction seems to prevent apoptosis induced by loss of ER activation.

In normal culture conditions, BiP levels were higher in E2-independent cells that were TAM-resistant or antiestrogen resistant (MCF7-RR and MCF-7/LCC9) when compared to those that remained antiestrogen sensitive (MCF-7/LCC1 and MCF-7) (16). In MCF7-RR and MCF-7/LCC9 cells, anti-estrogen treatment elevated BiP mRNA and protein levels to promote survival through distinct pathways: by integrating pro-survival signaling from *Tuberous sclerosis 2* (TSC2) and *AMP-activated protein kinase* (AMPK) with autophagy and UPR (16); and by inhibiting apoptosis through mTOR activation, anti-apoptotic BCL2 family proteins (80) and by reducing caspase-7 (81-83). Supporting this findings, BiP silencing in these cells induced antiestrogen sensitivity (16). On the contrary, BiP over-expression in antiestrogen sensitive MCF7 and LCC1 cells lead to a reduced proliferation rate even in the absence of antiestrogen treatment. The authors suggested that BiP may be involved in the growth-inhibition process and that this function is lost in resistant cells (16). Therefore, BiP appears to be necessary for the maintenance of a resistance phenotype since its over-expression was related with resistance to E2 starvation-induced apoptosis in MCF7-5C, mimicking hormonal therapy resistance (80) and its down-regulation with shRNAs re-sensitizes anti-estrogen resistant cells to anti-estrogen treatment (16).

Novel studies have recently shown that in LCC9 anti-estrogen resistant cells and tumors, reducing BiP with an antisense morpholino diminishes *de novo* fatty acid synthesis and mitochondrial fatty acid transport through down-regulation of SRFBP1

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transcription factor and its downstream genes SCD1, FASN and CPT1A (84).This results in accumulation of linoleic acid, linolenic acid and arachidonic acid due to reduced mitochondrial beta-oxidation and consequent lipotoxicity. Notably, while only reduction of BiP was necessary to reduce SRFBP1 and increase lipid accumulation, co-stimulation with TAM was needed to increase lipid peroxidation and ROS levels. This suggests that high BiP levels in anti-estrogen resistant cells could reduce dependency on ER signaling for survival. Interestingly, BiP morpholinos activated IRE1α-XBP1 branch, which is known to stimulate the SRFBP1 co-activator C/EBPα, as well as lipogenesis genes (85). Still, lipogenic activation by UPR branches is reduced upon chronic stress (85), which explains the results in LCC9 cells and tumors. Metabolic adaptations in endocrine resistance occur through expression of oncogenes like *Myc proto-oncogene protein* (MYC) (86) or *Early growth response protein 1* (EGR1) (86,87). For example, MYC is expressed at higher levels in LCC9 anti-estrogen resistant cells as compared to sensitive counterparts (86) and in glucose-deprived conditions the increase in BiP selectively actives UPR branches to resolve nutritional stress (86). Thus, this body of work has disclosed BiP as an important hub regulating adaptation to nutrient-deprivation and ER signaling that extends beyond the EnR.

T47-D and MCF-7 BC cells, as well as BC samples have higher basal BiP levels than non-neoplastic human mammary epithelial cells and normal mammary tissues, respectively (16,66,72,78,88). *In vivo* studies using a DMBA-induced mouse mammary tumor model that mimics the spectrum of TAM responses seen in patients, showed increased BiP expression in tumors with acquired antiestrogen resistance when compared with tumors showing complete response, or *de novo* resistance (16). DMBA tumors with acquired antiestrogen resistance up-regulated BiP in response to antiestrogen treatment, supporting the idea that BiP up-regulation is an adaptive response of resistant cells to endocrine therapy.

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BiP has also been detected in the cell surface of BC cells (89-91) and was associated with early stages of the disease, with progesterone receptor expression, highest p53 levels and with good prognosis in ERα-positive tumors (90). On the other hand, in triple negative BC, BiP cell surface expression was related with growth inhibition, apoptosis and reduced anti-BiP antibodies in mouse serum (89). Presently, the functional significance of BiP in the cell surface is unknown but may be related to non-genomic effects as shown in PC cells (see below).

- BiP in prostate cancer: in PC tissue samples BiP is over-expressed as disease progresses from early to metastatic androgen-independent state (64,65,92). BiP seems to be essential for PC cell survival, to allow cells to resolve EnR overload in response to the AR anabolic signaling in nutrient-deprived conditions (93). In fact, BiP temporal up-regulation can occur independently of EnR stress, and promotes acute adaptation to nutrient starvation by blocking autophagy (93). However, BiP-mediated autophagy was shown to be critical for the development of androgen-resistant PC (16,93). Therefore, it is not surprising that BiP is also associated with endocrine therapy resistance and its inhibition restored endocrine response in C42B castration resistant cells (15).

PC cells acquire resistance to endocrine therapy by translocating BiP from EnR lumen to the cell membrane (94-96). Although the molecular mechanisms involved in BiP translocation are not completely clear, in PC3 cells, the tumor suppressor *Proteinase-activated receptor 4* (PAR-4) (97,98) as well as the co-chaperone *Dna J-like protein 1* are required (99). In addition, lost or altered EnR amino acid retention motif KDEL in BiP C-terminal domain may also enhance its surface expression and this mechanism can be dependent or independent of EnR stress (100). As a cell membrane protein BiP mediates PI3-K/Akt signaling to stimulate proliferation, invasion and therapeutic resistance of PC cells (94-96).Therefore, BiP can be localized in the extracellular surface of late stage PC cells, but not in the surface of normal cells,

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opening a new opportunity for using this protein as a diagnostic biomarker and/ or as a drug target (92).

In summary, in endocrine-sensitive cells, BiP overactivation has an inhibitory effect. Yet, it is upregulated as BC and PC progresses, and is associated with endocrine-resistance. Whether BiP subcellular localization pays a role in promoting endocrine-resistance in both BC and PC remains to be elucidated.

<u>IRE1α/XBP-1 branch:</u>

- IRE1α branch in breast cancer: MCF-7 and T47-D cells treated with E2 rapidly and transiently increase XBP-1s levels as early as 2 hours after treatment (66,78). XBP-1s over-expression in these cell lines resulted in increased ERα expression and enhanced ERα transcriptional activity, favoring a hormone-independent growth and anti-estrogen resistance (18). This is probably because ERα:XBP-1 alter the expression of several anti-apoptotic genes, such as *Apoptosis regulator Bcl-2* (BCL2) and *Bcl-2-like protein 2 (*BCLW) to inhibit apoptosis as well as cell cycle genes to prevent cell cycle arrest in G0/G1 phase (18,101).

In normal culture conditions XBP-1s levels are higher in TAM resistant MCF-7RR cell than in their sensitive counterparts (20) and TAM sensitivity of these resistant cells could be re-established after treatment with the inhibitor of IRE1α/XBP1 activation, STF-083010 (102). Moreover, in endocrine resistant T47-D/ERαY537S mutant cells in which ERα is constitutively activated, XBP-1s was also constitutively high and BiP as well as $p58^{IPK}$ expression was up-regulated (13), while XBP-1 knock-down in anti-estrogen resistant LCC9 cells re-sensitized cells to antiestrogens (103). On the other hand, in anti-estrogen sensitive MCF-7 and T47-D cells ICI 182 780 or 4OH-TAM treatment inhibited the E2 mediated splicing of XBP-1 (66). XBP-1s is also able to confer E2-independence and resistance to aromatase inhibitors as well as anti-

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estrogens in both MCF-7 and T47-D cells (18). These findings strongly support the link between IRE1α activation, XBP-1s increase and anti-estrogen resistance.

XBP-1u can also interact with ERα and enhance its transcriptional activity. Interestingly, when analyzing ERE-Luc activation in MDA-MB-231 cells transfected with ERα and either XBP-1s or XBP-1u, treatment with the anti-estrogens ICI 182 780 or 4OH-TAM completely blocked the synergistic effects of XBP-1u on ERα transcriptional activity in the presence or absence of E2, whereas both ICI 182 780 and, to a lesser extent, 4OH-TAM reduced but did not abolish the ability of XBP-1s to transactivate ERα (101). Thus, an increase in XBP-1s/XBP-1u ratio may indicate a loss of XBP-1u co-repressor function in the presence of antiestrogens, leading to endocrine resistance. XBP-1s protein interaction with *hypoxia-inducible factor 1α* (HIF1α) increases cell tolerance to hypoxia, facilitating tumor growth by a mechanism independent of angiogenesis (104,105). HIF-1α is an ERα direct transcriptional target and both proteins share many target genes. HIF-1α is able to confer anti-estrogen resistance to MCF-7 cells (106). Therefore, XBP-1s co-activator function can enhance HIF-1α/ERα cross-talk to facilitate endocrine resistance.

The cross-talk between ERα and NF-κβ in endocrine resistance is well documented and has been reviewed elsewhere (107). These interdependencies have a significant impact on cell survival, especially in cells with elevated IKK/ NF-κβ activity such as breast (108) and prostate cancer cells (107), activating downstream NF-κβ signaling (101,103). XBP-1 was shown to regulate NF-kβ signaling. Indeed, anti-estrogen resistant cells up-regulate p65/RelA while, XBP-1 inhibition with siRNAs reduced NF-kβ gene reporter activation through downregulation of RelA/p65 mRNA levels (103). The authors used an ingenious approach to study if XBP-1s or XBP-1u was responsible for these effects and found that while both isoforms can activate NF-kβ, XBP-1u needs ERα expression, while XBP-1s can also induce p65/RelA expression independently of ERα signaling. Thus, while both isoforms can influence

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cell fate decisions by affecting the balance between apoptosis and pro-survival autophagy, XBP-1s is more potent in activating NF-κβ signaling (103). In addition, in combination with translation repression by PERK, IRE1α was able to maintain IKK basal activity, which is critical for maximal NF-κβ activation during UPR activation (109).

In opposition to ERα, which promotes BC cell proliferation, ERβ can, in certain conditions, counterbalance ERα effects and inhibit proliferation and survival (110,111). ERβ over-expression in BC cells has been associated with anti-metastatic and anti-proliferative responses (112), being its expression correlated with an improved response to endocrine therapy (113) and increased patient survival (66,114). ERβ1 isoform in both antiestrogen sensitive and resistant BC cells, activates death pathways through IRE1α/XBP-1 down-regulation while isoform ERβ2 does not alter XBP-1s activity nor BC sensitivity to stress inducers (115).

Changes in XBP-1s levels have been associated with poor prognosis and with anti-estrogen resistance (18,20,30,61,78,101,116,117). On the other hand, XBP-1u favors apoptosis via dominant-negative downregulation of XBP-1s in ERα-positive BC cells (30). In fact, a study of 100 BC samples from patients treated with adjuvant TAM showed that a higher XBP-1s/XBP-1u mRNA ratios were associated with grade 3 tumors, higher proliferation index and poor survival in ERα-positive cases as well as ERα- and PR-negative cases (30). XBP-1 mRNA expression has also been shown to predict a poor response to TAM therapy (30).

Taken together, these results point towards activation of IRE1α to enhance ERα activity and possibly endocrine resistance through up-regulation of XBP-1s which induces transcriptional activation of ERα target genes as well as NFKβ activation, stimulating both estrogen-dependent and independent cell cycle progression and inhibiting antiestrogen induced apoptosis (28). More studies are needed to understand the interplay between IRE1α/XBP-1s UPR branch and ERβ. However, XBP-1s appear

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as a key co-activator of ERα that is inhibited by ERβ1, thus counteracting ERα´s positive regulation of cell cycle and pro-survival genes which supports observations where ERβ over-expression increases endocrine sensitivity (113,118).

- IRE1α/XBP-1s in prostate cancer: in androgen-responsive LNCaP and PC3 cells, AR activation can directly upregulate the expression of IRE1α branch target genes, including IRE1α and XBP-1s themselves. This leads to activation of proliferative signaling and simultaneous inhibition of pro-apoptotic JNK signaling; an effect reversed by IRE1α or XBP-1 knock-down (61). In these experiments, IRE1α mRNA and protein increased in a time-dependent manner from 6h until 36h following R1881 treatment, thereafter, levels decayed but remained significantly higher than the control throughout the time-course study of 184 h. XBP-1s mRNA increase was evident after 12h and showed the same dynamics as IRE1α. In agreement with this, androgen withdrawal in CWR22 cells induces a significant decrease of IRE1α and XBP-1s levels by 72 hours, nearing basal levels at 120h, whereas XBP-1u levels were not affected, resulting in increased apoptosis of CWR22 cells and tumor regression (61). It has been reported that XBP-1s mRNA levels do not change in LNCaP cells after 6 hours treatment with the AR agonist Mibolerone (93), which is possibly due to the fact that XBP-1s increase occurs at later time points as discussed above.

In summary, BC and PC cells respond to ERα and AR activation by up-regulating the IRE1α/XBP-1 pathway. However, in PC cells, this response does not seem to be non-genomic/anticipatory as observed in BC, but a genomic and sustained effect. In endocrine resistant BC cells antiestrogen treatment possibly increases ERα/XBP-1s transcriptional activity, but in sensitive cells this is prevented by increasing the XBP1u/XBP-1s ratio. Currently, there are no studies reporting the effects of anti-androgens on the activation of this UPR branch.

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PERK/eIF2α arm:

- PERK/eIF2α arm in breast cancer: In T47-D cells, p-PERK levels are increased 15 minutes after E2 stimulation and decrease after 45 minutes. This is accompanied by an increase in p-eIF2αand in ATF4, resulting in a modest and transient decline of protein synthesis that is maintained for 40 minutes and without pro-apoptotic CHOP induction. In the same study ICI 182 780 blocked the inhibition of protein synthesis by E2 (66). ICI 182 780 induces ERα protein aggregation and its degradation by the proteasome (119). Therefore, upon ICI 182 780 treatment the cell´s clearing mechanism are being used and preventing the anticipatory response to E2 which may result in unresolved proteotoxic stress leading to ICI 182 780 sensitivity. In fact, long-term treatment of MCF-7/LCC1 antiestrogen sensitive cells with ICI 182 780 (6 days) promotes sustained EnR stress, causing a further activation of apoptotic signaling through PERK-CHOP activation and BCL2 down-regulation (19).

PI3K/Akt/mTOR pathway activation is a clinically relevant aspect of ERα-positive endocrine resistant BC (120-123). MCF-7 cells with constitutive Akt activation escape from growth inhibition induced by 4OH-TAM. This mechanism involves both ligand-dependent and independent activation of ERα in part by mTOR signaling pathway activation (124). Akt negatively regulates PERK through phosphorylation at threonine 799, this prevents induction of apoptosis under severe or chronic EnR stress (125) and could be one mechanism used by resistant cells to evade apoptosis. Moreover, PERK ablation impairs NRF-2 antioxidant response triggering DNA damage (126). On the other hand, under oxidative stress conditions, Akt promotes cell death (127) and this can be antagonized by PERK activation (125) and downstream NRF-2-regulated transcription of antioxidant enzymes (128,129) which has already been shown to protect BC cells from chemotherapy (130). Since during prolonged EnR stress PERK-eIF2α constitute a switch from pro-survival to pro-apoptotic signaling through ATF4, CHOP and NRF-2 up-regulation (131) and the cross-talk between UPR

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and PI3K/Akt/mTOR pathway regulates cell survival in response to different tumor micro-environment insults, stress resolution seems to be dependent on the cellular context and the hierarchical organization of the PERK and PI3-K pathway components.

NF-κβ over-expression can confer estrogen-independence and antiestrogen resistance because of an overlap in their target genes (132) and trans-repressive interaction between these two proteins (107). NF-κβ is a major stress-inducible anti-apoptotic transcription factor and eIF2αinactivation by PERK inhibits the synthesis of NF-κβ inhibitor IkB, thereby enhancing NF-κβanti-apoptotic activity in stressed BC cells (133,134). Moreover, NF-κβ subunit p65 can repress CHOP expression in BC cells, thus protecting cells against EnR stress-induced death (135,136) through the expression of pro-survival genes like BCL-2s, TRAF1/TRAF2 and SOD (137). Therefore, PERK activation could select BC cells for dependence on NF-κβ signaling and thus promote endocrine resistance.

Protein-tyrosine phosphatase 1B (PTP1B) reverts PERK phosphorylation in response to EnR stress (138). PTP1B specifically de-phosphorylates Tyr616, resulting in PERK inactivation and attenuation of this UPR branch (138). PTP1B is commonly over-expressed in BC (139,140), being correlated with ER (141). Therefore, it would be interesting to know if PTP1B is implicated in BC endocrine resistance through down-regulation of the pro-apoptotic PERK-CHOP pathway.

PERK and eIF2α were found overexpressed in BC samples and were significantly associated with high histological grade and with tumor-infiltrating lymphocytes (142). However, it is important to consider that most of the performed studies lack information about therapy and the phosphorylation state of these two proteins, being of interest to verify their expression pattern before and after endocrine therapy.

- PERK/eIF2α in prostate cancer: Androgen treatment in LNCaP cells had no short-term effect on total or phosphorylated PERK and eIF2α protein, while it reduced

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their levels after 24h treatment and until the end of the time-course experiment at 72h. Interestingly, ATF4 and CHOP protein levels increased in a time-dependent manner from 24h until 72h (61). The authors suggest that upon androgen treatment dephosphorylation of PERK and eIF2αresults in a general increase in protein synthesis, compensating the effects observed for mRNA levels. However, in this work CHOP increase wasn´t sufficient to trigger apoptosis as it was counterbalanced by strong activation of IRE1α/XBP-1 pathway (61). On the other hand, when LNCaP cells were cultured in serum-starvation, a rapid PERK/p-eIF2α induction occurred after 2h androgen treatment and was maintained above basal levels for up to 24h, while CHOP protein levels were also found to be upregulated after 24h of starvation (93). Taken together, these data are somewhat contradictory as to whether androgens activate or inhibit PERK/eIF2α branch; yet coincide with the observation that CHOP activation does not lead to increased apoptosis. Currently, there is no information regarding AR-mediated anticipatory/non-genomic activation of this arm, neither its regulation by AR antagonists, nor its function on castration-resistance phenotype.

ATF6 arm: ATF6 activation and its association with BC and PC has received much less attention than IRE1α or PERK. In T47-D BC cells, E2 increased ATF6 proteolysis transiently between 2 and 4 hours after treatment followed by expression of BiP and other chaperones (66). It is known from studies performed in mice and fibroblasts thatATF6 is activated under mild stress conditions to stimulate XBP-1u transcription and prepare cells for possible IRE1α branch activation if stress persists (39,143). In line with this, continued activation of ATF6 and IRE1α during chronic EnR stress in LNCaP cells and in prostate glands of ETS related gene (ERG) transgenic mouse model due to AR aggregation, induced survival pathways and selective pressure throughout the neoplastic process (60).

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6. Conclusions and perspectives

Although the mechanisms by which UPR participates in tumorigenesis and interferes with anti-cancer therapies are not completely clear, current information supports its role in up-regulating pro-survival signaling, being correlated with poor response and resistance to endocrine therapy, disease progression, metastasis, shorter time to recurrence and decreased overall survival in both BC and PC patients. Thus, it is important to understand the contribution of each UPR arm and their downstream proteins, in order to disclose targets to enhance therapy response. With this in mind, we have summarized the current knowledge about UPR activation in endocrine response of breast and prostate cancer (Table 1 and Figure 3).

Current knowledge on UPR function in endocrine response and resistance in PC cells is very limited 1. Future studies to provide new insights about the regulation of these pathways and their role in development of castration-resistance are needed. In addition, the effect of AR and ERα agonists and antagonists at different time points leads to a variety of effects on UPR branch activation. Therefore, studies are needed to understand how the diversity of responses are modulated by acute or chronic exposure to different agonists, antagonists and other endocrine regulators as well as how the mutational landscape of the cell contributes to shaping the UPR outcome. This will further our understanding of how selective UPR arm activation can influence to clonal evolution.

An increasing number of publications support the idea that BiP is a key participant in BC and PC tumorigenesis. Its up-regulation is correlated with decreased apoptosis, promotion of angiogenesis, tumor progression and development of therapy resistance. One possible explanation is that higher BiP levels confer a better proteotoxic resolution and would increase the amount of BiP bound to IRE1α, PERK and ATF6, this in turn would result in a milder UPR activation favoring survival responses rather than cytostasis or death. On the other hand, it was observed that BiP

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is involved in growth inhibitory mechanisms in endocrine therapy sensitive cancer cells that are lost in resistant cells, the mechanisms behind this remain to be disclosed. However, the fact that BiP subcellular distribution could impact non-genomic signaling directing cells toward apoptosis or survival needs to be explored.

ATF6 remains the less explored branch of UPR and its interaction with IRE1α/XBP-1s and PERK arms remain to be disclosed. It will also be interesting to study ATF6 threshold levels involved in differential UPR activation in high or mild stress conditions. These threshold levels may be important in the context of endocrine therapy resistance.

Since resistant BC and PC cases remain a major clinical problem, new therapeutic approaches and better predictors of therapeutic response are clearly needed. Due to UPR involvement in cell proliferation, apoptosis evasion and drug-resistance phenotypes targeting these pathways have recently emerged as a promising strategy in anti-cancer therapy. Thus, testing the expression levels of proteins of the UPR, such as BiP or XBP-1as biomarkers in BC and PC may be useful to predict therapy responsiveness and would reduce the exposure to therapeutic agents that are not likely to be beneficial. Additionally, combining existent BC and PC therapies with modulation of UPR network may be a promising strategy to sensitize resistant cancer cells to therapy and to improve clinical outcome.

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Author contributions

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All the authors made substantial contributions to the conception, design, critical discussion and writing of the manuscript.

Conflict of interest

Authors declare no conflict of interest.

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Fig.1- Unfolded protein response (UPR) pathways. (**A**) The UPR is composed of three different effector branches, protein kinase RNA-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme 1α (IRE1α) and activating transcription factor 6 (ATF6), which in unstressed conditions remain inactive by association with the EnR stressor sensor-binding immunoglobulin protein (BiP); (B)Initiation of UPR is an attempt to restore proteostasis in response to EnR stress provoked by metabolic changes, hypoxia, acidosis, nutrient deprivation and/or gene mutations. The three different UPR effectors are activated by dissociation from the complexes formed with BiP, leaving BiP free to interact with misfolded proteins within the EnR lumen. Activated PERK phosphorylates and inhibits the eukaryotic initiation factor 2α (eIF2α), which turns off protein synthesis. PERK also increases expression of activating transcription factor 4 (ATF4), which in turn induces the expression of anti-oxidative enzymes, promotes amino acid synthesis and protein folding. Dissociation from BiP leads to IRE1α homodimerization and autophosphorylation, activating its endonuclease activity and promoting the subsequent splicing of X-box binding protein 1 (XBP-1) mRNA. Spliced XBP-1 (XBP-1s) gives origin to a transcription factor that up-regulates genes involved in protein folding, lipid metabolism, quality control and endoplasmic-reticulum-associated degradation (ERAD). Finally, upon its release from BiP, ATF6 translocates to the Golgi where it is cleaved by proteases, releasing an N-terminal fragment that acts as a transcription factor to stimulate the transcription of XBP-1u and ERAD-associated genes.

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Fig. 2 – Molecular mechanisms of gene expression regulation mediated by Estrogen Receptor α (ERα) and Androgen Receptor (AR). ERα and AR are transcription factors which exert their molecular functions by regulating gene expression. In the genomic pathway, ERα and AR, illustrated here as nuclear factors (NF) bind their agonist, 17β-estradiol (E2) and dihydrotestosterone (DHT), respectively which leads to their dimerization. Nuclear translocation of receptor-ligand complexes results in 1) direct DNA binding along with coactivators (CA) to form a transcription complex; 2) indirect DNA binding by recruitment of other transcription factors (TF) like Fos/Jun or SP-1. In addition, ERα and AR can be activated by phosphorylation through other signaling pathways, like growth factor signaling, that results in their dimerization, DNA binding and gene regulation in a ligand-independent way. The genomic pathway induces the transcription of genes involved in cell proliferation and metabolism. In the non-genomic pathway ligand binding activates membrane-associated receptors or receptors located in the cytoplasm, promoting the activation of signaling cascades such as phospholipase C (PLC)/protein kinase C (PKCs), Ras/Raf/MAPK, phosphatidyl inositol 3 kinase (PI3K)/Akt and cAMP/ protein kinase A (PKA). The non-genomic pathway results in rapid physiological responses without direct gene regulation by NRs. ERα and AR actions may be blocked by endocrine therapy. Selective receptor modulators such tamoxifen (or its active metabolite 4-hydroxytamoxifen) or Flutamide promote ERα or AR binding, respectively, to DNA and recruit transcriptional co-repressors (CR) to inhibit gene expression. Selective receptor disruptors such as Fulvestrant or Bicalutamide, induce ERα or AR proteasomal degradation, respectively.

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Fig.3- Estrogens and anti-estrogens promote UPR activation in breast cells. (**A**) Upon estrogen treatment all the 3 branches of the UPR are activated preparing the cells for high protein synthesis demands that precedes cell proliferation. PERK, through eIf2α blocks translation and ATF6 and IRE1α branches are responsible for the induction of molecular chaperones to increase protein folding capacity and induction of endoplasmic-reticulum-associated degradation (ERAD) mechanisms that alleviate endoplasmic reticulum (EnR) overload. (**B**) In BC cells sensitive to endocrine therapy, BiP is upregulated in an attempt to resolve EnR stress caused by treatment, IRE1α/ XBP1 branch is downregulated and in parallel PERK pathway trough eIf2α / ATF4 /CHOP and JNK signaling are upregulated promoting the expression of pro-apoptotic proteins. mTOR signaling seems also to be slightly induced by treatment promoting autophagy that in high levels is also responsible for cell death. (**C**) Antiestrogen resistant BC cells in response to endocrine therapy activate UPR through BiP /IRE1α/ XBP-1 branch upregulation, ultimately promoting cell survival and antiestrogen resistance by increasing protein folding capacity and activation of ERAD mechanisms. In parallel with BiP, GRP94 is one of the EnR chaperones that is mostly upregulated upon therapy. Simultaneously, PERK through phosphorylation of eIf2α and blocking translation of NF-κβ inhibitor (IKBα) promotes pro-survival NF-κβ signaling and inhibition of pro-apoptotic CHOP. PERK activation also induces NRF-2 anti-oxidant transcription factor contributing for an anti-oxidative stress response. A pro-survival upregulation of a mTOR-independent autophagy program seems also to be activated contributing for cell survival.

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Figure 1

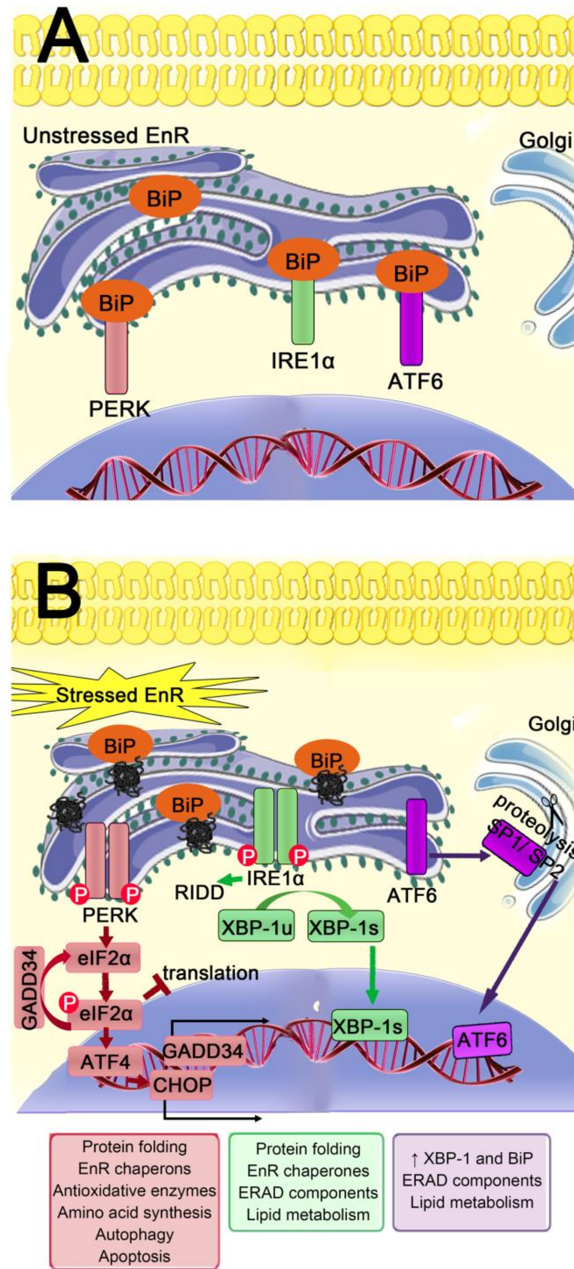

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Figure 2

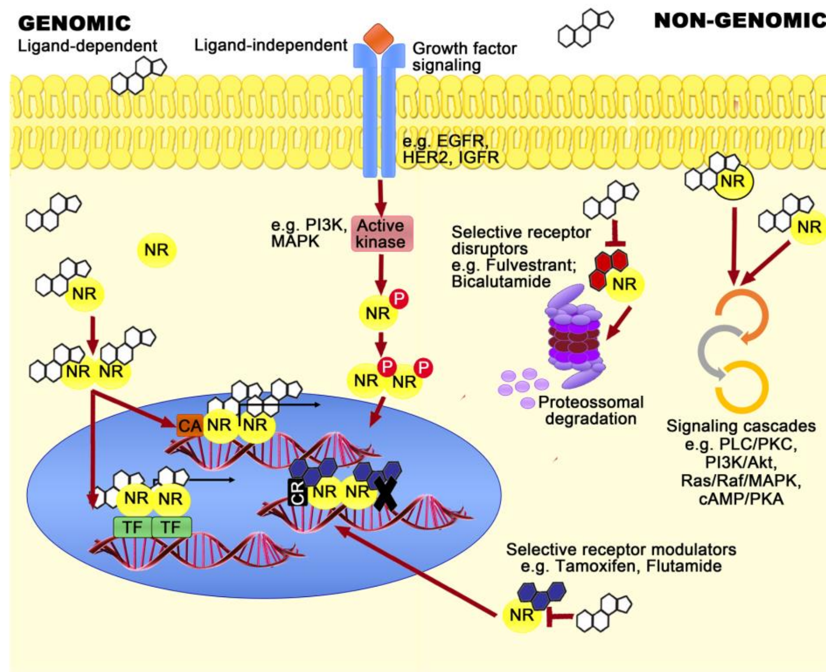

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Figure 3

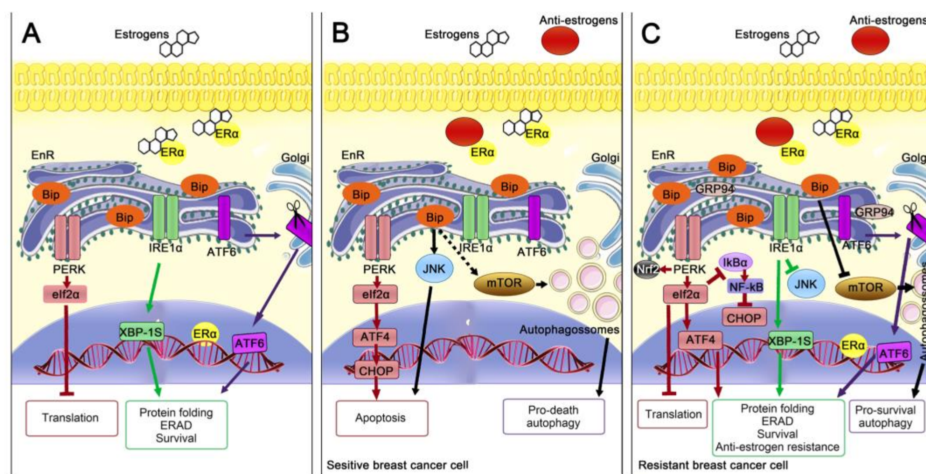

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Table 1. Influence of endocrine treatment on UPR activation in Breast and Prostate Cancer cells

UPR Branch	Condition	Sample	mRNA	Protein	References
		BreastCancer			
BiP /GRP94	EstrogenTreatment	T47D cells, MCF7 cells, MCF7 xenografts	BiP; GRP94	BiP; GRP94	(65,77)
	ICI or TAM treatment	LCC9 cells, LCC1/BiP+ cells, T47D cells, LCC1 cells, MCF7RR cells, TR5 cells	BiP	BiP	(19,77)
	Cancervs Normal	Non-treated BC tissue samples from patients	BiP	BiP; GRP94	(16,65,77,83)
IRE1α	EstrogenTreatment	T47D cells, MCF7 cells, MCF7 xenografts	IRE1α; XBP1-s	IRE1α; XBP1-s; XBP1-u	(65)
	ICI or TAM treatment	T47D cells, LCC1 cells	IRE1α; XBP1-s	XBP1-s	
	Cancervs Normal	Invasive ductal adenocarcinoma tissue samples of patients treated with TAM; Non-treated BC tissue samples from patients		IRE1α; XBP1	(65,77,116)
PERK	EstrogenTreatment	T47D cells, MCF7 cells, MCF7 xenografts	PERK; eif2α; ATF4	PERK; ATF4	(65)
	ICI or TAM treatment	LCC9 cells, LCC1/BiP+ cells, MCF7RR cells, TR5 cells,		PERK; CHOP	(16,19)
		T47D cells, LCC1 cells		PERK; eif2α; CHOP	(19,65)
	Cancervs Normal	Invasive ductal adenocarcinoma tissue samples of patients treated with TAM		eif2α	(65)
ATF6	EstrogenTreatment	T47D cells, MCF7 cells, MCF7 xenografts	ATF6		(65)

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	Cancervs Normal	Invasive ductal adenocarcinoma tissue samples of patients treated with TAM		ATF6	
		ProstateCancer			
BiP	AndrongenTreatment	LNCaPcells; VCaPcells		BiP	(15,60)
	Cancer vs Normal; Metastatic vs early stage castration resistant PC	Tissue samples frompatients*			(24,63,64)
IRE1α	AndrongenTreatment	LNCaPcells; CWR22 xenografts	IRE1α; XBP1-s; XBP1-u		(60)
	Cancervs Normal;	Tissue samples frompatients*		XBP1-s	
	Hormonetherapysensitive PC	Tissue samples frompatients		XBP1-s	
PERK	AndrongenTreatment	LNCaPcells; VCaPcells		PERK;eif2α; ATF4; CHOP	(60)

Upregulation in red; downregulation in green. *Without information about patients' treatment. ATF4, activating transcription factor 4; ATF6, activating transcription factor 6; BC, breast cancer; BiP, sensor-binding immunoglobulin protein; CHOP, C/EBP homologous protein; eIF2α, eukaryotic initiation factor 2α; IRE1α, inositol-requiring enzyme 1α; PC, prostate cancer; PERK, protein kinase RNA-like endoplasmic reticulum kinase; TAM, hydroxytamoxifen; XBP-1, X-box binding protein 1; XBP-1s, spliced XBP-1; XBP-1u, unspliced XBP-1.

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