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Carolina Lopes Melo

PROGNOSTIC SIGNIFICANCE OF PLCγ1 IN LUMINAL A BREAST CANCER: INSIGHTS INTO CELL CYCLE REGULATION AND ESTROGEN SIGNALING

Dissertação no âmbito do Mestrado em Investigação Biomédica, ramo de especialização em Oncobiologia, orientada pela Doutora Marta Sofia Alves Martins e apresentada à Faculdade de Medicina da Universidade de Coimbra.

Setembro de 2023

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Carolina Lopes Melo

Orientadora: Doutora Marta Sofia Alves Martins

Orientadora Interna: Doutora Salomé Pires Lourenço

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Agradecimentos

Em primeiro lugar quero agradecer ao Professor Luís Costa por me ter recebido como estudante de mestrado e dado a oportunidade de desenvolver este trabalho no seu laboratório. À minha orientadora, Doutora Marta Martins, por ter acreditado e confiado em mim para realizar este projeto. Obrigada pelas críticas, opiniões, transmissão de conhecimento, apoio e confiança depositada. À Raquel Duarte, que desde o primeiro dia foi um apoio incondicional. Obrigada pela disponibilidade para me orientar e ajudar neste trabalho, pela paciência, por todos os ensinamentos e conhecimentos transmitidos, pelos conselhos e amizade.

Às pessoas a quem mais devo esta tese, os que me acompanharam diariamente nesta caminhada, ouviram as minhas dúvidas existenciais e deram o seu apoio incondicional, dentro e fora do laboratório. À Raquel, minha companheira de todas as horas, fins-de-semana, feriados e madrugadas. Obrigada pela paciência, companheirismo, amizade e aulas de *moles*. Foi um prazer trabalhar ao teu lado. À Maria, minha companheira da *água salgada* porque o "pessoal da borda d'água é todo igual", és, também, uma inspiração. Obrigada *powerpuff girls* por todos os momentos que partilhámos este ano, choros, gargalhadas e muito *gossip, xoxo gossip girl.* Que *Sweet Dreams* nos dê sempre o empoderamento que precisamos. Ao Rúben, este amigo da vida e para a vida, com quem tenho o prazer de trabalhar lado a lado há 7 anos, é tão bom ver-nos crescer. Ao Coelho, a boa disposição em pessoa. Obrigada por trazeres leveza e alegria às nossas vidas. E ao Miguel, pela paciência, apoio e carinho. Obrigada por me ouvires nos momentos mais difíceis. Espero contar com vocês para o resto dos dias da minha vida.

Também quero agradecer aos restantes membros do Luís Costa Lab, por me terem recebido tão bem e por todo apoio ao longo deste ano, e ao nosso laboratório vizinho, Sérgio Dias Lab. Sérgio, obrigada por todos os conselhos, ajuda e motivação. Ana, obrigada pela boa disposição, partilha de artigos e discussão científica, mas também pela palavra amiga.

Não posso deixar de agradecer ao Professor Fernando e à Professora Diana que irão sempre fazer parte do meu percurso académico, profissional e também pessoal, esta tese também se deve a vocês. Obrigada por acreditarem em mim e me motivarem sempre para fazer mais e melhor.

Por fim, um agradecimento muito especial à minha família, nada disto seria possível sem vocês. Obrigada por todos os vossos sacrifícios e as oportunidades que me proporcionaram ao longo da minha vida. Sou eternamente grata.

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Abbreviations

γSA	PLCγ specific array
AKT	v-akt murine thymoma viral oncogene homolog
AI	Aromatase inhibitor
AITL	Angioimmunoblastic T-cell lymphoma
ATL	Adult T cell leukemia/lymphoma
BC	Breast cancer
BSA	Bovine serum albumin
Ca^{2+}	Calcium
cDNA	Complementary deoxyribonucleic acid
CDK	Cyclin-dependent kinase
CT	Threshold cycle
CTCLs	Cutaneous T-cell lymphomas
DAG	Diacylglycerol
DMFS	Distant metastasis-free survival
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DFS	Disease-free survival
E2	17β-estradiol
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
ERE	Estrogen-responsive element
ERK	Extracellular regulated kinase

ESMO	European society for medical oncology
ET	Endocrine therapy
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FDA	Food and drug administration
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FLWT	Full-length wild-type
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
h	Hours
HEPES	N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid
HER2/ERBB2	Epidermal growth factor receptor 2
HNSCC	Head and neck squamous cell carcinoma
HRP	Horseradish peroxidase
IHC	Immunohistochemistry
IP_3	Inositol 1,4,5-trisphosphate
JAK	Janus kinase
KO	Knockout
LRFS	Local relapse-free survival
KRAS	Kirsten rat sarcoma viral oncogene homolog
MAPK	Mitogen activated protein kinase
min	Minutes
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
OS	Overall survival
OSCC	Oral squamous cell carcinoma
PAR	Parental

pCR	Pathological complete response
PDGF	Platelet derived growth factor
PDGFR	Platelet derived growth factor receptor
Pen/Strep	Penicillin-streptomycin
PH	Pleckstrin domain
PI3K	Phosphatidylinositol 4,5-bisphosphate 3-kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PLCγ/PLCG	Phospholipase C gamma
RPM	Rotations per minute
RT	Room temperature
RT-qPCR	Quantitative reverse transcription PCR
RTK	Receptor tyrosine kinase
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SERD	Selective estrogen receptor down-regulator
SERM	Selective estrogen receptor modulator
SH2/SH3	Src homology 2/3
STAT	Signal transducer and activator of transcription
TBS	Tris-buffered saline
TCEP	Tris(2-carboxyethyl)phosphine hydrochloride
TCGA	The cancer genome atlas
TGF-α	Transforming growth factor α
TNBC	Triple-negative breast cancer
UPR	Unfolded protein response
VEGF	Vascular endothelial growth factor

VEGFR	Vascular endothelial growth factor receptor

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Abstract

Phospholipase C gamma 1 (PLCγ1) is an essential mediator of cell signaling, activated downstream of receptor tyrosine kinases (RTKs). Dysregulation of PLCγ1 signaling has been associated with tumor progression, with PLCγ1 being overexpressed in several human tumors, including breast cancer (BC), which is the most common cancer and the leading cause of cancer-related deaths among women worldwide.

Previous studies have described PLCγ1 overexpression as a risk factor in patients with early Luminal A BC. Since Luminal A BC is characterized by estrogen receptor (ER) expression, the standard treatment for these patients is endocrine therapy and, in some cases, combination with CDK4/6 inhibitors. However, not all patients respond to therapy, due to innate or acquired therapy resistance. Therefore, the development of new biomarkers capable of distinguishing patients who will respond to therapy is essential for improving breast cancer treatment approaches.

In this thesis we aimed to understand the prognostic value of PLCγ1 expression and function in Luminal A BC, through the crosstalk between PLCγ1 and the ER signaling pathway. To this end, we developed several *in vitro* assays for Luminal A BC, with downregulation or overexpression of PLCγ1. The effect of PLCγ1 expression in Luminal A BC was assessed by cell viability assays and cell cycle analysis. To understand the interaction between PLCγ1 and ER, the different assays were performed with and without estradiol stimulation.

Overall, our results did not show a correlation between PLCγ1 and the ER signaling pathway, nor a significant impact of PLCγ1 on the viability of Luminal A BC cell lines. Nevertheless, PLCγ1-deficient cells showed impaired cell cycle progression with loss of CDK6 and CDK2 expression. However, this dysregulation of the cell cycle did not affect the response to CDK4/6 inhibitors in our models.

Future research is needed to understand the role of these signaling pathways in Luminal B cell lines, as preliminary results from our group have shown that PLCγ1 expression may have a different prognostic value between Luminal A and B BC.

Keywords: Phospholipase C gamma 1; Breast Cancer; Estrogen Receptor alpha; Cell Cycle Checkpoints; Target therapy.

Resumo

A fosfolipase C gamma 1 (PLCγ1) é um mediador essencial de sinalização celular, ativado a jusante dos recetores tirosina-quinases (RTK). A desregulação da sinalização da PLCγ1 tem sido associada à progressão tumoral, estando a PLCγ1 sobre expressa em vários tumores humanos, incluindo o cancro da mama (CM), que é o cancro mais comum e a principal causa de morte por cancro entre as mulheres em todo o mundo.

Estudos anteriores descreveram a sobreexpressão da PLCγ1 como um fator de risco em doentes com CM Luminal A. Uma vez que o CM Luminal A é caracterizado pela expressão do recetor de estrogénio (RE), o tratamento padrão para estes doentes é a terapia endócrina e, em alguns casos, a combinação com inibidores CDK4/6. No entanto, nem todos os doentes respondem à terapia, devido à resistência inata ou adquirida. Assim, o desenvolvimento de novos biomarcadores capazes de distinguir os pacientes que responderão à terapia é essencial para melhorar as abordagens de tratamento do CM.

Nesta tese pretendemos compreender o valor prognóstico da PLCγ1 no CM Luminal A, através da interação entre a PLCγ1 e a via de sinalização do RE. Com este objetivo, desenvolvemos vários ensaios *in vitro* de CM Luminal A, com regulação negativa e sobreexpressão de PLCγ1. O efeito da expressão da PLCγ1 no CM Luminal A foi avaliado por ensaios de viabilidade celular e análise do ciclo celular. Por fim, para compreender a interação entre o PLCγ1 e o RE, os vários ensaios foram desenvolvidos com e sem estimulação de estradiol.

Em geral, os nossos resultados não mostram uma relação entre a PLCγ1 e a via de sinalização do RE, nem um impacto significativo da PLCγ1 na viabilidade das linhas celulares de CM Luminal A. No entanto, as células com reduzida expressão de PLCγ1 mostraram um défice na progressão do ciclo celular com perda de expressão de CDK6 e CDK2. Ainda assim, esta desregulação do ciclo celular não afetou a eficácia da terapia dirigida com inibidores da CDK4/6.

Outros estudos são necessários para compreender o papel destas vias de sinalização em linhas celulares Luminal B, uma vez que resultados preliminares do nosso grupo demonstraram que a PLCγ1 pode ter um valor prognóstico diferente entre os CM Luminal A e B.

Palavras-chave: Fosfolipase C gamma 1; Cancro da Mama; Recetor de Estrogénio alpha; Regulação do ciclo celular; Terapia dirigida.

1 Introduction

1.1 Phospholipase C enzymes

The phospholipase C (PLC) family members are intracellular enzymes involved in signal transduction. The most well-established cellular signaling mediated by all PLC enzymes is the phosphoinositide signaling pathway, in which PLC hydrolyzes the membrane phospholipid phosphatidylinositol 4,5-biphosphate (PIP_2) into two secondary messengers, diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3) (1). DAG and IP_3 are responsible for the regulation of multiple cellular processes, through the activation of protein kinase C (PKC) and the release of Ca^{2+} from the endoplasmic reticulum into the cytoplasm, respectively (2).

Mammalian PLCs are divided into seven families (β, γ, δ, ε, η, ζ and XD) that cover 14 PLCs isoenzymes (PLCβ1, PLCβ2, PLCβ3, PLCβ4, PLCγ1, PLCγ2, PLCδ1, PLCδ3, PLCδ4, PLCε, PLCη1, PLCη2, PLCζ and PLC-XD) (3,4). The common structure of all PLCs consists of a pleckstrin homology (PH) domain, four tandem EF hand domains, a catalytic triosephosphate isomerase (TIM) barrel-like domain split into X/Y boxes by an X–Y linker, and a C2 domain (5). The exceptions for this common structure are PLC-ζ, that lacks the PH domain, and PLC-XD, considered an atypical PLC, characterized only by a conserved X in the catalytic domain (6).

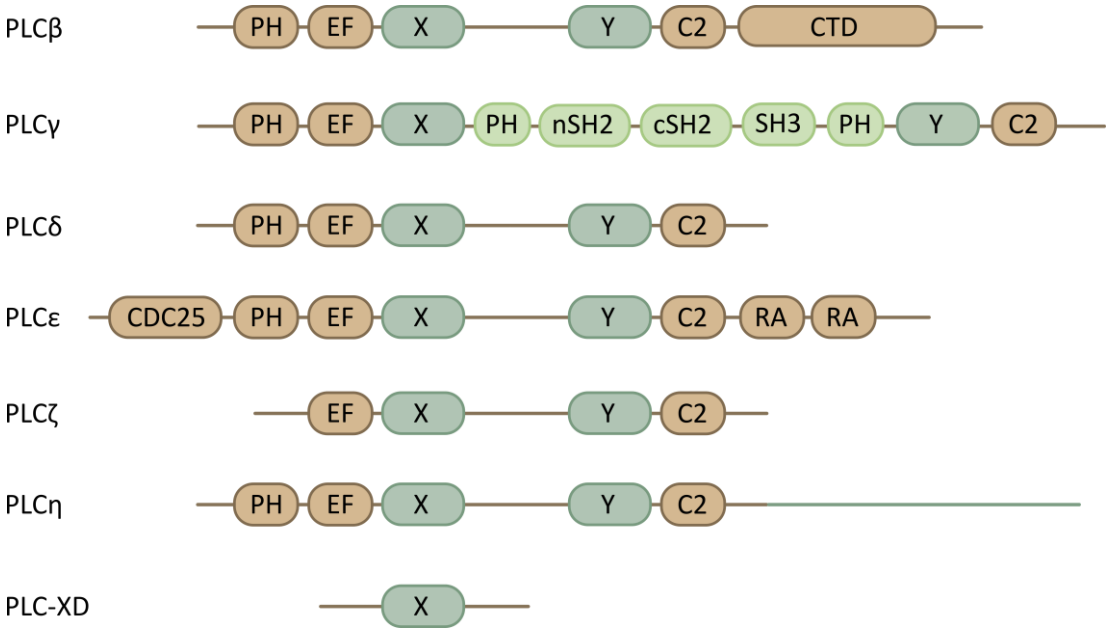


Figure 1.1. Structure and domains of the seven PLC families. Except for PLC-ζ and PLC-XD, they share a common structure composed of an N-terminal PH domain, four tandem EF repeats, the TIM barrel catalytic domain, and a C2 domain. The catalytic domain consists of X and Y regions and the linker between them. PLCβ has a C-terminal CTD domain. PLCγ contains a split-PH domain, nSH2, cSH2, and an SH3 domain between its X–Y linker. PLCε has an N-terminal

CDC25 domain and two C-terminal RA domains. PLCη possesses an extended C-terminal tail. PLC-XD is a single-domain protein with only the X region in the catalytic domain.

The PH domain engages with PIP_2 and various proteins like the heteromeric G protein subunit Gβγ, facilitating the translocation and activation of PLC. Additionally, the EF hand and C2 domains interact with Ca^{2+}, enhancing PLC enzymatic activity (7). However, enzymes involved in signal transduction frequently require selective intermolecular interactions mediated by modular domains, such as the Src homology 2 (SH2) domain, which recognizes phosphotyrosine motifs generated by post-translational modifications, and the Src homology 3 (SH3), which recognizes polypeptide motifs characterized by specific composition (8).

1.1.1 PLCγ family

Within PLC families, PLC gamma (PLCγ) enzymes are key components of signaling networks. The X-Y linker of this family contains a specific array of domains (γSA), comprising a C-terminal SH2 (cSH2), an N-terminal SH2 (nSH2), an SH3 domain and a ‘‘split’’ PH (spPH) domain, inserted between the X/Y boxes (8). PLCγ1 and PLCγ2 have similar structure and regulation, however, PLCγ1 is ubiquitously expressed and is involved in cell growth and differentiation in response to receptor tyrosine kinases (RTK), while PLCγ2 is mostly expressed in hematopoietic cells, and acts downstream of soluble tyrosine kinases recruited by B cell receptors in acute responses (3,9).

In the inactive form of PLCγ1, the cSH2 domain is folded on top of the catalytic core, inhibiting the phosphorylation of this enzyme, which is in the cytoplasm in its inactive form. For its activation, PLCγ1 is recruited to the plasma membrane by the binding of the nSH2 domain to a phosphotyrosine residue of the RTK cytoplasmic tail. PLCγ1 is subsequently phosphorylated at Tyr783, resulting in an intramolecular association with the cSH2 domain, which leads to a conformational change, leaving the catalytic core free for PIP_2 hydrolysis (4,5,10).

Given that PLCγ1 is basally autoinhibited by its X–Y linker, in particular by its cSH2 domain, mutations in the cSH2 domain or in its catalytic core can lead to a constitutive activation of PLCγ1. The PLCγ1 catalytic core is electronegatively charged, and some residues are crucial for the interaction with cSH2. For instance, the mutation of D1019 residue to an oppositely charged amino acid (D1019K) completely affects its ability to interact with the cSH2 domain and, consequently, activates the enzyme constitutively (5,8,11).

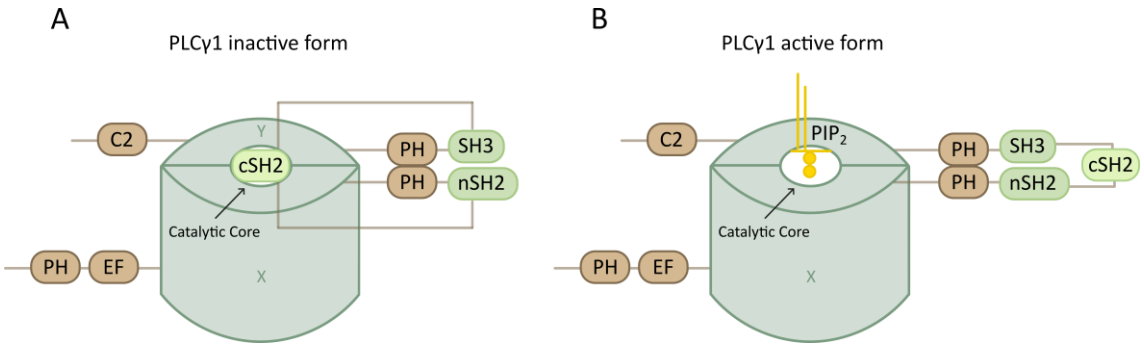


Figure 1.2. PLCγ1 3D structure. (A) PLCγ1 inactive form, with the cSH2 domain folded on top of the catalytic core, inhibiting the phosphorylation of this enzyme. (B) PLCγ1 active form suffers a conformational change, where cSH2 domain leaves the catalytic core free for PIP_2 hydrolysis.

1.1.2 PLCγ1 physiology

PLCγ1 can be activated by extracellular stimuli such as neurotransmitters, hormones, lipids, and growth factors, which signal through RTKs, such as epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), fibroblast growth factor receptor (FGFR), and vascular endothelial growth factor receptor (VEGFR). Once activated, PLCγ1 not only leads to DAG production and Ca^{2+} release, but also interacts with several molecules and consequently regulates multiple cell signaling processes, including cell proliferation (12), angiogenesis (13), receptor endocytosis (14) and cell motility (15), contributing to the maintenance of cellular homeostasis.

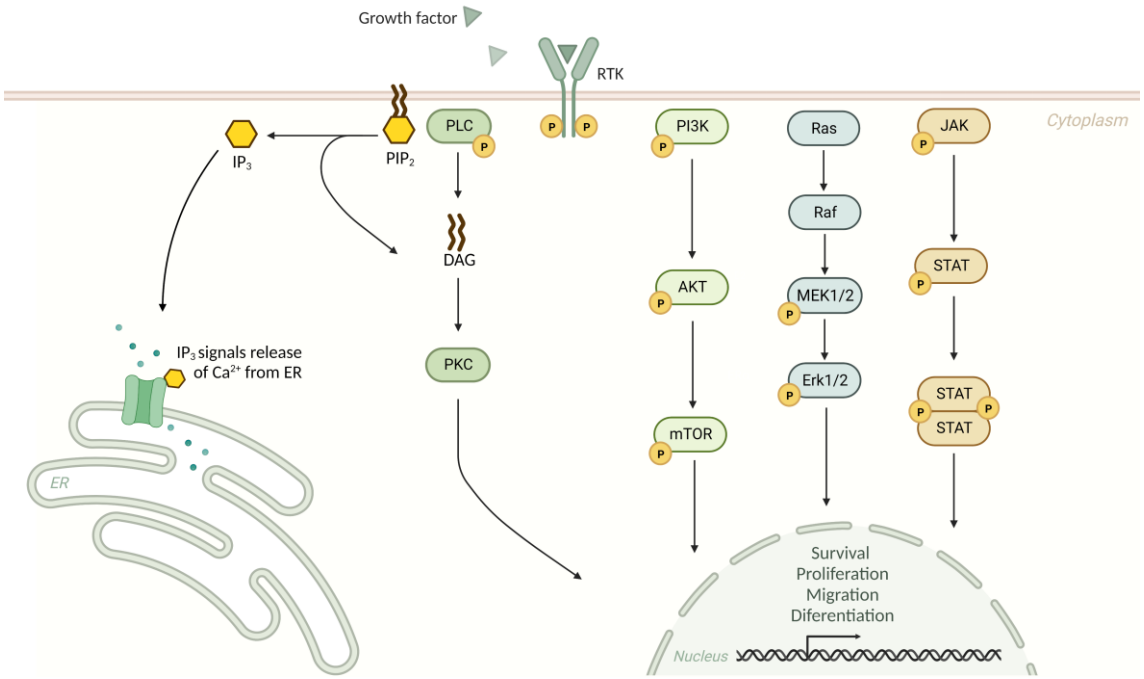


Figure 1.3. Activation and function of PLCγ1. In response to extracellular stimuli, PLC is activated by direct binding to RTKs. Activation of these receptors also stimulates other signaling pathways, including PI3K/AKT, MAPK/ERK and

JAK/STAT. Active PLC hydrolyzes PIP_2 into IP_3 and DAG. IP_3 causes Ca^{2+} release from the endoplasmic reticulum into the cytoplasm and DAG activates PKC and other DAG-dependent proteins. In addition, active PLC also interacts with the signaling pathways described above, promoting cell survival, proliferation, migration, and differentiation. Created with BioRender.com.

1.1.2.1 Cell proliferation

Cell proliferation plays a crucial role in embryonic development, tissue growth and repair, and the maintenance of organisms (16). As a process by which cells divide and multiply, it is tightly regulated to ensure proper control and balance of cell growth. Key players in the regulation of cell proliferation, cyclins and cyclin-dependent kinases (CDKs) control cell cycle transition, progression, and arrest (17).

Cell cycle is triggered by the binding of CDK4/6 to D-type cyclins (D1, D2 and D3), in response to mitogenic, hormonal, and growth factor signals. This CDK4/6-cyclin D complex phosphorylates the retinoblastoma protein (Rb) allowing the release of the E2F transcription factor (18). Cyclin E1 (*CCNE1*) and cyclin E2 (*CCNE2*) are E2F-target genes, which are subsequently activated, forming a G1/S checkpoint complex by binding to CDK2, leading to hyperphosphorylation of Rb and promotion of S-phase entry and DNA synthesis (19).

Inhibition of cell cycle progression can occur through the INK4 family (including $p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$, and $p19^{INK4D}$) and the CIP/KIP family (consisting of $p21^{Waf1/Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$). These CDK inhibitors avoid inappropriate cell division by effectively suppressing cell cycle progression (20).

Multiple studies have demonstrated that PLCγ1 can induce cell cycle progression. Microinjection of purified PLCγ1 into quiescent (G0) mouse fibroblasts induced DNA synthesis and PLCγ1 inhibition blocked PDGF-induced DNA synthesis, independent of PLCγ1 lipase activity (12,21). Nonetheless, other studies revealed that PLCγ1 lipase activity is crucial to induce mitogenesis. Wang *et al.* showed that PLCγ1 without its catalytic domain inhibits cell proliferation and only DAG and PKC can reverse this effect and Lee *et al.* also showed a reduction in cell proliferation by inhibiting PLCγ1 lipase activity (22,23). Moreover, Take *et al.* revealed that PLCγ1 is required for the expression and translocation of CDK4 to the nucleus and for the nuclear export of p27, in FGF-induced cell cycle progression, of corneal endothelial cells (23).

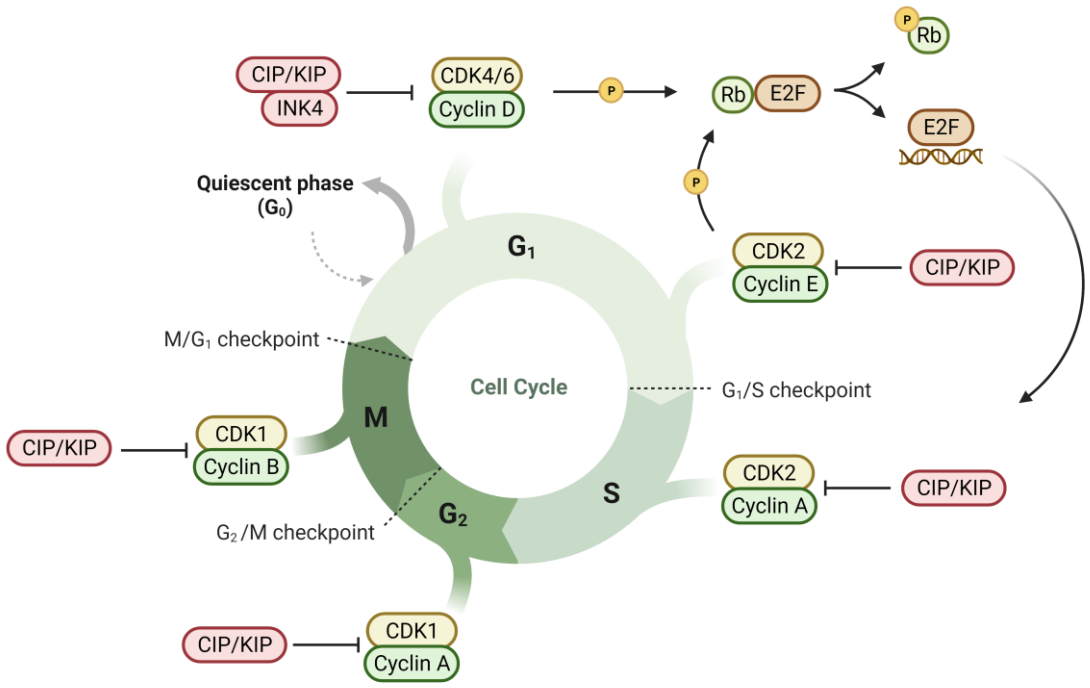


Figure 1.4. Cell Cycle in eukaryotic cells. Following a mitogenic stimulus, CDK4/6 binds to cyclin D, which results in the phosphorylation of Rb, releasing it from the Rb/E2F complex. E2F then transcribes target genes for cell cycle progression, such as cyclin E, which binds to CDK2, activating the G1/S checkpoint complex, leading to hyperphosphorylation of Rb and promoting entry into S phase and DNA synthesis. Created in Biorender.com

1.1.2.2 Angiogenesis

Concerning angiogenesis, PLCγ1 has been extensively implicated as a critical component in VEGF signaling. This is evident from studies showing that PLCγ1-deficient mice exhibit early embryonic mortality at approximately day 9, due to impaired angiogenesis and erythropoiesis (13,24). Moreover, zebrafish embryos with a catalytic inactive PLCγ1 mutation show defects in artery formation, and when PLCγ1 lipase activity is inhibited, it reduces Ca^{2+} transport in the ventricular cardiomyocytes, causing the loss of their spontaneous contractile activity (25,26).

1.1.2.3 Cell motility

Cell motility is involved in several biological processes, such as tissue repair and regeneration, immune response, and the formation of complex tissues and organs at the embryonic stage (27). During cell motility, actin polymerization is particularly critical. This process leads to the creation of cell protrusions essential for adhesion to the extracellular matrix, establishing the direction of migration and initiating cell tracking (28).

Actin polymerization is regulated by a complex network of signaling pathways and associated proteins, including Arp2/3 complex, cofilin and profilin (29). Arp2/3 complex nucleates actin filaments, whereas cofilin and profilin are actin-binding proteins. Cofilin enhances actin

depolymerization, by promoting the recycling of actin monomers, making them available for subsequent actin polymerization. Profilin promotes actin polymerization and contributes to filament elongation (30).

Studies showed that PLCγ1 is required for cell motility through its involvement in cytoskeletal remodeling, either by its lipase activity or by interaction with other molecules. PIP_2 acts as an inhibitor of the binding between cofilin/profilin and actin. Consequently, PLCγ-mediated PIP_2 hydrolysis is crucial for cofilin and profilin activity (27,31,32). In addition, PKC phosphorylates and activates focal adhesion kinase (FAK) and profilin, while Ca^{2+} release stimulates myosin activity and modulates focal adhesions (33,34). Jones *et al.* showed that depletion of PLCγ1 resulted in the inability of endothelial cells and fibroblasts to form cellular protrusions and undergo cell spreading and elongation in response to integrin engagement, which reveals the importance of PLCγ1 in cell motility (35).

Besides PLCγ1 lipase activity, some authors described that the SH3 domain of PLCγ1 directly interacts with Rac1 and AKT in response to EGF, inducing cytoskeletal reorganization that facilitates cell motility (15,36,37). Furthermore, integrin-induced activation of PLCγ1 leads to the activation of Pyk2, a non-receptor tyrosine kinase, and recruitment of paxillin, facilitating cell motility (38).

1.2 PLCγ1 in Cancer

Based on the understanding of its physiological functions, PLCγ1 has been implicated in several key hallmarks of cancer (39). Consequently, the dysregulation or aberrant activation of PLCγ1 can contribute to tumorigenesis, highlighting its potential role as a driver in tumor progression.

1.2.1 Tumor progression

PLCγ1 has emerged as a crucial player in the progression of several cancers mainly due to its activation by growth factor receptors that are frequently overexpressed in cancer cells (40). The hyperactivation of PLCγ1 induced by growth factors plays a significant role in promoting cell proliferation and migration (41). The release of Ca^{2+} from intracellular stores can activate multiple signaling pathways, including the PI3K/AKT and MAPK/ERK pathways, along with PKC that also stimulates the MAPK/ERK pathway, resulting in the activation of transcription factors responsible for tumor progression (42).

Studies by Xie *et al.* revealed that PLCγ1 translocates to the nucleus in response to EGFR activation, inducing cell mitogenesis in squamous cell carcinoma (43). Furthermore, Song *et al.* demonstrated that PLCγ1 facilitates lung cancer cell proliferation, particularly in *KRAS*-mutant lung cancer, and loss of PLCγ1 reduces cell viability in *KRAS*-mutant lung cancer cell lines (44).

PLCγ1 is widely recognized as a tumor metastasis driver in several types of cancer. In ovarian cancer cells, inhibition of the PLCγ1 pathway had little effect on cell growth but significantly decreased cell migration (45). In prostate cancer cells, Mamoune *et al.* revealed that PLCγ1 mediates tumor invasion by increasing urokinase receptor (uPAR) transcription (46). In head and neck squamous cell carcinoma (HNSCC), PLCγ1 promotes tumor cell invasion and, consequently, metastasis development (47). Recent findings have demonstrated an important role of PLCγ1 in gastric cancer, where loss of PLCγ1 suppresses hepatocellular carcinogenesis and liver cancer growth in mouse models (48–50).

1.2.2 Dysregulation of PLCγ1 expression in cancer

PLCγ1 encoding gene (*PLCG1*) harbors somatic mutations in some cancers, implicating its role in tumorigenesis. Additionally, several cancers have shown aberrant expression of PLCγ1, evident at both the mRNA and protein levels (42,51).

1.2.2.1 Somatic mutations in *PLCG1*

Somatic mutations in *PLCG1* gene have been mostly found in hematologic malignancies and angiosarcomas (52). The recurrent PLCγ1-S345F mutation has been identified in approximately 20% of angioimmunoblastic T-cell lymphomas (AITLs) and cutaneous T-cell lymphomas (CTCLs), and 36% of adult T cell leukemia/lymphomas (ATLs), being the most frequently mutated gene in ATL (53,54).

PLCγ1-R707Q mutation is more frequent in angiosarcomas and is located in the highly conserved autoinhibitory SH2 domain (55–57). These mutations often lead to constitutive activation of the PLCγ1 protein, causing increased basal activity. Consequently, this aberrant signaling contributes to uncontrolled cell growth (58).

1.2.2.2 Aberrant expression of PLCγ1

PLCG1 gene upregulation or PLCγ1 protein overexpression have been observed in several cancers, such as glioblastoma (59), non-small cell lung cancer (NSCLC) (60), squamous cell carcinoma (43,47), colorectal cancer (61), prostate cancer (62), gastric cancer (48), and breast cancer (BC) (63) when compared to normal tissue.

In the clinical setting, higher PLCγ1 expression has been correlated with worse clinical outcome. Particularly, elevated PLCγ1 expression has been observed in advanced stages of lung adenocarcinoma (64), and it has been linked to worse overall survival (OS), disease-free survival (DFS), local relapse-free survival (LRFS), distant metastasis-free survival (DMFS), and pathological complete response (pCR) in oral squamous cell carcinoma (OSCC) (65,66). Moreover, BC tumor samples show a significant increase in PLCγ1 expression when compared to normal mammary gland, with moderately and poorly differentiated breast tumors exhibiting higher levels than well-differentiated tumors (63,67).

1.3 Breast Cancer

1.3.1 Epidemiology and risk factors

BC is the world's most incident and prevalent cancer, with about 2 million new cases identified per year and 7 million active cases. Moreover, it is the leading cause of cancer-related death in women, accounting for 684 996 deaths (15.5%) in 2020. In Portugal, BC accounts for 26.4% of newly identified cancer cases and 33.7% of active cases, remaining the type of cancer with the highest incidence and prevalence (68).

Only a small fraction, approximately 10%, of BC cases are hereditary and connected to family history. The *BRCA1* and *BRCA2* genes harbor the most prevalent germline mutations associated with BC development, with an average cumulative lifetime risk of around 70% (69,70). This means that about 90% of BC cases are sporadic, and lifestyle risk factors, such as changes in reproductive patterns, menopausal hormone therapy, physical inactivity, tobacco and alcohol consumption, as well as obesity, may be linked to the high incidence of BC cases (69). However, it's important to note that the majority of diagnosed sporadic BC cases are not associated with any specific lifestyle risk factor (71).

1.3.2 Molecular subtypes

The heterogeneity of BC is one of the underlying reasons for its persistent high prevalence (72). Hence, the identification of different BC subtypes holds significant clinical relevance as it allows a more personalized treatment approach and a better prognosis assessment (73).

BC can be divided according to the expression of hormone receptors, including estrogen receptor (ER) and progesterone receptor (PR), and the expression of human epidermal growth factor receptor 2 (HER2/ERBB2), into three subtypes: hormone receptor positive (luminal A and B), HER2-positive, and triple negative breast cancer (TNBC)(74).

Luminal BC is characterized by the presence of hormone receptors (ER and/or PR) and can be further subdivided into Luminal A and Luminal B. Luminal A represents the majority (50%) of all BCs and is HER2-negative, with low expression of cell proliferation marker Ki-67. Luminal B is less frequent (15%) and can be HER2-positive or-negative, however, has a high Ki-67 expression and tends to have a poor prognosis with high recurrence (69).

HER2-positive BC is characterized by the *HER2/ERBB2* oncogene overexpression, which is present in 15 to 20% of BCs, and is associated with increased invasiveness and risk of recurrence (75). Nowadays, these patients receive anti-HER2 targeted therapies, that include monoclonal antibodies (trastuzumab, pertuzumab) or tyrosine kinase inhibitors (TKIs) (lapatinib, neratinib, tucatinib) (76).

Finally, TNBC is highly aggressive with the worse prognosis, mainly due to the absence of targetable receptors, being negative for both hormone receptors and HER2 (77–79).

1.3.3 Estrogen signaling

About 70% of all BCs express ER, which plays a central role in mediating estrogen signaling (80). The ERα and ERβ receptors are classical members of the nuclear hormone receptor superfamily. ERα is encoded by the *ESR1* gene, while ERβ is encoded by the *ESR2* gene (81). Although ERα is well established in BC development and progression, the role of ERβ in tumorigenesis remains controversial among researchers (82).

Upon activation, ER promotes the transcription of specific target genes crucial for cellular processes such as proliferation, differentiation, and survival. Therefore, the dysregulation of ER expression and signaling leads to uncontrolled cell growth. Cyclin D1 gene (*CCDN1*) provides an excellent example of an ER target gene, playing a crucial role in cell cycle progression, particularly in regulating the G1/S transition through the Cyclin D1-CDK4/6 axis (83,84).

Estrogens, such as 17β-estradiol (E2), are the most abundant circulating hormones that promote cell proliferation through ER binding. E2 is mainly synthesized in the ovaries of premenopausal women and its synthesis begins with cholesterol, the precursor to all steroid hormones, which is catalyzed into pregnenolone. This compound is subsequently transformed into progesterone or androstenedione. Androstenedione is then converted into androgens, which are converted into estrogens by the aromatase enzyme (85).

Estrogen signaling is activated through two main types of pathways: the classical (genomic) and alternative (nongenomic) pathways (86). In the classical pathway, E2 enters the cell and binds to the ligand-binding domain of the ER. E2-ER complex dimerizes and translocates to

the nucleus, where it interacts with coregulatory proteins and specific DNA sequences known as estrogen-responsive elements (EREs) (87). These interactions promote the transcription of several genes implicated in BC progression (88).

In addition to its activation through E2 binding, ER can also be activated by phosphorylation through the alternative pathway. In this pathway, growth factors trigger ER phosphorylation at specific serine or tyrosine sites through RTK or G-protein-coupled receptors (GPCR), which immediately initiates the activation of growth factor signaling pathways, including PI3K/AKT and MAPK/ERK. For example, activation of EGFR promotes ERα phosphorylation at Ser118 by ERK. After phosphorylation, ER dimers translocate to the nucleus, where they also promote the transcription of various genes (70,89).

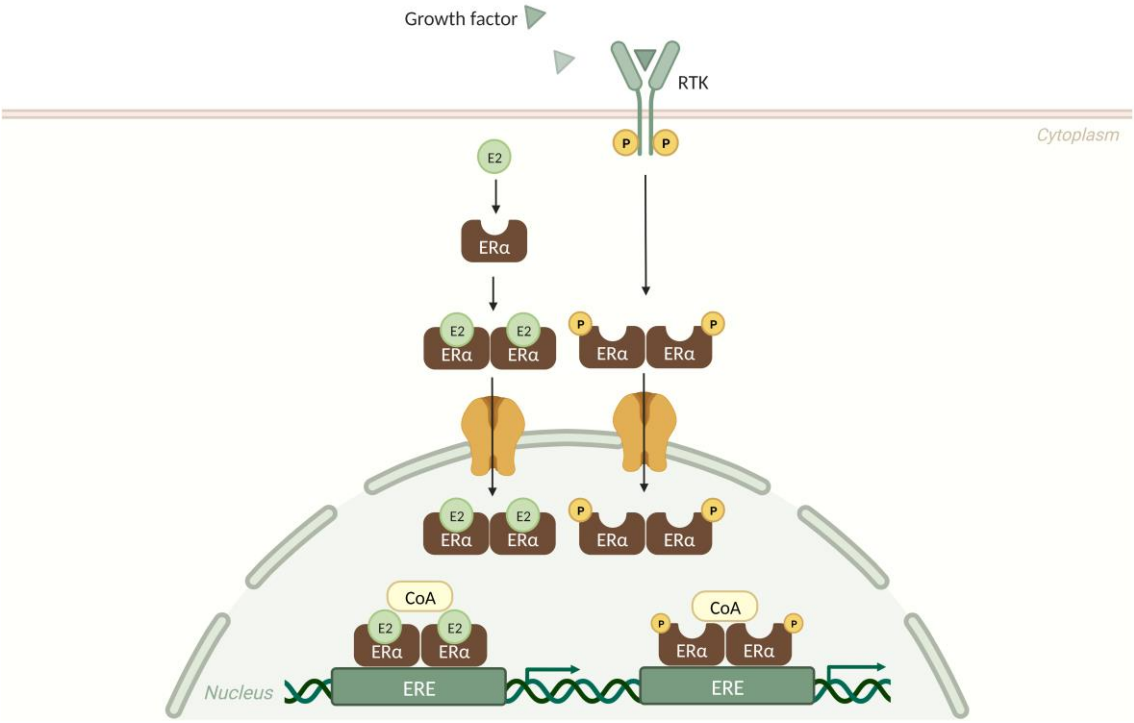


Figure 1.5. Estrogen signaling. In the classical pathway, the binding of E2 leads to ER activation and dimerization. ER dimers translocate to the nucleus where interact with EREs in the regulatory regions of target gene promoters. ER binding to DNA is followed by the recruitment of coactivators (CoA) and transcription initiation. In the alternative pathway, protein kinases belonging to signal transduction cascades activated by cell surface receptors, as RTKs, phosphorylate residues in ER, triggering its activity. Activated ER can interact with cell surface receptors and intracellular kinases, promoting its activation and resulting in nongenomic responses. Created with BioRender.com.

1.3.4 ER-positive BC treatment

Endocrine therapy is the standard treatment for ER-positive BC, which includes selective estrogen receptor modulators (SERMs), selective estrogen receptor degraders (SERDs) and aromatase inhibitors (AI) (90). SERMs competitively bind to both ERα and ERβ, disrupting co-

activator binding and inhibiting ER transcriptional activity. SERDs act as ER antagonists, binding competitively to ER and causing reduced ER translocation to the nucleus, enhanced ER degradation, and downregulation (79). AIs, including letrozole, anastrozole, and exemestane, work by inhibiting the aromatase enzyme, which converts androgens to estrogens, thereby reducing estrogen production (81).

Tamoxifen was the first SERM approved by the Food and Drug Administration (FDA) and has been the first-line treatment ever since, particularly for premenopausal women. Tamoxifen is recommended for the treatment of early-stage, locally advanced and metastatic ER-positive BC and has been shown to significantly improve OS (91). Fulvestrant is the only FDA-approved SERD, recommended for postmenopausal patients with advanced disease and has shown comparable efficacy to tamoxifen in first-line treatment for metastatic disease (90).

Following the European Society for Medical Oncology (ESMO) guidelines, Luminal A tumors have shown significant response to endocrine therapy alone, except in cases of high tumor burden where chemotherapy may be considered as an adjuvant treatment. On the other hand, Luminal B tumors often receive adjuvant chemotherapy followed by endocrine therapy (92).

Despite the success of endocrine therapy, many patients eventually develop resistance (acquired resistance), and some never respond (innate resistance) (91). The mechanisms of endocrine resistance are usually related to alterations in ER, in particular the lack/loss of ER expression (93). Modulation of ER expression may be due to ER mutations, modifications in ER coactivators/co-repressors, transcription factors, nuclear receptors, and epigenetic modulators. Additionally, interactions between the ER and RTKs, along with intracellular kinases could play a role in this modulation. Perturbations in cell cycle regulators, stress-triggered signaling, alterations in tumor microenvironment, as well as changes in nutritional stress and metabolic regulation might also contribute to these changes (88,94).

The development of combinations of endocrine therapy with targeted therapies, such as PI3K/AKT/mTOR and CDK4/6 inhibitors, is currently a key focus of clinical research in patients who have demonstrated disease recurrence or progression (95).

In the case of Luminal A, FDA-approved CDK4/6 inhibitors such as palbociclib, ribociclib and abemaciclib are being used as first-line treatment in combination with endocrine therapy for locally advanced or metastatic tumors (20). CDK4/6 inhibitors sensitivity is associated with increased levels of cyclin D1 and pRb, as well as decreased p16. ER-positive BCs often show amplification of *CCND1* and/or overexpression of cyclin D1 protein, as well as higher levels of pRb.

On the other hand, they usually express low levels of the p16, which explains why CDK4/6 inhibitors have shown efficacy in the treatment of ER-positive BCs (84,96).

Resistance to tamoxifen has been associated with cyclin D1 amplification. Interestingly, CDK4/6 inhibitors have demonstrated efficacy in tamoxifen-resistant cells as monotherapy and, when combined with tamoxifen, have shown the potential to enhance sensitivity to the latter in resistant cells (96). However, certain signaling pathways, like PI3K/AKT, can enhance the stability of cyclin D1 expression, potentially leading to endocrine therapy resistance (83).

The emerging role of CDK4/6 inhibitors as combination therapy provides a promising option to overcome resistance and improve outcomes for BC patients. However, it's important to note that not all cases of ER-positive BC respond to CDK4/6 inhibitors (82). Given the complexity of signaling pathways and resistance mechanisms, there is an emerging need to uncover novel biomarkers for improved characterization and stratification of these ER-positive BC patients (93).

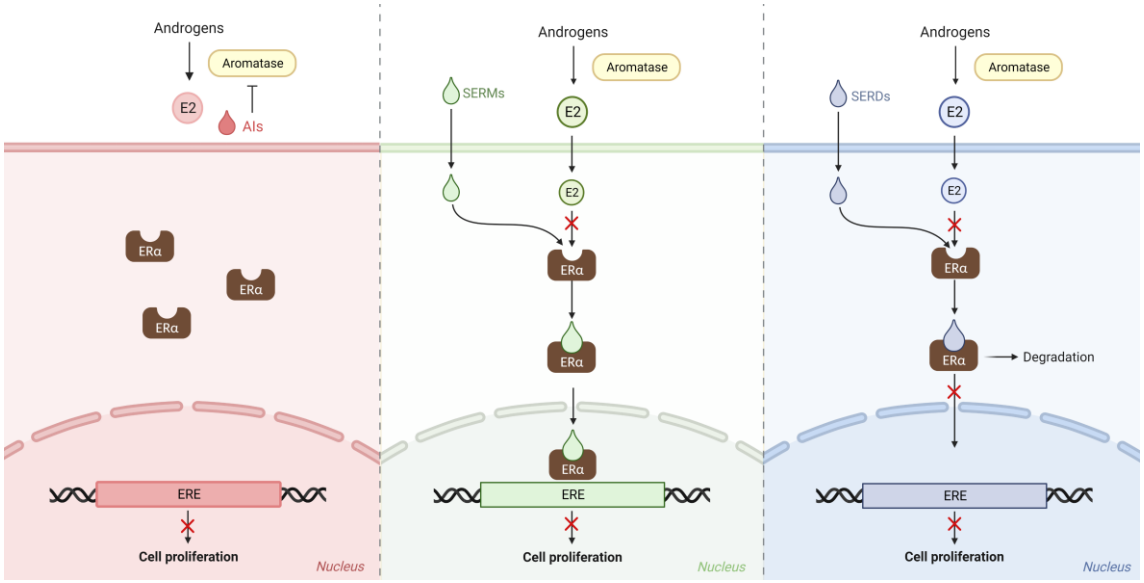


Figure 1.6. Endocrine therapy mechanism. Aromatase converts androgens produced in diverse tissues into estrogens (E2). After E2 binding, ER dimerizes and translocates to the nucleus, promoting gene expression. AIs block estrogen production by inhibiting the aromatization of androgens to estrogens. SERMs compete with estrogens for ER binding. SERM bounded ER is an inactive ER complex that cannot associate with co-activators, partially inhibiting transcription. SERDs also compete for ER binding, but these complexes have reduced capability of nuclear translocation, leading to ER degradation. Created with BioRender.com.

1.3.5 Biomarkers

BC cases are highly unique and distinct from one another, requiring personalized treatment based on the molecular features of each patient's tumor. Precision treatment is expected to increase efficacy, reduce toxicity and lead to more cost-effective care for patients (97).

A crucial goal of precision treatment for newly diagnosed BC patients is to avoid unnecessary and ineffective adjuvant chemotherapy (98). By identifying patients with low-risk profiles who may have good outcomes without the need for adjuvant chemotherapy, it becomes possible to spare patients from unnecessary and potentially harmful side effects. This approach not only improves their quality of life, but also leads to more cost-effective healthcare (99). To achieve personalized treatment, the establishment of reliable biomarkers is essential. These prognostic and predictive biomarkers play a crucial role in anticipating patient outcomes and selecting the most appropriate therapy (72).

Prognostic biomarkers, such as Ki-67, help estimating patient outcomes by allowing the identification of high- and low-risk groups, and help tailor the treatment strategy according to the aggressiveness of the tumor (100). Some prognostic biomarker tests, such as Oncotype DX and MammaPrint, based on RNA quantification of multiple genes, are already being applied in clinical practice (101,102).

Predictive biomarkers play a crucial role in driving precision treatment. These biomarkers have the potential to transform a treatment that shows low efficacy in an unselected group of patients into a highly effective treatment for specific biomarker-defined subgroups (103). Currently, predictive biomarkers are available for two main types of systemic treatment in BC: anti-HER2 and endocrine therapies.

HER2 is not only a prognostic biomarker but also a predictive biomarker, assessed in all newly diagnosed cases of invasive BC (76). This biomarker is essential for selecting patients for treatment with anti-HER2 targeted therapies, which has significantly improved the outcomes for this specific subgroup of BC patients who previously faced a poor prognosis (104).

As early as the 1970s, ER expression emerged as a predictive biomarker, in addition to its important value as a prognostic biomarker. Tumors expressing ER responded well to endocrine therapy, while those lacking the receptor typically did not benefit from the treatment (105). These findings led to the incorporation of ER expression assessment as a mandatory step for predicting response to endocrine therapy in all stages of BC management (106).

While predictive biomarkers for anti-HER2 and endocrine therapies have been successfully identified, resistance to these therapies is a major challenge that requires further investigation of additional biomarkers. These ongoing efforts are essential to improve the accuracy and efficacy of BC therapies.

1.3.6 PLCγ1 in BC

PLCγ1 overexpression has been identified as a risk factor for BC patients and multiple studies revealed a critical role for PLCγ1 in BC (107). In TNBC cell lines, AKT binds and phosphorylates PLCγ1, releasing the G2/M checkpoint and allowing the entry in the M-phase of the cell cycle. These authors found that both PI3K/AKT and PLCγ1 pathways are crucial for the G2/M transition triggered by FGFR (108). Moreover, it has been described that PLCγ1 modulates the PI3K/AKT pathway to promote tamoxifen-resistant BC cell growth and survival (91).

PLCγ1 has long been recognized as a metastasis promoter. Nie *et al.* reported that hyperactivation of PLCγ1, along with PI3K/AKT, correlated with increased metastatic plasticity and invasion of BC cell line-derived brain metastasis (109). Sala *et al.* demonstrated that PLCγ1 is required for the development and progression of metastasis, through its activation along with the small GTP-binding protein Rac, which leads to a rearrangement of the cytoskeleton. Furthermore, PLCγ1 knockdown strongly inhibited lung metastasis and reverted metastasis formation (110).

Lattanzio *et al.* analyzed the expression of PLCγ1 and its phosphorylated forms Y783 and Y1253 by immunohistochemistry (IHC) in a large cohort of BC samples and found a significant association between high PLCγ1, PLCγ1-Y783 and PLCγ1-Y1253 expression with decreased DFS. High expression of phosphorylated PLCγ1 was also associated with decreased distant relapse-free survival (DRFS) in patients treated with chemotherapy (107). However, a few years later, the same authors found differences between the molecular subtypes of BC. Interestingly, high expression of PLCγ1, PLCγ1-Y783 and PLCγ1-Y1253 is significantly correlated with poorer DFS in Luminal A BC patients, but not in Luminal B, HER2-positive or TNBC. Moreover, they found a correlation between high levels of PLCγ1-Y783 and lower DRFS in pre/perimenopausal Luminal A BC patients undergoing endocrine therapy (111). These results indicate that PLCγ1 may play an important role in Luminal A BC. Given that Luminal A BC is ER-positive, this effect may be related to the ER signaling pathway.

In ER-positive BC, the E2-ERα complex induces rapid anticipatory activation of the unfolded protein response (UPR), which results in protein folding and promotes survival, proliferation, angiogenesis and resistance to chemotherapy and endocrine therapy (112). This interaction occurs through the binding of the ERα to PLCγ1, which causes phosphorylation of PLCγ1 and consequently Ca^{2+} release. This sustained Ca^{2+} efflux leads to strong and sustained activation of the UPR (113). Knockdown or inhibition of PLCγ1 strongly inhibits the estrogen-mediated UPR activation (114).

Previous results from our group showed impaired proliferation of ER-positive BC cell lines, probably a consequence of decreased ERα signaling and ERα-mediated gene transactivation resulting from PLCγ1 depletion. These findings suggest a crosstalk between PLCγ1, and ER signaling and highlight the potential role of PLCγ1 as a prognostic biomarker in Luminal A BC, indicating its relevance in predicting disease outcomes and potentially serving as a target for future therapeutic strategies.

2 Objectives

Considering the advances in targeted therapies for ER-positive BC patients and the current challenges of innate and acquired resistance, the work presented in this thesis aimed to identify new biomarkers and treatment options to improve BC patient outcomes.

PLCγ1 has been recognized as a mediator of tumor development and progression, and is overexpressed in Luminal A breast tumors, correlating with poor prognosis.

To this end, we aimed to understand the interplay between PLCγ1 and ER signaling:

- Unravel the molecular mechanisms underlying the crosstalk between PLCγ1 and ER signaling pathways in Luminal A BC cell lines.
- Investigate how the interaction between PLCγ1 and ER influences cellular processes, including proliferation and migration.

Study the impact of PLCγ1 on therapeutic response:

- Investigate how PLCγ1 influences the response of Luminal A BC cell lines to targeted therapies.
- Assess the efficacy of combining PLCγ1 down-regulation with existing targeted treatments to overcome resistance and improve therapeutic outcomes.

By achieving these goals, the work presented in this thesis aimed to reveal the complex crosstalk between PLCγ1 and estrogen signaling in ER-positive BC. This knowledge will contribute to the identification of novel biomarkers, resistance mechanisms and ultimately lead to the development of innovative therapeutic strategies to improve patient outcomes in BC treatment.

3 Material and Methods

3.1 Cell Culture

Luminal A, ER-positive Breast Cancer cell lines used in this work were MCF7 and T47D cell lines (115,116). Both cell lines were purchased from the American Type Culture Collection (ATCC) and were cultured in Dulbecco's Modified Eagle Medium (DMEM; #41966029; Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS; #10270106; Gibco), 1% (v/v) penicillin/streptomycin (Pen/Strep; #15140122; Gibco) and 0.01 mg/ml human recombinant insulin (#12585014, Gibco). Cells were maintained at 37°C in a 5% CO_2-95% air atmosphere and passaged in sterile conditions when reaching 80% of confluence.

3.1.1 Cell lines infections

To study estrogen signaling, MCF7 and T47D cell lines were transduced with an Estrogen Response Element Reporter (pGreenFire 2.0 #TR455PA-P; System Biosciences), encoding pGF2-ERE-rFluc-T2A-GFP-mPGK-Puro. Briefly, cells were seeded in 60 mm petri dishes at a density of $6.5x10^5$/petri dish in 3 ml of complete growth medium, until they achieve a confluence of 80%. Then, cells were infected with virus particles of the pGreenFire 2.0 vector in complete medium supplemented with 5 µg/ml polybrene. Selection of stable clones of MCF7 and T47D cell lines started 2 days after infection with 1 µg/mL and 3 µg/mL puromycin dihydrochloride (#sc-108071, Sigma-Aldrich), respectively. The efficiently of infection was confirmed by Western blot, using anti-GFP antibody.

3.1.2 Cell lines transfections

3.1.2.1 PLCγ1 Knock-Out (KO)

For a stable PLCγ1 KO, MCF7 and T47D cell lines were seeded in a 6-well plate at a density of $5x10^5$ cells/well in 1.5 ml of complete growth medium. At 80% of confluence, cell line was co-transfected with 2.5 µg of PLCγ1 CRISPR/Cas9 KO Plasmid (sc-400472-KO-2; Santa Cruz Biotechnology) and PLCγ1 HDR Plasmid (sc-400472-HDR-2; Santa Cruz Biotechnology), using Lipofectamine reagent 3000 (#L3000015, Invitrogen) following manufacturer's instructions. Briefly, the mix solution was incubated for 15 min at room temperature (RT), followed by replacement of medium by fresh antibiotic-free medium containing the DNA-lipid complexes for 6h. Selection of stable clones of MCF7 and T47D cell lines started 2 days after infection with

1 µg/mL and 3 µg/mL puromycin dihydrochloride (#sc-108071, Sigma-Aldrich), respectively. PLCγ1 KO was confirmed by Western blot.

Previously pGreenFire 2.0 lentivector infected cell lines, were also co-transfected with PLCγ1 CRISPR/Cas9 KO Plasmid and HDR Plasmid as described before. Since pGreenFire 2.0 lentivector infected cell lines are already resistant to puromycin, the stable clones were selected by cell sorting RFP positive cells (Flow Cytometry; BD FACSAria III).

3.1.2.2 PLCγ1 variants

MCF7 and T47D cell lines were transfected with PLCγ1 variants using Lipofectamine reagent 3000 (#L3000015, Invitrogen) following manufacturer's instructions. pTriex4 human full-length (FLWT) PLCγ1 and mutant D1019K were gently provided by Dr. Matilda Katan (UCL, UK; ref. 15). Mutants of PLCγ1 comprising deletion of amino acids 545–759 (ΔSH2) and 791–870 (ΔSH3) were previously constructed by *in vitro* mutagenesis using the NZYMutagenesis Kit (#MB012, nzytech) according to manufacturer's instructions. All constructs include an N-terminal Hisx6 tag followed by an S-tag.

3.1.3 Crosstalk between PLCγ1 and ERα signaling

To understand the crosstalk between PLCγ1 and ERα signaling, the PLCγ1 signaling pathway was studied after 17β-estradiol (E2) stimulation. Cells were cultured in phenol red-free DMEM-F12 medium (# 11039021, Gibco), supplemented with 10 mmol/L HEPES (#15630080, Gibco) to maintain pH, and 5% charcoal stripped FBS (csFBS) (#12676029, Gibco). Cells were stimulated with 1 µM E2 (#E2758, Sigma-Aldrich) and lysed in lysis buffer containing 25 mM Tris pH 7.5 (Sigma), 500 mM EDTA (Sigma), 1% Triton X-100™ (VWR), 25 nM TCEP (Sigma), protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail 2 (Sigma), at the following timepoints: 0, 1, 5, 15, 30 min and 3h after E2 stimulation.

3.1.4 Growth factor-independent activation of AKT and ERK pathways

To study the role of PLCγ1 in PI3K/AKT and MAPK/ERK signaling pathways under serum deprivation conditions, the cells were cultured in 60 mm petri dish with DMEM without FBS for 24h and then lysed in lysis buffer.

3.2 Proliferation Curve

Cells were plated in 60 mm petri dish at an initial density of $1x10^5$, in DMEM supplemented with 10% (v/v) FBS, 1% (v/v) Pen/Strep and 0.01 mg/ml insulin. After 12h, 24h and 48h, cells were harvested and counted using a hemocytometer on an inverted bright field microscope.

3.3 Colony Formation Assay

Cells were seeded in a 6-well plate at a density of 5000 cells/well and were grown for 15 days. Media with or without drugs was changed every two days. Colonies were fixed with formaldehyde 3.7% (m/v) for 10 min and stained with 2% (w/v) crystal violet (#HT90132, Sigma-Aldrich). For quantification, crystal violet was solubilized in 1% (m/v) SDS pre-warmed at 37˚C with agitation for 30 min. Absorbance was measured at 570 nm in Infinite M200 microplate reader (Tecan).

3.4 Wound Healing ("scratch") assay

BC cells were seeded in 6-well plates until they reached a confluent monolayer. Once confluent, a linear scratch was made with a P1000 pipette tip, creating a wound across the well diameter. The media was replaced to remove debris and cells in suspension. Cells were incubated with 5 µM mitomycin-C and bright-field images of each well were acquired on an inverted microscope at the following timepoints: 0h, 24h and 72h. The wound closure was quantified using the ImageJ software (imagej.nih.gov/ij/).

3.5 DNA extraction

3.5.1 Bacterial transformation

For bacterial transformation of PLCγ1 FLWT and mutants (ΔSH2 and ΔSH3) DNA, 60 µl of NZY5α Competent Cells (#MB00401; nzytech) were mixed with 10 to 100 ng of plasmid DNA on ice for 30 min. To introduce the DNA into competent cells, they were heat-shocked for 1 min, in a 42°C water bath and placed in ice again for 2 min. 0.9 ml of S.O.C (Super Optimal broth with Catabolite repression) medium was added to cells, and they were agitated at 225 RPM, for 1h. Finally, 50 µl of transformed cells were spread on LB agar plates containing kanamycin antibiotic (Kanamycin sulfate from Streptomyces kanamyceticus; #SLBB0945V; Sigma-Aldrich) and incubate overnight at 37°C. Then, transformed cells were grown for LB Broth containing 50 µg/ml of kanamycin antibiotic and incubate overnight under agitation at 37°C for DNA extraction.

3.5.2 Plasmid DNA purification from *Escherichia coli* strains

For plasmid DNA purification from *Escherichia coli* cells, we used the NZYMaxiprep kit (#MB05101; nzytech), which is designed for the rapid, large-scale preparation of highly pure plasmid DNA from recombinant *Escherichia coli* strains. Plasmid DNA binds selectively to nzytech columns charged with a silica-based anion-exchange resin. All contaminants, such as proteins, RNA, salts, nucleotides and oligos (<40-mer) are washed from the column. In the elution step, the positive charge of the resin is neutralized by a pH shift to slightly alkaline conditions and pure plasmid DNA is eluted in a high-salt elution buffer.

3.6 Co-immunoprecipitation

For immunoprecipitation experiments, cells were first co-transfected with PLCγ1 FLWT and mutants (ΔSH2 and ΔSH3), and pEGFP-C1-ER alpha plasmid (#28230; Addgene), using Lipofectamine reagent 3000 (Invitrogen) following manufacturer's instructions.

Cells were lysed in 50 mmol/L Tris pH 7.5 (Sigma), 150 mmol/L NaCl (VWR), and 1% NP-40 (Sigma-Aldrich) and digested with 5U DNase I (#EN0521, Promega) before preclearing with Protein G Dynabeads (#10003D, Invitrogen) at 4°C for 30 min. Samples were diluted in immunoprecipitation buffer (20 mM HEPES pH 7.0, 10 mM KCl, 1.5 mM $MgCl_2$, 0.2% (v/v) Tween20, 10% (v/v) glycerol, 1 mM DTT) and incubated with anti-S-Tag antibody (#12774; Cell Signaling Technology) overnight at 4°C. The protein complexes were pulled down using Protein G Dynabeads for 4h at 4°C and washed three times in washing buffer (20 mM HEPES pH 7.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2% (v/v) Tween20, 10% (v/v) glycerol, 1 mM DTT). Protein samples were eluted in 4x Laemmli buffer and resolved by Western blot analysis. 10% of the total cell lysates was used as input samples.

3.7 Cell Fractionation

For cell fractionation into cytoplasm and nucleus, cells were lysed in a subcellular fractionation buffer (20 mM HEPES pH7.4, 10 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT supplemented with protease inhibitor cocktail (#4693159, Roche) and phosphatase-inhibitor cocktail 2 (#P5726, Sigma). Lysates were agitated for 30 min at 4°C. After centrifugation at 720 x *g* for 5 min at 4°C, the supernatants were separated as the cytoplasmic fraction. Pellets correspond to nuclei fraction and were washed three times, lysed in nuclear lysis buffer (50 mmol/L Tris HCL pH 8 (Sigma), 150 mmol/L NaCl (VWR), 1% NP-40 (Sigma-Aldrich), 0.5% sodium deoxycholate, 0.1% SDS, 10% glycerol and protease inhibitor cocktail (Roche)) and

centrifuged for 10 min at 15000 x *g*. Equal amounts of protein extracts were resolved by western blot.

3.8 Western-Blot

Total protein extracts were prepared by lysing cells in lysis buffer containing 25 mM Tris pH 7.5 (Sigma), 500 mM EDTA (Sigma), 1% Triton X-100™ (VWR), 25 nM TCEP (Sigma), protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail 2 (Sigma). After 10 min of incubation on ice, the extracts were centrifuged at 13800 x *g* for 10 min at 4°C. Supernatants were transferred to a new tube and protein concentration were quantified using Quick Start™ Bradford Protein Assay (Bio-Rad), measuring the absorbance at 562 nm. Protein concentration was determined by comparing it to a standard curve of known BSA concentrations. 4x SDS-PAGE Sample Buffer was added to 10 µg total protein extract and denatured for 10 min at 96˚C. Proteins were separated by electrophoresis in 10% SDS-polyacrylamide gel and transferred to nitrocellulose membranes using the iBlot 2 Dry Blotting System (Life Technologies).

The following specific primary antibodies were incubated overnight at 4˚C: rabbit monoclonal anti-PLCγ1 (D9H10) (#5690, Cell Signaling), rabbit monoclonal anti-phospho-PLCγ1 (Tyr783) (D6M9S) (#14008, Cell Signaling) rabbit monoclonal anti-ERα (D6R2W) (#132585, Cell Signaling), mouse monoclonal anti-phospho-ERα (Ser118) (16J4) (#2511, Cell Signaling), rabbit monoclonal anti-Cyclin D1 (92G2) (#2978, Cell Signaling), rabbit monoclonal anti-α-tubulin (11H10) (#2125, Cell Signaling), rabbit monoclonal anti-Lamin B1 (D9V6H) (#13435, Cell signaling), rabbit monoclonal anti-S-Tag (D2K2V) XP (#12774, Cell Signaling), rabbit monoclonal anti-AKT (pan) (11E7) (#4685, Cell Signaling), rabbit monoclonal anti-phospho-AKT (Ser473) (D9E) (#4060, Cell Signaling), rabbit monoclonal anti-p44/42 MAPK (ERK1/2) (137F5) (#4695, Cell Signaling), rabbit monoclonal anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) (#4370, Cell Signaling), rabbit polyclonal anti-phospho-PKC (pan) (βII Ser660) (#9371, Cell Signaling), rabbit monoclonal anti-phospho-Rb (Ser807/811) (D20B12) (#8516, Cell Signaling), rabbit monoclonal anti-p21 Waf1/Cip1 (12D1) (#29475, Cell Signaling), rabbit monoclonal anti-CDK2 (78B2) (#2546, Cell Signaling), rabbit monoclonal anti-CDK4 (D9G3E) (#12790, Cell Signaling), mouse monoclonal anti-CDK6 (DCS83) (#3136, Cell Signaling), rabbit monoclonal anti-p27 Kip1 (D69C12) (#3686T, Cell Signaling), mouse monoclonal anti-p53 (Bp53-12) (#sc-263, Santa Cruz Biotechnology) and mouse monoclonal anti-β-Actin (AC-15) (#ab6276, Abcam).

On the following day, membranes were incubated with horseradish peroxidase-conjugated (HRP) specific secondary antibodies for 2h at RT (anti-mouse-HRP IgG #7076 and anti-rabbit-HRP IgG #7074, both from Cell Signaling). Protein-antibody complexes were detected using Novex ECL

Chemiluminescent Substrate Reagent Kit (#WP20005, Invitrogen) and chemiluminescence signal was detected in Amersham Imager 680 and 800.

3.9 RNA isolation, cDNA Synthesis and RT-qPCR

The RNA was extracted using the NZY Total RNA Isolation kit (#MB13402; nzytech) according to the manufacturer's instructions. Cells were lysed in a lysis buffer containing guanidine thiocyanate, which inactivates cellular RNases and loaded into a column with a silica membrane. Membranes were washed to clean impurities and treated with DNase to prevent DNA contamination. Columns were washed three times and total RNA was eluted in RNase-free water and quantified using Nanodrop™2000 (Thermo Scientific).

Then, cDNA was synthesized using 1 µg of total RNA and NZYM-MuLV First-Strand cDNA Synthesis Kit (nzytech) according to the manufacturer's instructions. For that, the annealing reaction was performed by mixing RNA, Oligo(dt) 18 primer mix and annealing buffer. The mixture was incubated for 5 min at 65˚C and then placed on ice for 1 min. The reverse-transcription reaction was performed by adding NZYM 2x Master Mix (no oligos) and NZYM-MuLV RT enzyme mix to the tubes and incubated for 50 min at 37˚C. The reaction was inactivated by heating at 85˚C and then chilled on ice. RNA template was degraded by incubating with RNase for 20 min at 37˚C.

Transcript levels of individual genes were assayed by qPCR, using Power SYBR® Green PCR Master Mix (Applied Biosystems) in RT-PCR ViiA 7 (384 well), according to the manufacturer's instructions. Reactions were run in triplicate. Cycling conditions were the following: holding at 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 seconds, 55˚C for 40 seconds and 70˚C for 30 seconds. Relative mRNA expression levels were normalized to endogenous *GAPDH* and calculated using the $2^{-\Delta\Delta CT}$ method. Specific primers used were *ERS1* and Human *GAPDH* (PPH00150E-200).

3.10 Flow Cytometry: Cell Cycle

For cell cycle analysis, cells were collected, fixed in ice-cold 66% ethanol, and kept at 4°C until analysis. Cell cycle analysis was performed using the Propidium iodide (PI) flow cytometry kit (#ab139418, Abcam), following the manufacturer's protocol; and a BD LSRFortessa flow cytometer (BD Biosciences). Analysis was made using FlowJo V10 software.

3.11 Palbociclib Response

Cells were cultured in a 96-well plate and treated with 0, 0.01, 0.1, 0.5, 1 or 2.5 µM of palbociclib (#PD0332991, Sigma-Aldrich) and then incubated in 5% CO_2 at 37°C. The medium with

palbociclib was changed every 48h and the cell viability was assessed after 1 week of incubation, by adding 1:10 AlamarBlue reagent (Invitrogen) and fluorescence was measured 2h after incubation (excitation 560 nm; emission 590 nm) in Infinite M200 microplate reader (Tecan).

3.12 Statistical Analysis

GraphPad Prism version 8.0.1 was used to perform statistical analysis. Data is presented as the mean ± standard deviation (SD) of the indicated number of independent experiments (n) or triplicates in case of n=1.

Non-parametric Mann-Whitney test and parametric t-test were used to compare Parental with KO cells, as indicated in the figure legends. The level of statistical significance was set as nonsignificant (ns); *, $P < 0.05$; **, $P < 0.01$ and *** $P < 0.001$. For all the statistical analyses, P value (P) is from a two-tailed test with a confidence interval of 95%.

4 Results

4.1 PLCγ1 modulates ER-positive BC cells viability

We started by investigating the role of PLCγ1 in ER-positive BC cell lines: MCF7 and T47D. To achieve this, we have used CRISPR/Cas9 technology to deplete PLCγ1 expression in both cell lines (Fig.4.1A), which were further evaluated regarding the effect of PLCγ1 on proliferation and cell migration.

To study the impact of PLCγ1 on cell proliferation, cells were cultured under normal conditions (in complete medium, 5% CO2) and counted at 12h, 24h and 48h post seeding. No significant differences in proliferation were observed in both cell lines. However, MCF7 cells deficient in PLCγ1 show an impairment in proliferation when compared to control cells, with a 2-fold increase in cell number being observed in PLCγ1 KO cells after 48 hours, while parental MCF7 cells had a 3-fold increase (Fig.4.1B).

In parallel, MCF7 and T47D parental and PLCγ1 KO cells were seeded at low density and left to grow for 15 days, under normal conditions, to assess their colony-forming ability. PLCγ1-deficient MCF7 cells showed a significant reduced ability to form colonies when compared to MCF7 parental cells (*$p<0.05$), whereas T47D PLCγ1 KO cells showed no significant differences. MCF7 PLCγ1 KO showed a 34.6 ± 11.5% impairment while T47D PLCγ1 KO showed a 13.8 ± 2.3% decreased ability to form colonies compared with parental cells (Fig.4.1C).

To assess PLCγ1 role in cell migration, parental and PLCγ1 KO cells were left to grow to confluency and a wound was made at the bottom of the plate to evaluate the capacity of cells to migrate and close the wound. PLCγ1 KO leads to a compromised migration of MCF7 cells, although not statistically significantly. The parental cells migrated 2-fold more than PLCγ1-deficient MCF7 cells, since after 72h MCF7 cells had closed 79.9% of the wound while MCF7 PLCγ1 KO cells only closed 33.8 ± 0.7%. On the other hand, T47D cell line did not show a difference between the migration of parental and PLCγ1 KO cells. After 72h T47D cells had closed 32.7 ± 6.8% of the wound and T47D KO cells had closed 38.9% (Fig.4.1D).

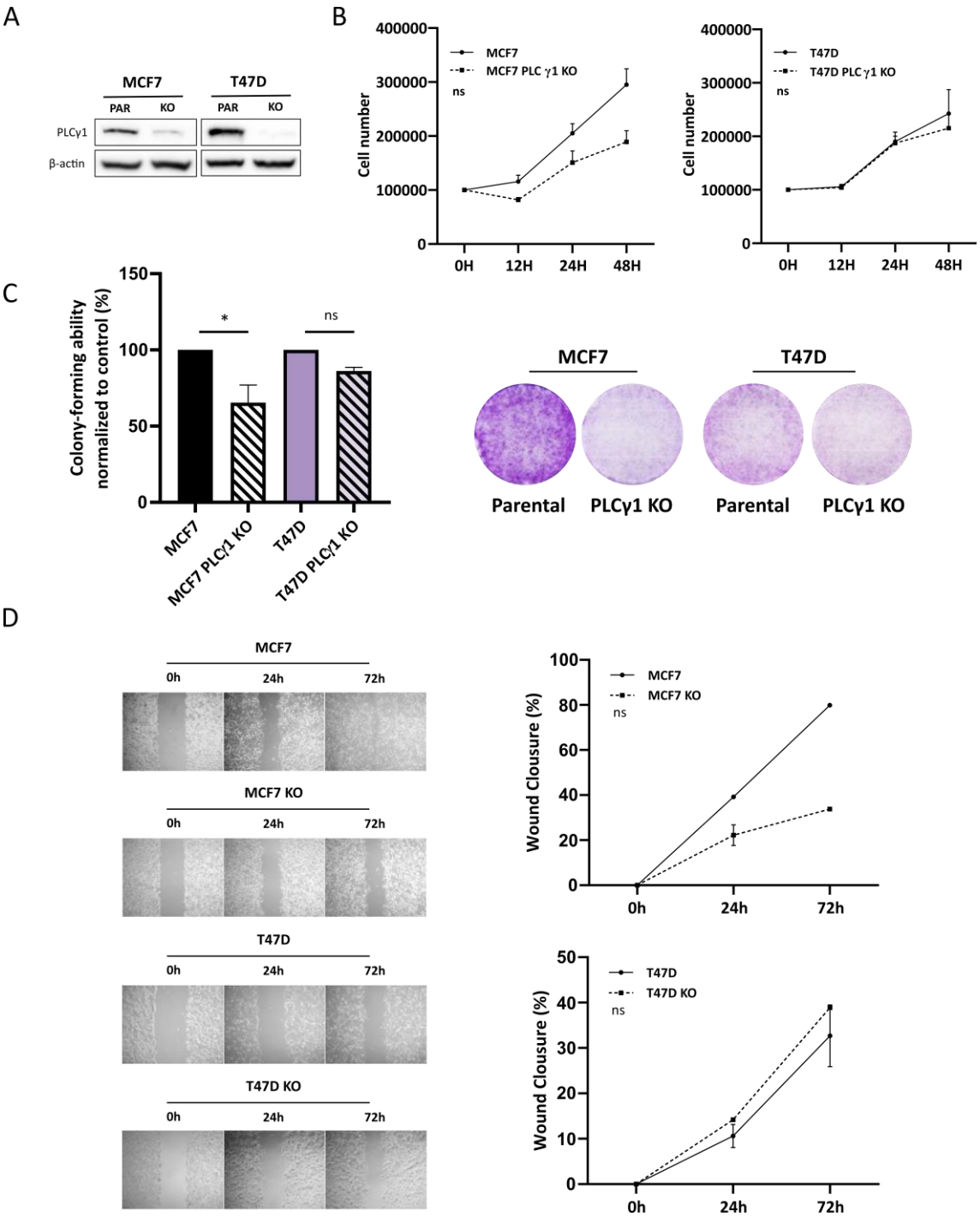


Figure 4.1. Role of PLCγ1 in Luminal A, ER-positive BC cells proliferation and migration. (A) Western blot analysis of PLCγ1 KO in MCF7 and T47D cell lines (B) Proliferation curve of MCF7 and T47D parental and PLCγ1 KO cells. Cells were plated at an initial density of $1x10^5$ and after 12h, 24h and 48h, were harvested and counted on an inverted bright field microscope. Data is presented as the mean ± SD, statistical Mann-Whitney test showed no statistical significance between parental and PLCγ1 KO cells (n=1, in triplicate). (C) In the colony-formation assay, cells were seeded for 15 days, stained with crystal violet, and quantified by measuring the absorbance at 570 nm. PLCγ1 KO cells are normalized to MCF7 and T47D and data is presented as the mean ± SD. Statistical analysis was performed using the Mann-Whitney test, that showed statistical significance between MCF7 parental and PLCγ1 KO cells (*$p \leq 0.05$), however no statistical significance was observed between T47D parental and PLCγ1 KO cells (n=3). (D) Cell migration was assessed by wound

healing assay. Wound closure was captured on an inverted bright field microscope, and the percentage of wound closure (%) was calculated 24h and 48h later using ImageJ software. Data is presented as the mean ± SD, statistical Mann-Whitney test showed no statistical significance between parental and PLCγ1 KO cells (n=1). Statistical results: *p ≤ 0.05, **p ≤ 0.01, ***p≤ 0.001.

4.2 PLCγ1 KO impairs cell cycle progression

To explore the impact of PLCγ1 in MCF7 cells proliferation, we performed a cell cycle analysis in MCF7 parental and PLCγ1 KO asynchronized cells, where we assessed cell distribution in the different phases of the cell cycle.

The results showed 2-fold increase of PLCγ1-deficient MCF7 cells in a quiescent state (G0) compared to parental cells, and the number of MCF7 parental cells in the G2 phase is 2.8-fold higher compared to the MCF7 PLCγ1 KO cells (Fig.4.2A). The data suggest that PLCγ1 KO not only affects cell cycle entry, but also delays cells from entering the G2 phase. The disruption of the cell cycle mediated by PLCγ1 KO may be explained by the decrease of CDK6, CDK2 and consequently the decrease of phosphorylated Rb (p-Rb) in MCF7 cells. Furthermore, when we overexpressed PLCγ1 (OE), we observed an increase in p-Rb expression (Fig.4.2B).

To further understand where PLCγ1 may act in the cell cycle, cells were synchronized and harvested in G1 phase, after 24h of FBS starvation; and were subsequently desynchronized through additions of FBS and harvested 24 hours after. PLCγ1 KO cells synchronized in G1 phase showed a 2-fold increase in ERα expression, followed by a decrease in CDK6 expression, along with increased expression of p53, p27, p21 and interestingly, E2F1. After 24 hours of cell cycle entry, PLCγ1 is phosphorylated at Tyr783, cyclin D1 and CDK4 increase in both parental and PLCγ1 KO cells. However, CDK6 expression increases only in parental cells, remaining unexpressed in PLCγ1-deficient cells. Also, CDK2 expression increases 1.6-fold in parental cells compared to PLCγ1 KO cells and p21 expression has a 2-fold increase in PLCγ1 KO cells, which could account for the KO-mediated cell cycle disruption of PLCγ1. However, levels of p-Rb are slightly higher in PLCγ1 KO cells and there is a loss of E2F1 expression 24h after cells enter the cycle (Fig.4.2C, D, E).

To validate the influence of PLCγ1 on cell cycle mediators, we transfected parental cells with a constitutively active PLCγ1 mutant (D1019K) and overexpressed PLCγ1 in PLCγ1 KO cells, to confirm whether these cells recover the expression of some cell cycle mediators. Curiously, in the MCF7 cell line CDK4 and p-Rb show an expression pattern similar to p21, with higher expression in the absence of PLCγ1. This experiment was also carried out with the T47D cell line, which showed an opposite pattern of CDK4 expression compared to the MCF7 cell line, where CDK4 expression is higher in the parental cells, but decreases when PLCγ1 is constitutively active

(D1019K). In addition, in the T47D cell line we were able to observe CDK6 expression in PLCγ1 KO cells. (Fig.4.2F, G).

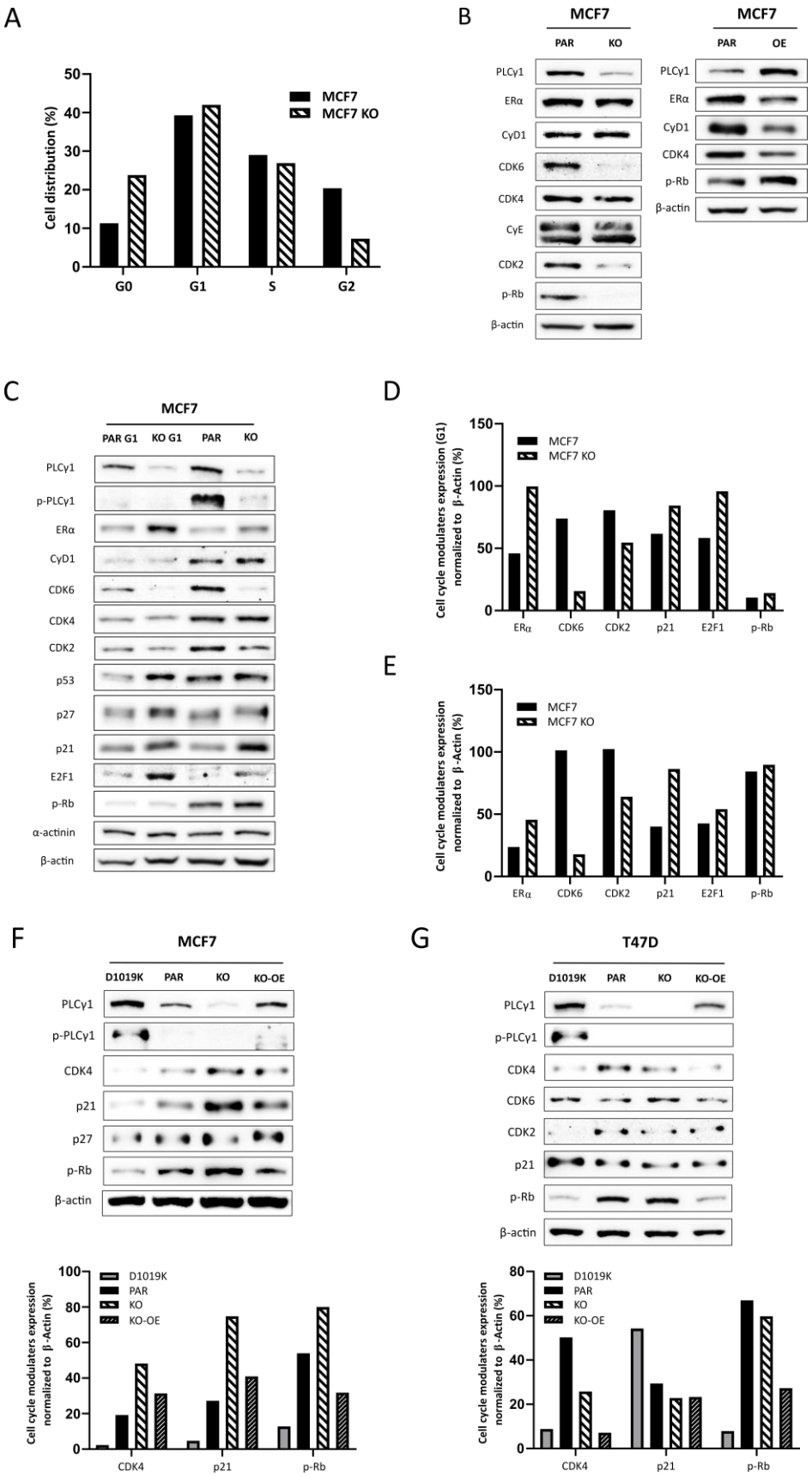


Figure 4.2. PLCγ1 modulates cell cycle progression and several cell cycle mediators. (A) Cell cycle analysis by quantification of DNA content with PI staining in MCF7 parental and PLCγ1 KO asynchronized cells (n=1). (B) Western blot analysis of MCF7 parental, PLCγ1 KO and PLCγ1 (overexpression) OE asynchronized cells (n=1). (C) Western blot analysis of MCF7 parental and PLCγ1 KO cells synchronized in G1, 24h of FBS starving (PAR G1; KO G1) and 24h after cells entering the cycle (FBS addition) (PAR; KO) (n=1). (D) Quantification of the expression of cell cycle mediators in synchronized cells (G1), normalized to the loading control, β-actin. (E) Quantification of cell cycle mediators' expression 24 hours after cells enter the cycle, normalized to loading control, β-actin. (F)(G) Western blot analysis of asynchronized MCF7 and T47D cell lines expressing constitutively active PLCγ1 (D1019K), parental, PLCγ1 KO and recovery of PLCγ1 expression by over-expression of PLCγ1 in PLCγ1 KO cells (n=1).

4.3 PLCγ1 role in response to palbociclib therapy

Previous studies documented that resistance to CDK4/6 inhibitors is associated with CDK6 amplification, and CDK6 depletion can restore sensitivity to CDK4/6 inhibitors in MCF7 cell line (83). Given that PLCγ1 KO results in a reduction of CDK6 levels in MCF7 cells, we hypothesized that PLCγ1 KO could enhance the sensitivity of cells to a CDK4/6 inhibitor, such as palbociclib. To test our hypothesis, MCF7 and T47D parental and PLCγ1 KO cells were treated with different concentrations of palbociclib, and their viability was measured after seven days of treatment. The results showed that PLCγ1 does not influence the viability of MCF7 and T47D cells treated with palbociclib (Fig.4.3A)

A colony formation assay was also established, where cells were treated with 0.5 μM of palbociclib for 15 days, to assess their ability to form colonies in these conditions. MCF7 cells treated with palbociclib exhibited a 1.9-fold decrease in colony forming ability, while MCF7 PLCγ1 KO treated cells displayed a 2.3-fold decrease in comparison to untreated cells. Regarding T47D cell line, parental treated cells showed a 2-fold decrease and PLCγ1 KO treated cells exhibited a 2.2-fold decrease compared to untreated cells. PLCγ1 KO combined with palbociclib results in a 3-fold reduction in cell viability compared to the control. However, this decrease in viability caused by PLCγ1 deletion does not involve a synergistic effect with the treatment (Fig.4.3B).

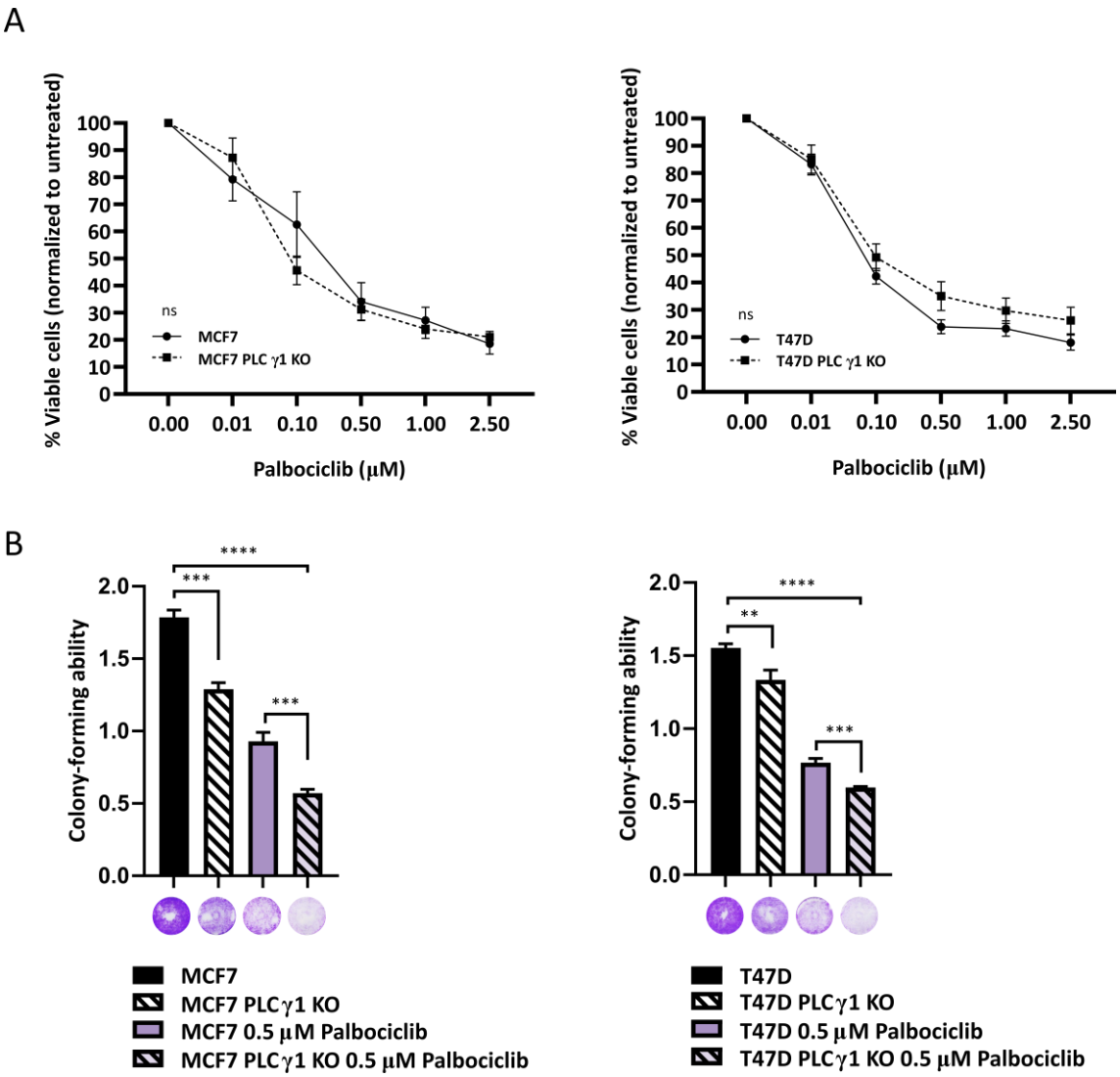


Figure 4.3. PLCγ1 does not affect palbociclib sensitivity. (A) Viability assay of MCF7 and T47D parental and PLCγ1 KO cells treated with palbociclib at indicated concentrations for seven days. Results are normalized to untreated control. Data is presented as the mean ± SD, statistical unpaired t-test showed no statistical significance between parental and PLCγ1 KO cells (n=3). (B) Colony formation assay of MCF7 and T47D parental and PLCγ1 KO cells treated with 0.5 μM of palbociclib for 15 days and respective quantification by measuring absorbance at 570 nm (n=3). Data is presented as the mean ± SD. Statistical analysis was performed using unpaired t-test: *p ≤ 0.05, **p ≤ 0.01, ***p≤ 0.001.

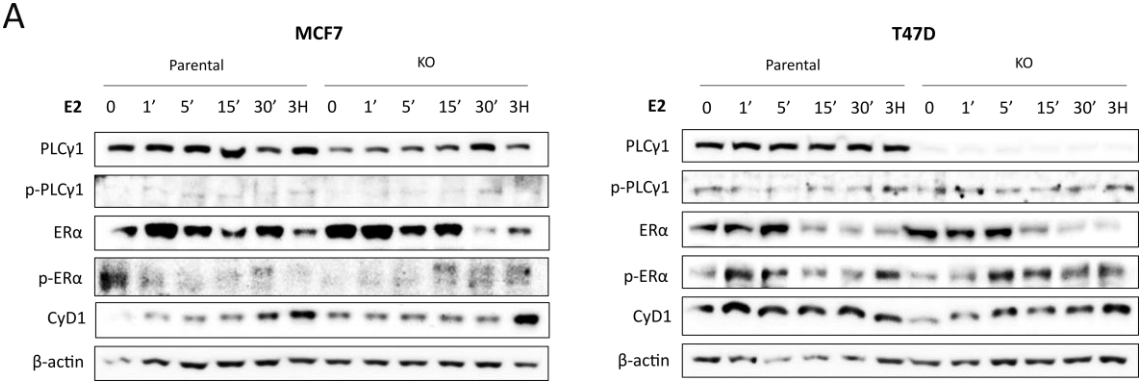
4.4 Crosstalk between PLCγ1 and ERα signaling

ERα signaling is crucial for the proliferation and growth of Luminal BC tumors (117). Hence, we investigated whether the reduction in viability of these cells mediated by PLCγ1 KO could be related to modulation of ERα signaling. For that, we stimulated cells with 1 nM of E2 for different time points. Western blot analysis suggests that the absence of PLCγ1 did not impact ERα signaling, as the expression pattern of ERα remained unaltered, along with its transcriptional target cyclin D1 (Fig.4.4A).

However, PLCγ1 was previously found in the nucleus of BC patient cells (107) and it is known that ERα needs to translocate to the nucleus to promote transcription of target genes. With this in mind, we investigated whether PLCγ1 translocates to the nucleus upon E2 stimulation. To accomplish this, we isolated cytoplasmic and nuclear fractions from MCF7 and T47D cells at different time points following E2 stimulation. The results show the presence of PLCγ1 and its phosphorylated activated form (Y783) in the nucleus, exhibiting a cyclic pattern upon E2 stimulation. In MCF7 cell line, PLCγ1 expression in the nucleus is higher at 5 and 30 min after E2 stimulation, with phosphorylation at 15 min. In T47D cell line, PLCγ1 is found in the nucleus in the absence of E2 and one minute after stimulation with E2 PLCγ1 is phosphorylated. Total PLCγ1 expression is recovered after 5 min, and it is phosphorylated again 30 min after stimulation (Fig. 4.4B).

Given that PLCγ1 translocates to the nucleus upon E2 stimulation, we hypothesized that PLCγ1 might interact directly with ERα. To determine whether such an interaction occurs, and which domain is involved, we performed co-immunoprecipitation (co-IP) experiments using T47D cells with PLCγ1 full-length wild-type (FLWT) and PLCγ1 mutants lacking the SH2 domain (ΔSH2) and the SH3 domain (ΔSH3), both in the presence and absence of E2. Western blot analysis revealed that ERα co-immunoprecipitated with the PLCγ1ΔSH2 isoform in the absence of E2 (Fig. 4.4C).

A



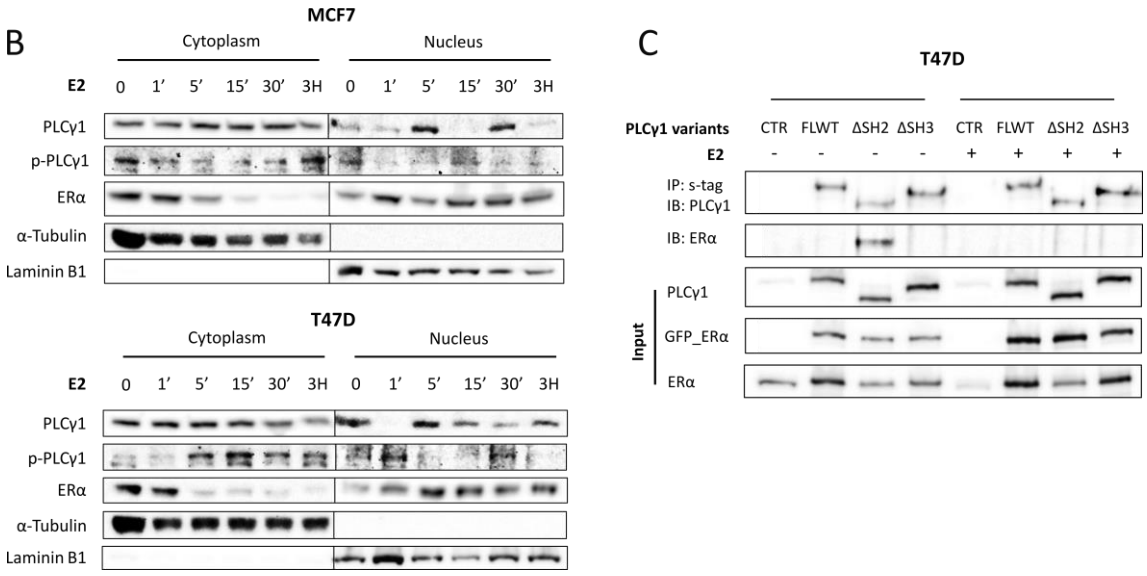


Figure 4.4. Crosstalk between PLCγ1 and ERα signaling upon E2 stimulation. (A) Western blot analysis of PLCγ1 and ERα signaling of MCF7 and T47D parental and PLCγ1 KO cells stimulated with 1nM E2 at the indicated time points, β-actin was used as loading control. (B) Cellular fractionation of MCF7 and T47D cells stimulated with 1nM E2 at the indicated time-points. α-tubulin and Lamin B1 were used as cytoplasmatic and nuclear fractions loading controls, respectively. (C) Western blot analysis of co-immunoprecipitated PLCγ1 in T47D cells transfected with PLCγ1 FLWT and mutants (ΔSH2 and ΔSH3), in the presence or absence of E2.

Simultaneously, we evaluated the impact of PLCγ1 depletion on ERα transcriptional activity. To accomplished that, we generated stable MCF7 and T47D parental and PLCγ1 KO cells expressing an ERE reporter, capable of inducing GFP expression upon ERα activation.

Western blot analysis shows that PLCγ1-deficient MCF7 cells have at least 2-fold higher GFP expression at any period of E2 stimulation, compared to parental MCF7 cells. GFP expression in PLCγ1-deficient T47D cells appears to be independent of E2 stimulation, since T47D PLCγ1 KO cells have 5-fold higher GFP expression than parental T47D cells in the absence of E2; however, when cells are treated with E2, parental T47D cells recover GFP expression, being higher than PLCγ1 KO cells at 6h and 24h after stimulation with E2 (Fig.4.5A). These results suggest that PLCγ1 is inhibiting ERα transcriptional activity.

To investigate whether PLCγ1 was regulating ERα expression, we measured the *estrogen receptor t1* (*ERS1*) mRNA expression, and the RT-qPCR results revealed that PLCγ1 did not influence *ERS1* expression in both cell lines (Fig.4.5B).

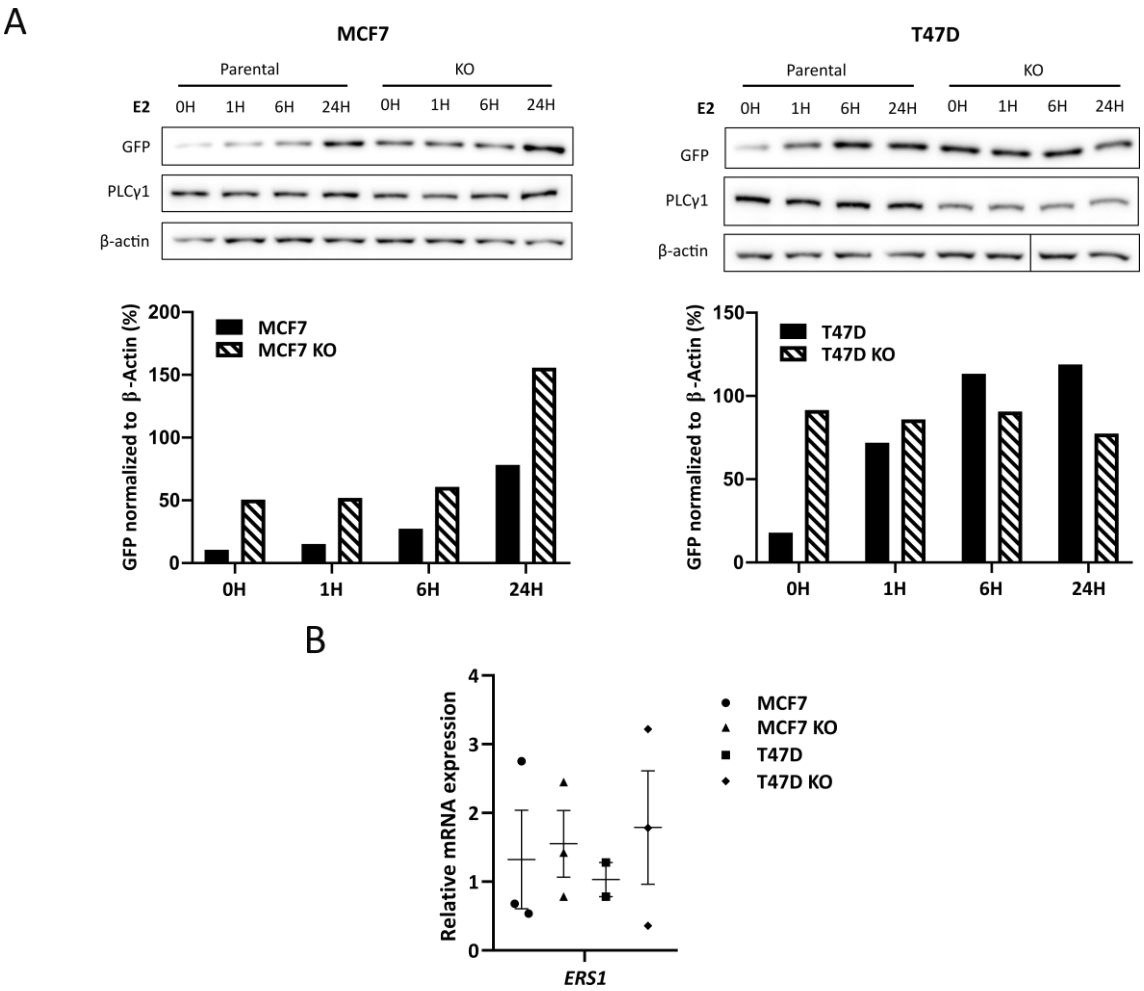


Figure 4.5. Role of PLCγ1 in ERα transcriptional activity. (A) Western blot analysis of MCF7 and T47D parental and PLCγ1 KO expressing an ERE reporter, encoding GFP activation, stimulated with 1nM E2 at the indicated time points, and respective GFP expression quantification, normalized to the loading control, β-actin. (B) RT-qPCR of *ERS1* mRNA expression, *GAPDH* was used as the control (n=1, in triplicate).

4.5 Growth factor-independent activation of AKT and ERK pathways

Previously, we observed a 2-fold upregulation in the expression of both ERα and E2F1 in PLCγ1 KO cells when cultured in a medium deprived of nutrients, hormones, and growth factors (Fig.4.2C). *In vivo*, the tumor microenvironment is very heterogeneous and BC cells often face stressful conditions, including nutrient limitations. However, these cells often adapt to these conditions by activating alternative survival mechanisms (118). In this context, an increase in E2F1 expression may indicate that the cells are compensating for the reduction in available nutrients by activating survival signaling pathways, including the AKT and ERK pathways, to maintain cell cycle progression.

To test our hypothesis, we performed a western blot analysis of AKT and ERK pathways after a 24-hour FBS starvation period, and we assessed the activation status of these pathways both in the presence and absence of PLCγ1.

Western blot analysis revealed that even in the absence of growth factors, PLCγ1 can be phosphorylated and trigger ERK phosphorylation, exhibiting a 2.2-fold higher expression in the parental cells. However, the depletion of PLCγ1 (PLCγ1 KO) resulted in the activation of the AKT pathway, showing a 1.3-fold increase in AKT phosphorylation (p-AKT) in PLCγ1-deficient cells (Fig.4.6A,B).

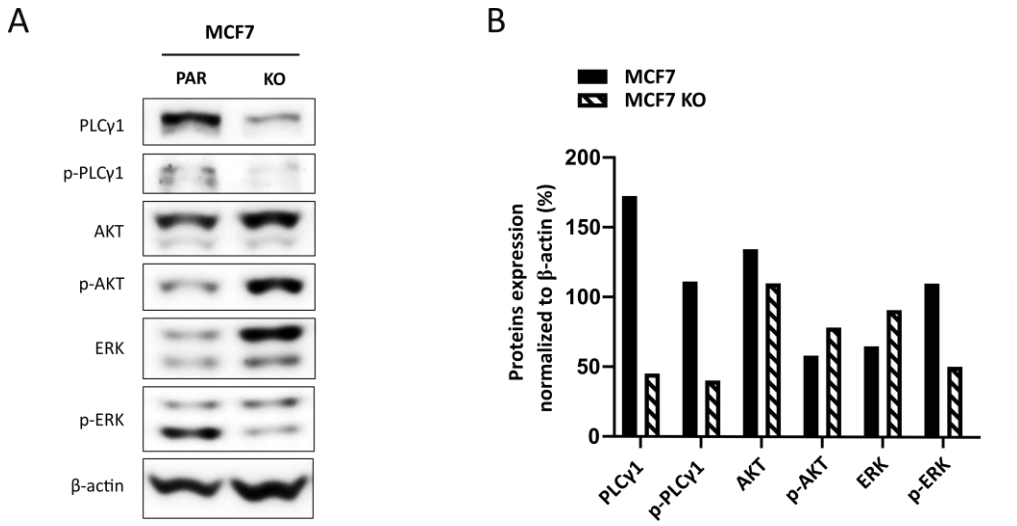


Figure 4.6. PLCγ1 KO promotes growth factor-independent AKT signaling pathway activation. (A) Western blot analysis of MCF7 parental and PLCγ1 KO (n=1). (B) Proteins expression, normalized to the loading control, β-actin.

5 Discussion

PLCγ1 is an important signal transduction molecule, activated downstream of RTK, which contributes to several cellular processes, maintaining cellular homeostasis (6,12–15). Upon activation, PLCγ1 directly interacts with many molecules and signaling pathways in addition to its lipase activity that lead to cellular Ca^{2+} efflux and PKC activation (1,10).

Although numerous studies have shown that PLCγ1 drives tumor cell proliferation, motility and invasion, several reports suggest a neutral or opposite role of PLCγ1 in tumor progression. Indeed, the role of PLCγ1 in cell proliferation remains controversial, with reports showing opposing roles of PLCγ1 in growth factor-induced proliferation (37,119–122).

PLCγ1-deficient mice suffer early embryonic mortality around day 9, as shown by Ji *et al* (24). This suggests that PLCγ1 is crucial for embryonic development. However, the same authors revealed intriguing results in which fibroblasts derived from these embryos not only grew to a higher saturation density compared to wild-type cells *in vitro*, but also increased DNA synthesis in response to EGF (120). Chen *et al.* demonstrated that the activation of PKC by PLCγ1 leads to the phosphorylation and subsequent inhibition of PLCγ1, creating a negative feedback loop that downregulates EGFR-induced mitogenic signaling. (119). Choi and colleagues showed that PLCγ1 forms a ternary complex with Jak2 and protein tyrosine phosphatase 1B (PTP-1B) and negatively regulates growth hormone-induced phosphorylation of Jak2, which results in a downregulation of STAT5 phosphorylation, transcriptional activation, and cell proliferation (121).

The contradictory effects of PLCγ1 on cell proliferation described by these authors can be explained through its interactions with various signaling molecules. These interactions can trigger both proliferative and growth-inhibitory signals due to the complex nature of protein-protein interactions (9,40). Furthermore, the depletion of PLCγ1 alone can be compensated by the activation of alternative signaling pathways that act in parallel (120).

Here we show that PLCγ1 depletion appears to impair proliferation and migration of the MCF7 cell line, but not of the T47D cell line. Although the MCF7 and T47D cell lines represent the Luminal A subtype of BC ($ERα^+$, PR^+, and $HER2^-$), several studies have reported discrepancies at the molecular level between the cell lines (115,116). One study compared the proteomic profiles of the two cell lines using two-dimensional gel analysis and mass spectrometry and concluded that 164 proteins are differently expressed. Proteins involved in cell proliferation appear to be more up-regulated in T47D than in MCF7, while proteins involved in transcription repression and

apoptosis regulation are more up-regulated in MCF7 than in T47D (123). This could account for a more proliferative phenotype of the T47D cell line compared to the MCF7 cell line.

The apparent contradictory role of PLCγ1 may also be due to different basal bioenergetic parameters between the cell lines. It has been described that T47D cells have a higher mitochondrial reserve capacity, suggesting that these cells adapt better to stress than MCF7 cells by altering mitochondrial functions that favor survival and prevent apoptosis (124). This could explain why the absence of PLCγ1 does not have an impact on the T47D cell line.

Using the MCF7 cell line, we found that PLCγ1 KO impairs cell cycle progression. The absence of PLCγ1 affected cells entry into cell cycle, with more cells in a quiescent state, and delayed cells from entering the G2 phase. The disruption of the cell cycle mediated by PLCγ1 KO may be explained by the decrease of CDK6, CDK2 and consequently the decrease of Rb phosphorylation (p-Rb). It has been described that loss of CDK6 results in a prolonged exit from quiescence in hematopoietic stem cells and a delay in G1 progression in lymphocytes (125,126).

The loss of CDK6 and CDK2 in MCF7 PLCγ1 KO cells is consistent when these cells are synchronized in G1 phase and 24h after the cells enter the cell cycle. CDK6 and CDK2 are regulated by p21 and p27, which are both increased in G1 phase, and p21 remains increased 24h after cells enter the cycle (127). This could account for CDK6 and CDK2 downregulation in PLCγ1 KO cells. However, this negative regulation does not impact cell cycle progression as p-Rb is equally expressed in parental and PLCγ1 KO cells, in addition there is higher expression of E2F1 in PLCγ1 KO cells.

This cycle progression phenotype may be due to the expression of CDK4, which is the homologous enzyme of CDK6 (17). Indeed, CDK4 expression was shown to be higher in MCF7 PLCγ1 KO cells when compared to parental cells, and when PLCγ1 was overexpressed in KO cells, CDK4 expression decreased. Furthermore, cells expressing constitutively active PLCγ1 (D1019K) showed an almost complete loss of CDK4. This suggests that PLCγ1 inhibits CDK4, in contrast to CDK6 and CDK2.

One hypothesis is that PLCγ1-mediated cell cycle progression may promote CDK6-cyclinD1 binding rather than CDK4-cyclinD1. In this case, the absence of PLCγ1 would increase CDK4 to compensate for the loss of CDK6 and still be able to proceed with the cell cycle. It has already been described that the lack of function of CDK4 can be compensated for by increasing the level of CDK6 in mouse models (128). CDK4 and CDK6 are expressed in most cell types and can compensate for each other due to functional redundancy (129).

Dysregulation in components of the cyclin D-CDK4/6 axis is common in BC and can be influenced by several mitogenic signaling pathways including the ER, RTK and the downstream signaling pathways (20). Yang *et al.* found that prolonged exposure of the MCF7 cell line to CDK4/6 inhibitors resulted in CDK6 amplification, and that forced overexpression of CDK6 was sufficient to induce resistance to CDK4/6 inhibitors and ER downregulation. Moreover, the knockdown of CDK6 restored sensitivity to CDK4/6 inhibitors (83).

With this in mind, we aimed to understand whether PLCγ1 KO cells were more sensitive to a CDK4/6 inhibitor, palbociclib, as they show a decreased CDK6 expression. In this work, PLCγ1 did not influence the sensitivity of MCF7 and T47D cells to palbociclib. In a prolonged exposure assay to palbociclib, PLCγ1 KO combined with palbociclib resulted in a significative reduced cell viability compared to the control, however it does not appear to involve a synergistic effect. This could be attributed to the compensatory role of CDK4, as previous findings have suggested that CDK4 amplification may indicate a poor response to CDK4/6 inhibitors (130).

Estrogen-driven cell cycle progression in BC is mediated, in part, through transcriptional regulation of cyclin D1 along with suppression of cell cycle inhibitors, such as p27 and p21. Therefore cyclin D1 is typically overexpressed in ER-positive BC (20). We did not observe a difference in cyclin D1 expression, however, and surprisingly, ERα expression was significantly higher in PLCγ1 KO cells. Although the relationship between ERα and PLCγ1 is still not fully understood, recent studies revealed that ERα binds to PLCγ1 to promote UPR pathway activation in ER-positive BC cells, which leads to tumor progression (113,114). In this study, PLCγ1 appears to not affect estrogen signaling. After stimulation with E2 at different time-points no differences in ERα or cyclin D1 expression were observed between parental and KO cells.

However, we show that PLCγ1 and its phosphorylated activated form (Y783) translocates to the nucleus upon E2 stimulation, exhibiting a cyclic pattern. In MCF7 cells, PLCγ1 appears in the nucleus 5 min after stimulation, 10 min later it is phosphorylated and loses expression of its total form, which only reappears 30 min after stimulation. In T47D cells, PLCγ1 is found in the nucleus in the absence of E2 and 1 min after stimulation with E2 PLCγ1 is phosphorylated. Total PLCγ1 expression is recovered after 5 min, and it is phosphorylated again 30 min after stimulation.

These findings can be independent of estrogen signaling, since PLCγ1 is found in the nucleus without E2 stimulation. PLCγ1 has previously been found in the nucleus of tumor cells from BC patients, and has been observed to translocate to the nucleus after EGF stimulation, but its correlation with estrogen signaling has never been explored (43,107). Since PLCγ1 is a multidomain protein that interacts with several proteins through its SH2 and/or SH3 domains

(5,8), we investigated whether PLCγ1 could interact directly with ERα and which domain would be involved.

Here we found that PLCγ1 in its full-length form does not bind to ERα either in the presence or absence of E2; however, the mutated form of PLCγ1 with SH2 domain deletion binds to ERα in the absence of E2. The SH2 domain is critical for PLCγ1 function and responsible for recognizing specific motifs generated by posttranslational modification (8). This mutated form of PLCγ1 is not found *in vivo* and does not explain a correlation with ERα, therefore more studies are needed in order to further explain our results.

ERα drives ER-positive BC growth by promoting the expression of oncogenic genes within EREs (81,131). In cell lines expressing a reporter capable of inducing GFP expression in response to ERα transcriptional activity, we found that PLCγ1 KO cells exhibited 2-fold higher GFP expression after stimulation with E2. Nevertheless, we also found that PLCγ1 KO cells expressing the ERE reporter vector had upregulated PLCγ1 expression. This would explain the lack of effect on ERα transcriptional activity but not its increase. Therefore, these results suggest that PLCγ1 is inhibiting ERα transcriptional activity. However, we found that PLCγ1 has no impact on *ERS1* expression.

ER signaling in BC cells can also interact with other signaling pathways, including PI3K/AKT and MAPK/ERK, which results in cross-interaction of signaling cascades and enhanced tumor cell survival and proliferation (132,133). It is well established that PLCγ1, PI3K/AKT and MAPK/ERK signaling pathways interact with each other, and all these signaling pathways have been implicated in endocrine therapy resistance, either through or independently of ERα activity (91,132,133). Although these signaling pathways are commonly triggered by growth factors, they can play a crucial role in preventing apoptosis in stressful situations, such as nutrients depletion. Within the BC microenvironment, the insufficient vasculature leads to the development of nutrient deprived conditions (118).

Here we show that in serum-depleted medium, the absence of PLCγ1 leads to the upregulation of ERα, among other proteins including p53, p21 and E2F1. Under nutrient deprivation conditions, it has been demonstrated that the activation of p21 by p53 is a protective mechanism against the induction of apoptosis (134). Furthermore, it has been observed that AKT directly phosphorylates p21, leading to its retention in the cytosol, where p21 has been shown to inhibit apoptosis (135). We also observed the activation of AKT signaling pathway, through AKT phosphorylation, in PLCγ1 KO cells. Previous studies have already shown that AKT can be activated in response to stress, even in serum-depleted medium, thus promoting cell survival and proliferation (136)

Our findings suggest that, under conditions of serum deprivation, the absence of PLCγ1 promotes the activation of survival mechanisms, such as the activation of p21 by p53 and/or AKT, thus inhibiting apoptosis. In addition, the AKT signaling pathway may be leading to positive regulation of E2F1 and cell cycle progression. This indicates that, in the presence of PLCγ1, cells do not need to activate these survival mechanisms, and PLCγ1 may play an important role in preventing apoptosis and promoting cell survival under conditions of nutrient deprivation.

Taken together, further research is needed to unravel the role of PLCγ1 in response to stress and nutrient deprivation. This understanding could provide insights into the mechanisms underlying therapeutic resistance, since the ability of tumor cells to adapt to unfavorable conditions in the tumor microenvironment represents a critical component of resistance to therapy.

6 Conclusion and Future Perspectives

BC treatment has faced great advances in recent years, mainly due to a better understanding of the signaling pathways involved in tumor progression, which has allowed not only the development and implementation of targeted therapies, but also the clinical use of novel biomarkers capable of predicting patient's outcome. However, there are still many patients who do not fit into any stratification and are sometimes subjected to therapies that are not effective. In this context, the investigation of new biomarkers and more precise therapeutic options is extremely important.

In this study we proposed to explore new approaches to improve BC patient's outcomes by identifying novel biomarkers. Given the role of PLCγ1 in tumor progression and its potential as a prognostic biomarker in Luminal A BC patients, the main goal of this project was to unravel the crosstalk between PLCγ1 and estrogen signaling in ER-positive BC and potentially understand the mechanisms of resistance.

Importantly, our hypothesis was not confirmed by my results since we could not find a link between PLCγ1 and estrogen signaling. However, we found that PLCγ1 KO decreases the expression of CDK6, which is important in cell cycle progression. Yet, this cell cycle dysregulation had no impact on sensitivity of cells to CDK4/6 inhibitors. Future studies are important to understand the impact of PLCγ1 KO-mediated CDK6 loss on tumor progression. For instance, it would be interesting to use cell lines resistant to endocrine therapy and CDK4/6 inhibitors and evaluate the potential of PLCγ1 KO to restore sensitivity to these therapies.

Our findings also suggest that PLCγ1 may play a crucial role in preventing apoptosis and promoting cell survival when cells are deprived of nutrients. To better understand the role of PLCγ1 in response to stress and nutrient deprivation, further investigations are needed, such as studying the impact of PLCγ1 on apoptosis, which can be measured by caspase 3/7 activity. The ability of tumor cells to adapt to adverse conditions in the tumor microenvironment is a key factor contributing to resistance to therapy. Therefore, unraveling the role of PLCγ1 in these processes is promising for the development of new therapeutic strategies aimed at disrupting these survival mechanisms and increasing the effectiveness of cancer treatments.

Additionally, preliminary results from our group showed that PLCγ1 can have different prognostic value between Luminal A and B BC. Analysis of The Cancer Genome Atlas (TCGA) data showed that high PLCγ1 expression conferred better prognosis to Luminal B patients, in contrast

to Luminal A patients, where PLCγ1 relates with worse prognosis. While this work focused on Luminal A BC, it's worth noting that in Luminal B BC, several of these pathways may exhibit distinct activation patterns, potentially leading to more encouraging findings. This intriguing distinction between Luminal A and B BC requires an in-depth exploration of the interplay between PLCγ1 and estrogen signaling in Luminal B cell lines.

BC is not only heterogeneous but also has a complex microenvironment, where PLCγ1 interacts with diverse signaling pathways depending on the surrounding environment, thus playing the role of a mediator that promotes cell growth or arrest cell division. To achieve a comprehensive understanding of the functions of PLCγ1 in cancer progression, in-depth mechanistic investigations are mandatory, with a particular focus on unraveling the interactions between PLCγ1 and its binding molecules. The identification and elucidation of these binding proteins and the regulatory mechanisms linked to PLCγ1 has the potential to open promising directions for the development of novel and effective cancer treatments.

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