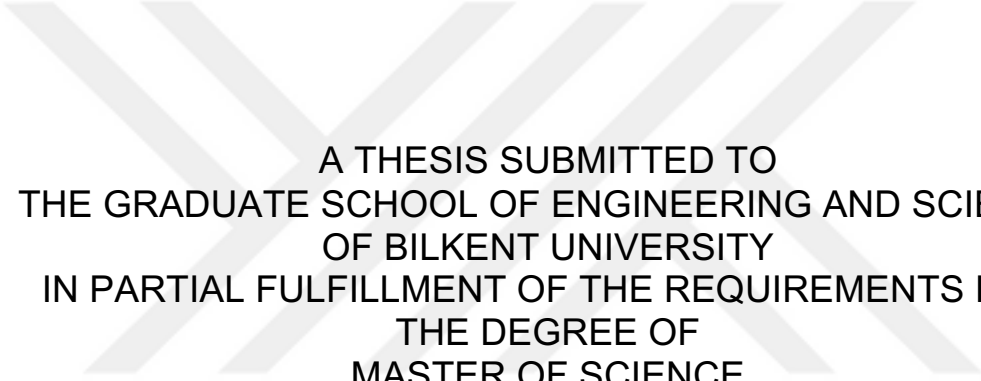




Ayşe'me...

"Homo sum, humani nihil a me alienum puto."

**CHARACTERISATION OF RESISTANCE MECHANISMS TO
ALPELISIB FULVESTRANT COMBINATION IN ADVANCED
STAGE LUMINAL A BREAST CANCER *IN VITRO* & *IN VIVO***



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IN
MOLECULAR BIOLOGY AND GENETICS

By
Melda Özge Oğuz
December 2025

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December 2025

We certify that we have read this thesis and that, in our opinion, it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

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ABSTRACT

CHARACTERISATION OF RESISTANCE MECHANISMS TO ALPELISIB FULVESTRANT COMBINATION IN ADVANCED STAGE LUMINAL A BREAST CANCER *IN VITRO* & *IN VIVO*

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M.S. in Molecular Biology and Genetics

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December 2025

Mutations in the PI3K pathway are prevalently observed in Luminal A-type breast cancer, characterized by HR positivity and HER2 negativity. While PI3K inhibitors, such as alpelisib, are FDA-approved for the treatment of *PI3KCA*-mutant breast cancers, PI3K pathway inhibition can activate compensatory signaling, leading to drug resistance. The objective of this study is to identify potential resistance mechanisms associated with the FDA-approved combination of alpelisib and fulvestrant in the treatment of advanced-stage *PIK3CA*-mutant breast cancer.

In this context, MCF7 and T47D cell lines with acquired resistance to the alpelisib/fulvestrant combination were generated and subjected to comparative transcriptome analysis to identify potential resistance networks. Transcriptomic analysis revealed overactivation of FGFR signaling as a key mechanism of resistance. To elucidate the functional impact of FGFR-dependent resistance mechanisms, resistant cells and 3D tumor spheroid models were treated with the combination of alpelisib/pemigatinib, revealing synergistic growth-inhibitory activity of pemigatinib, an FDA-approved pan-FGFR inhibitor, when combined with alpelisib in alpelisib/fulvestrant-resistant cell lines and 3D tumor spheroids. Concurrent inhibition of the FGFR pathway and p110 α led to sustained tumor regression in drug-resistant tumor xenografts *in vivo*.

Overall, our study identifies FGFR signaling as a key mediator of resistance to combined alpelisib and fulvestrant treatment in PI3KCA-mutant luminal A breast cancer. These findings suggest that targeting the FGFR pathway in combination with PI3K inhibition may represent a potential strategy to overcome drug resistance in advanced-stage refractory luminal A breast cancer.



Keywords: breast cancer, PI3K signaling pathway, FGFR signaling pathway, drug resistance

ÖZET

İLERİ EVRE LUMİNAL A MEME KANSERİNDE ALPELİSİB FULVESTRANT KOMBİNASYON TEDAVİSİNE DİRENÇ MEKANİZMALARININ *IN VITRO* VE *IN VIVO* KARAKTERİZASYONU

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PI3K yol ağı mutasyonları, hormon reseptörü (HR) pozitifliği ve HER2 negatifliği ile karakterize edilen Luminal A tipi meme kanserlerinde yaygın olarak gözlenmektedir. Alpelisib gibi PI3K inhibitörleri, PIK3CA-mutant meme kanserlerinin tedavisi için FDA tarafından onaylanmış olsa da, PI3K yol ağı inhibisyonunu telafi edici (kompansatuvar) sinyal yollarının aktivasyonuna yol açarak ilaç direncine neden olabilmektedir. Bu bağlamda, sunulmakta olan çalışmanın amacı, ileri evre PIK3CA-mutant meme kanserinin tedavisinde FDA onaylı alpelisib ve fulvestrant kombinasyonu ile ilişkili potansiyel direnç mekanizmalarını belirlemektir.

Bu kapsamda, alpelisib/fulvestrant kombinasyonuna kazanılmış direnç geliştirmiş MCF7 ve T47D hücre hatları oluşturulmuş ve olası direnç yürütücülerini tanımlamak amacıyla karşılaştırmalı transkriptom analizi gerçekleştirilmiştir. Transkriptomik analizler, FGFR sinyal yolunun aşırı aktivasyonunun direnç gelişiminde temel bir mekanizma olduğunu ortaya koymuştur. FGFR-bağımlı direnç mekanizmalarının fonksiyonel etkisini aydınlatmak amacıyla, dirençli hücreler ve 3B (üç boyutlu) tümör sferoid modelleri, alpelisib/pemigatinib ilaç kombinasyonuna maruz bırakılmıştır. Bu çalışmalar, FDA onaylı pan-FGFR inhibitörü pemigatinibin, alpelisib ile kombinasyon halinde, alpelisib/fulvestrant dirençli hücre hatlarında ve 3B tümör sferoidlerinde sinerjistik bir büyüme baskılayıcı etki

gösterdiğini ortaya koymuştur. FGFR yol ağının ve p110 α 'nın eşzamanlı inhibisyonu, *in vivo* ortamda ilaç dirençli tümör ksenograftlarında devam eden tümör gerilemesine yol açmıştır.

Sonuç olarak, bu çalışma PIK3CA-mutant luminal A tipi meme kanserinde, alpelisib ve fulvestrant kombinasyonuna karşı gelişen ilaç direncinin temel bir aracısı olarak FGFR sinyal yol ağını tanımlamaktadır. Bu bulgular, PI3K inhibisyonu ile birlikte FGFR yol ağının hedeflenmesinin, ileri evre ve tedaviye dirençli luminal A meme kanserinde ilaç direncini aşmaya yönelik potansiyel bir strateji olabileceğini göstermektedir.



Anahtar Kelimeler: meme kanseri, PI3K sinyal yol ağı, FGFR sinyal yol ağı, ilaç direnci

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ABBREVIATIONS

AI – aromatase inhibitor

AKT – protein kinase B

CDH1 – cadherin 1 (E-cadherin)

CDK4/6 – cyclin-dependent kinases 4 and 6

DCIS – ductal carcinoma in situ

DUSP4 – dual-specificity phosphatase 4

EGFR – epidermal growth factor receptor

ER – estrogen receptor

ERK – extracellular signal-regulated kinase

ESR1 – estrogen receptor 1

FDA – Food and Drug Administration

FGFR – fibroblast growth factor receptor

FOXA1 – forkhead box A1

FOS – FBJ murine osteosarcoma viral oncogene homolog

FRS2 – fibroblast growth factor receptor substrate 2

GATA3 – GATA binding protein 3

GSEA – gene set enrichment analysis

HER2 – human epidermal growth factor receptor 2

HR – hormone receptor

IDC – invasive ductal carcinoma

ILC – invasive lobular carcinoma

LCIS – lobular carcinoma in situ

MAP3K1 – mitogen-activated protein kinase kinase kinase 1

MEK – mitogen-activated protein kinase kinase

mTOR – mechanistic target of rapamycin

NTRK2 – neurotrophic receptor tyrosine kinase 2

NSCLC – non-small cell lung cancer

p110 α – phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha

p110 β – phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit beta

p85 – phosphoinositide-3-kinase regulatory subunit alpha

p90RSK – 90 kDa ribosomal S6 kinase
PD-L1 – programmed death-ligand 1
PDX – patient-derived xenograft
PI3K – phosphoinositide 3-kinase
PIK3CA – phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
PR – progesterone receptor
PTEN – phosphatase and tensin homolog
RAF – rapidly accelerated fibrosarcoma kinase
RAS – rat sarcoma small GTPase
RICTOR – rapamycin-insensitive companion of mTOR
RTK – receptor tyrosine kinase
SERD – selective estrogen receptor degrader
SERM – selective estrogen receptor modulator
TEL – translocation-ETS-leukemia (ETV6)
TDLU – terminal ductal lobular unit
TNBC – triple-negative breast cancer
TP53 – tumor protein p53
XPB1 – X-box binding protein 1

1. INTRODUCTION

1.1. Breast cancer: an overview

Breast cancer remains a major health concern globally, accounting for an estimated 2.3 million new diagnoses and 666,000 cancer-related deaths each year, making it the most commonly diagnosed cancer type in women (Bray et al., 2024). It is a heterogeneous disease stratified by diverse histopathological, clinical, and molecular characteristics of the tumor, which enables better prediction of treatment outcomes and allows the selection of an optimized treatment strategy for the patient. However, traditional histopathology parameters and marker expression of signature genes currently relied upon as predictors of therapeutic response are inadequate, particularly in advanced-stage, refractory disease, where the acquisition of drug resistance promotes the activation of compensatory mechanisms (Nolan, Lindeman, and Visvader, 2023).

Depending on whether the cancer cells have spread from the breast ducts or lobules into the surrounding stroma (Figure 1.1), human breast carcinomas are histopathologically classified into two subgroups: pre-invasive and invasive carcinoma. Furthermore, based on the mammary structure of origin, pre-invasive breast cancers are subdivided into ductal carcinoma *in situ* (DCIS), which comprises 80% of the preinvasive tumors, and

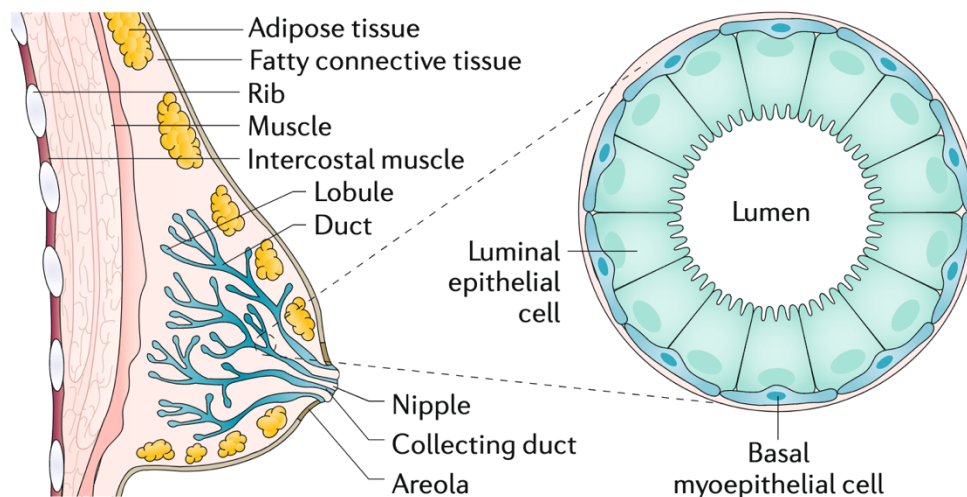


Figure 2.1: Schematic representation of the breast structure. The terminal ductal lobular unit (TDLU), comprised of breast lobules and ducts, is the functional unit of the breast where most breast malignancies occur. Physiologically, breast ducts are composed of an epithelial bilayer of luminal epithelial cells and basal myoepithelial cells, which are surrounded by a stroma rich in fibroblasts, adipocytes, and immune cells (modified from Harbeck et al., 2019). See copyright permission in Appendix C.

lobular carcinoma *in situ* (LCIS); whereas invasive tumors are classified as invasive ductal carcinoma (IDC), constituting 60-75% of invasive cases and invasive lobular carcinoma (ILC) (Nolan, Lindeman, and Visvader, 2023; Harbeck et al., 2019).

In addition to the aforementioned morphological characterisation, based on their expression levels of the estrogen receptor (ER), progesterone receptor (PR), and human epithelial growth factor 2 (HER2), human breast carcinomas are broadly stratified into three clinical subtypes: ER+, HER2+, and triple negative breast cancer (TNBC). ER+ tumors comprises 70% of all breast malignancies, while HER2+ cancers, further subdivided into HER2+ER+ and HER2+ER-, account for 15% of all breast cancer cases. TNBC subtype is characterised by ER, PR, and HER2 negativity and is frequently attributed as the most aggressive clinical subtype, constituting a disproportionate amount of breast cancer-related mortalities due to high rates of distant metastasis and disease relapse in patients (Nolan, Lindeman, and Visvader, 2023; Harbeck et al., 2019).

1.1.1. Molecular subtypes of breast cancer

The advent of transcriptome-level analysis of tumor molecular profiles has provided further insights into the profound heterogeneity among tumor samples and demonstrated the inadequacy of morphological or clinical stratification for predicting therapeutic response (Herschkowitz et al., 2007). This has led to the classification of five intrinsic molecular subtypes to refine patient response in breast cancer, namely luminal A, luminal B, HER2-enriched, basal-like, and claudin-low, each exhibiting distinct biological features. Luminal A tumors, which constitute approximately 28% of intrinsic molecular subtypes, are ER+PR+ and are characterized by high expression of luminal signature genes, including *ESR1*, *GATA3*, *XBP1*, and *FOXA1*. Although luminal A-type malignancies harbor the least number of mutations, interestingly, the highest number of recurrently mutated genes are encountered in this intrinsic subtype. The genes recurrently mutated in luminal A-type ductal carcinomas include *PIK3CA*, *GATA3*, *MAP3K1*, and *CDH1*. The luminal B intrinsic subtype is characterized as ER+ tumors with less pronounced luminal gene expression and higher proliferation gene expression. HER2-enriched (HER2-E) tumors are a distinct molecular subtype that exhibit intermediate levels of luminal gene expression and amplified HER2 expression. Although the terms are not interchangeable,

a high proportion of basal-like tumors are TNBC and are characterized by basal cytokeratin and EGFR overexpression, with concurrent low luminal A signature expression. Claudin-low tumors represent the least prevalent intrinsic subtype with mesenchymal features, lacking ER, PR, and HER2 expression, and exhibiting low expression of proliferative genes (Nolan, Lindeman, and Visvader, 2023; Harbeck et al., 2019).

1.1.2. Oncogenic signaling pathways in breast carcinogenesis

Breast carcinogenesis is primarily driven by distinct, and often concurrent, oncogenic aberrations that converge on signaling cascades regulating cellular proliferation and survival. Although there are multiple genes reported to be commonly mutated in breast cancer, such as *PIK3CA* and *TP53*, concordant with the convergence of genetic alterations, infrequently mutated genes should also be taken into account when interrogating potential biomarkers and therapeutic targets in breast cancer. Despite extensive research that elucidated oncogenic pathways in breast cancer (Figure 1.2), there remains a pressing need to better understand the crosstalk between distinct signaling pathways and compensatory mechanisms, especially in the acquisition of drug resistance.

1.1.2.1. ER pathway

The steroid hormone estrogen plays a significant role in both normal breast physiology and carcinogenesis. Encoded by the *ESR1* gene, estrogen receptor α (ER α) is attributed to be the chief oncogenic driver in ER+ luminal breast cancers and therefore is considered to be a major therapeutic target in breast cancer treatment. *GATA3* and *FOXA1* are chief transcriptional regulators of ER function (Figure 1.2), and loss-of-function mutations in *GATA3*, encountered in 10-15% of luminal breast cancers, are associated with worse prognosis (Mehra et al., 2005). Crosstalk between ER signaling and growth factor receptor signaling cascades primarily through PI3K/AKT/mTOR and RAS/RAF/MAPK pathways modulates ER α activity and thereby may contribute to acquired endocrine therapy resistance (Nolan, Lindeman, and Visvader, 2023; Harbeck et al., 2019).

1.1.2.2. PI3K/AKT/mTOR pathway

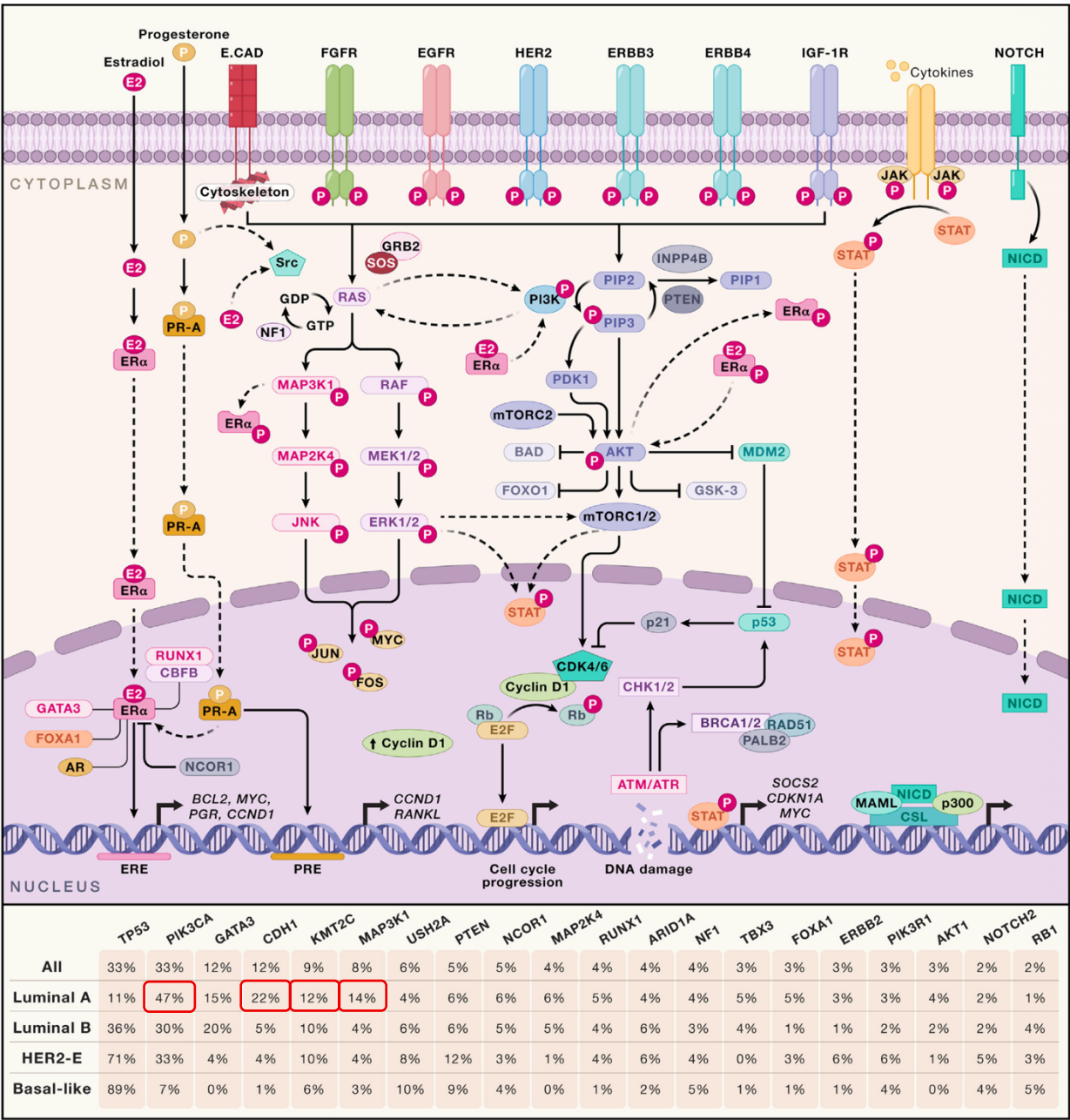


Figure 1.2: Aberrant signaling networks in breast oncogenesis. Schematic of key signaling pathways represented in breast carcinogenesis, underscoring signaling crosstalk and mutation rates for commonly altered genes in each molecular subtype. The top recurrently mutated genes, more prominently represented in luminal A-type tumors, are indicated in red boxes. P (pink), phosphorylation; ER, estrogen receptor; PR, progesterone receptor; ERE, estrogen response element; PRE, progesterone response element; ECAD, E-cadherin; FGFR, fibroblast growth factor receptor; EGFR, epidermal growth factor receptor; HER2, human epidermal growth factor type 2; IGF-1R, insulin-like growth factor type 1 receptor; NICD, Notch intracellular domain (adopted from Nolan, Lindeman, and Visvader, 2023). See copyright permission in Appendix C.

The PI3K/AKT/mTOR pathway is a key regulator of cellular growth and survival, transducing signals from upstream receptor tyrosine kinases (RTKs). The components of the PI3K pathway are prevalently altered in breast cancer through mutations in *PIK3CA*, *AKT1*, *PTEN*, and *PIK3R1* (Figure 1.2); however, the mutation patterns may occur as subtype-specific. *PIK3CA* mutations are commonly harbored in luminal and HER2-E tumors, whereas *PTEN* loss and alterations in other components are more frequent in basal-like tumors (Nolan, Lindeman, and Visvader, 2023; Harbeck et al., 2019). Convergent genetic alterations affecting PI3K signal transduction may emerge coincidentally across multiple pathway components during breast cancer therapy (Juric et al., 2015), underscoring the specific role of the PI3K pathway in acquired resistance and necessitating the development of robust therapies targeting the components of this pathway.

1.1.2.3. MAPK signaling pathway

The MAPK pathway is a signaling cascade that is activated downstream of receptor tyrosine kinases through RAS activity. *RAS* is one of the most frequently mutated genes across distinct human malignancies; however, the incidence of *RAS* mutations in breast cancers is rare. Instead, aberrant MAPK activity usually arises from overactivation of upstream receptor tyrosine kinases such as HER2. Genetic alterations in *MAP3K1* and *MAP2K4* are relatively more common in luminal breast cancers and often concurrent with *PIK3CA* mutations (Figure 1.2). Moreover, *PIK3CA* mutations can result in overactivation of MAPK signaling (Ramirez-Ardila et al., 2017), indicating a substantial crosstalk between the two pathways in breast oncogenesis.

1.1.3. Current targeted therapies in ER+ breast cancer treatment

Currently, rather than the clinicopathological features alone, breast cancer treatment decisions are mainly guided by molecular profiling and the determination of intrinsic tumor subtype. Molecular biomarkers such as Ki67 and PD-L1, and genomic assays such as RNA-based prognostic tests are routinely utilized in clinical settings to help refine prognosis in patients. For instance, RNA-based assays are particularly essential in the identification of post-menopausal patients with luminal B-type disease who may benefit

from chemotherapy. Combinational therapy approaches that aim to target both the primary oncogenic drivers and cooperating signaling pathways have evidently improved patient outcomes across all breast cancer subtypes, leading to the emergence of extended trials of combined therapeutics (Nolan, Lindeman, and Visvader, 2023).

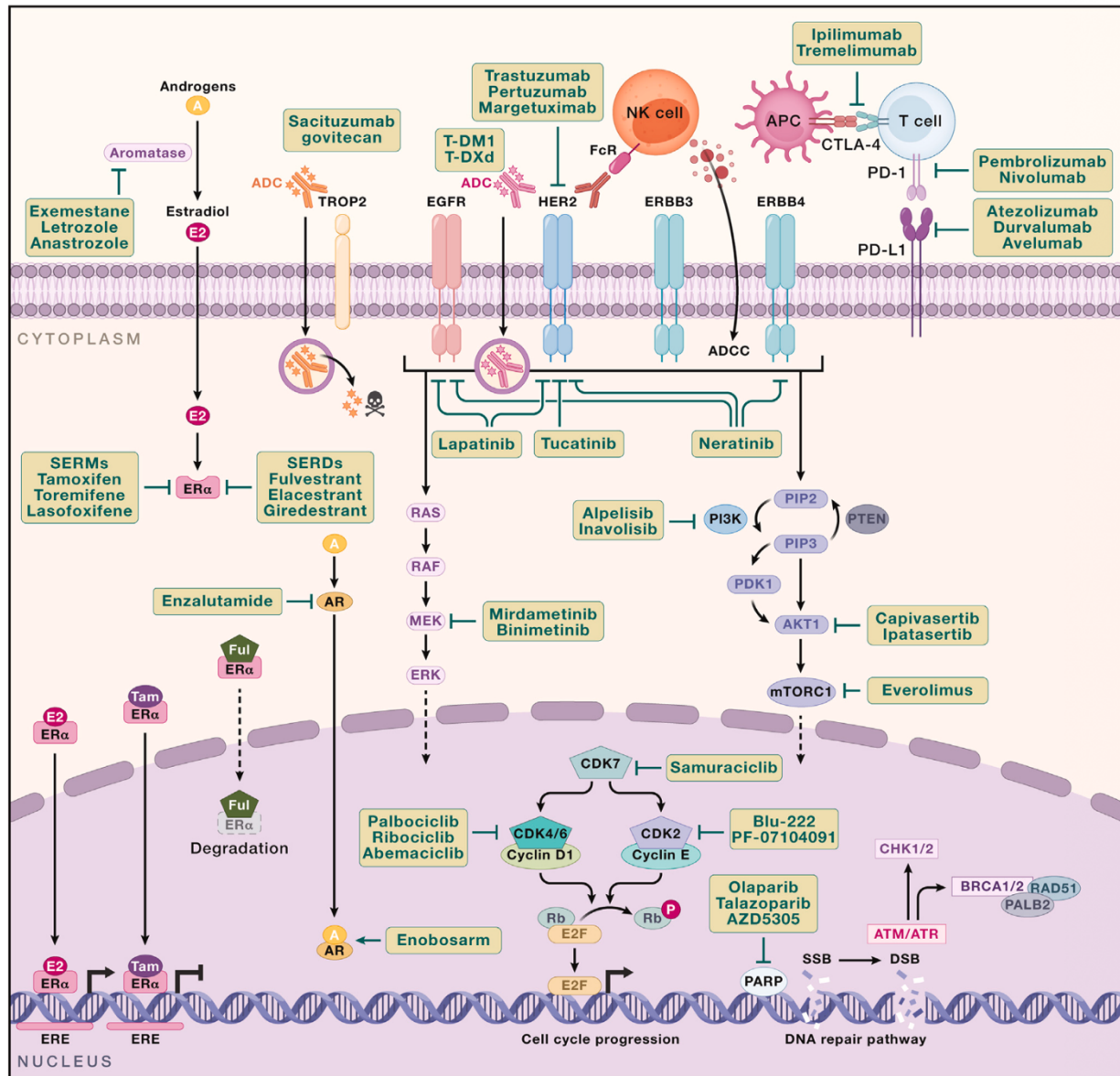


Figure 1.3: Current therapies in breast cancer. Illustration depicting key targeted therapeutics and their targets. MEK inhibitors are under investigation, while p110 α inhibitors alpelisib and inolisib, AKT inhibitor capivasertib, and mTORC1 inhibitor everolimus are approved for clinical use. Selective ER modulators (SERMs), such as tamoxifen, aromatase inhibitors (AIs), such as letrozole, and selective ER degraders (SERDs), including fulvestrant, are first-line treatments in clinical practice for ER+ breast cancer. Targeting CDK4/6 (palbociclib, ribociclib, abemaciclib) in combination therapies has been shown to improve patient outcomes (adopted from Nolan, Lindeman, and Visvader, 2023). See copyright permission in Appendix C.

Endocrine therapy has transformed clinical practice in the treatment of ER+ breast cancers and remains the foundation of most single and combination treatments. Standard endocrine therapy options involve selective estrogen receptor modulators (SERMs) such as tamoxifen, toremifene, and lasofoxifene, and aromatase inhibitors, including exemestane, letrozole, and anastrozole (Figure 1.3). However, ovarian ablation or suppression strategies are increasingly being utilized in addition to endocrine therapy for premenopausal women to prevent disease relapse. Fulvestrant (Bross et al., 2002) is a selective estrogen receptor degrader (SERD) frequently used in advanced-stage breast cancer, usually with progression after a first-line endocrine treatment (Xiong et al., 2025). Consequent to the signaling crosstalk between estrogen receptor and the regulation of cell-cycle, incorporation of CDK4/6 inhibitors such as palbociclib, ribociclib (Slamon et al., 2024), and abemaciclib (Rastogi et al., 2024) (Figure 1.3) to endocrine therapy as a standard treatment regimen for metastatic ER+ breast cancer has significantly improved progression-free survival (PFS) rates in patients. However, resistance to combined endocrine therapy and CDK4/6 inhibition is prevalent and is often attributed to compensatory activation of the PI3K/AKT pathway (McAndrew and Finn, 2022).

Acquired *ESR1* mutations, not evident during the primary disease, arise after prolonged exposure to AIs or SERMs and represent a primary resistance mechanism in endocrine therapy. Routine monitoring of the circulating tumor DNA (ctDNA) for the presence of *ESR1* mutations and switching to SERDs such as fulvestrant earlier in the clinical trajectory could delay the progression of the disease. Activation of the PI3K/AKT/mTOR pathway as a bypass of endocrine therapy represents another major mechanism of resistance. Consequently, the combined treatment of the AI exemestane with the mTORC1 inhibitor everolimus has shown promising results in delaying tumor progression (Baselga et al., 2012), and the combination of the AKT inhibitor capivasertib with fulvestrant has recently been approved by the FDA (Turner et al., 2023). Although prior studies with pan-PI3K inhibitors were not effective in providing patient benefit, targeted inhibition of the p110 α isoform with a selective inhibitor, alpelisib, in combination with fulvestrant treatment in patients with PIK3CA mutations has shown favorable outcomes (André et al., 2019; Nolan, Lindeman, and Visvader, 2023; Xiong et al., 2025).

1.1.3.1. Fulvestrant/alpelisib combination treatment in advanced stage ER+ *PIK3CA* mutant breast cancer

Alpelisib (BYL719) is a p110 α isoform-specific small molecule PI3K inhibitor that exhibits nearly 50-fold selectivity for the p110 α over other isoforms. The phase III SOLAR-I clinical trial evaluated the efficacy of alpelisib in combination with fulvestrant in patients with advanced-stage HR+, *PIK3CA* mutant breast cancer who have shown progression either on or after prior endocrine therapy. While no survival benefit was observed in the *PIK3CA* wild-type cohort, combination therapy significantly improved progression-free survival (PFS) to 11 months in patients with *PIK3CA* mutations. However, therapy-related adverse grade ≥ 3 events were comparably more frequent in the alpelisib arm (34.9% vs. 16.7%) (André et al., 2019). Guided by the SOLAR-1 clinical trial results, in 2019, the FDA approved the use of alpelisib/fulvestrant combination in postmenopausal women with advanced-stage HR+ refractory breast cancer (Narayan et al., 2021).

1.1.3.2. Drug resistance mechanisms to fulvestrant/alpelisib treatment

Despite its clinical efficacy, acquired resistance to p110 α inhibition is inevitable and previously reported to arise from multiple distinct mechanisms that primarily promote reciprocal activation of the PI3K pathway (Tarman, 2020). Persistent mTORC1 signaling has been considered as the primary bypass mechanism in acquired resistance to alpelisib in both preclinical models and patient biopsies (Elkabets et al., 2013). Consequently, concurrent inhibition of PI3K and mTOR with the dual inhibitor apitolisib has been evaluated in clinical trials; however, it was associated with limited tolerability in patients due to severe toxicities (Makker et al., 2016). Loss of the tumor suppressor PTEN has also been identified as a major driver in acquired alpelisib resistance (Juric et al., 2015; Razavi et al., 2020) in metastatic lesions from patients progressing on alpelisib treatment. Furthermore, compensatory activation of the p110 β isoform (Leroy et al., 2016) has also been proposed as a route for reciprocal PI3K pathway activation in alpelisib resistance, further highlighting PI3K pathway rewiring upon the acquisition of resistance. However, as with the mTOR combination, therapeutically targeting both p110 α and p110 β isoforms with pan-PI3K inhibitors (Krop et al., 2016) has also been associated with adverse effects in patients.

Collectively, these findings indicate that acquired resistance to p110 α inhibition is multifactorial, driven by convergent signalling crosstalk and feedback regulation, underscoring the currently unmet need to identify actionable drug resistance mechanisms to enhance the durability of clinical responses.

1.2. FGFR signaling pathway

FGF-FGFR signaling is paramount for diverse cellular processes, including proliferation, survival, metabolism, and differentiation, and plays a critical role in development and tissue homeostasis. Aberrant activity in FGFR signaling has been linked to tumorigenesis in distinct malignancies, including non-small-cell lung cancer (NSCLC), cholangiocarcinoma, and breast cancer (Chen, 2025).

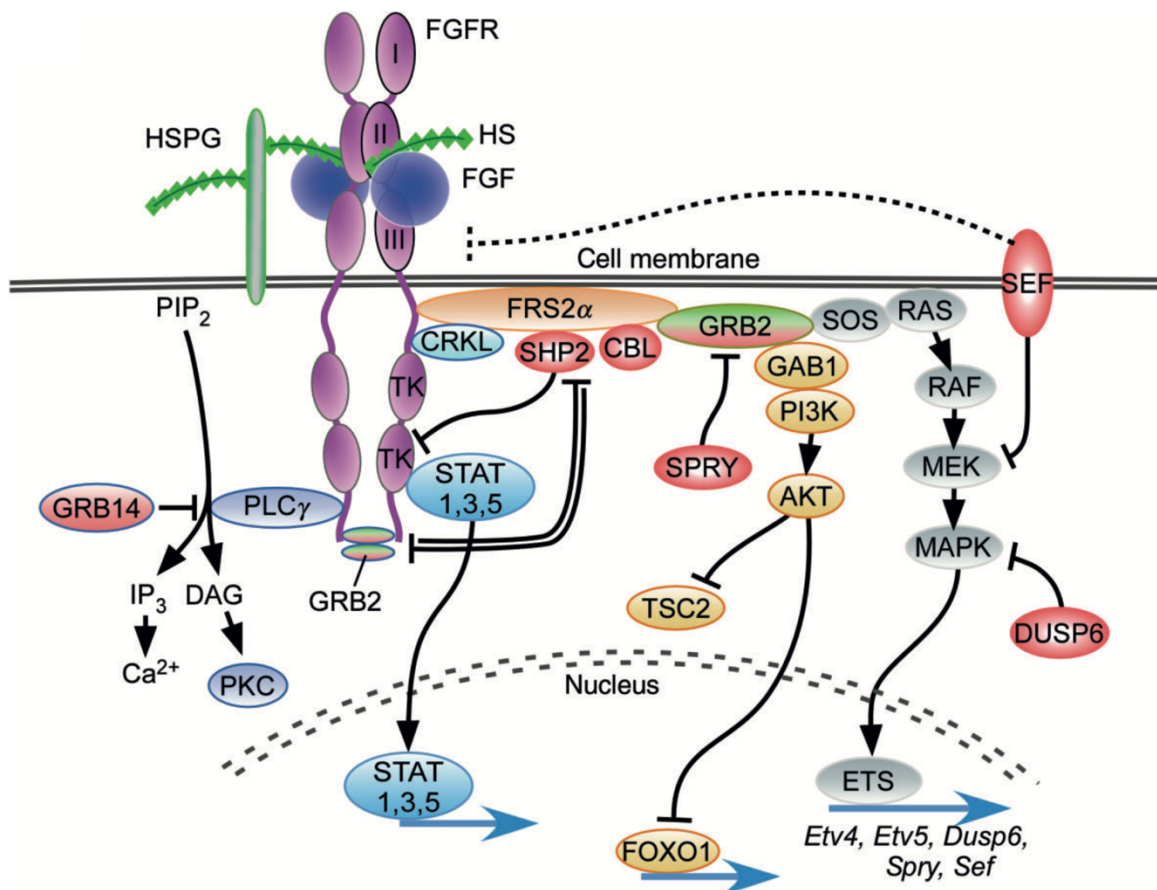


Figure 1.4: Fibroblast growth factor receptor (FGFR) pathway. Schematic representation of major components of the FGFR pathway (modified from Ornitz and Itoh, 2015). Ornitz and Itoh (2015) is an open-access source protected under © Attribution-NonCommercial-NoDerivatives 4.0 International license, and therefore sharing, copying, or redistributing the material is free.

Fibroblast growth factors (FGFs) comprise a family of proteins with distinctive roles in signaling transmission and can be classified as canonical FGFs (FGF1–10, FGF16–18, FGF20, and FGF22), endocrine FGFs (FGF19/FGF15, FGF21, and FGF23), and FGF-homologous factors (FGF11–14). Fibroblast growth factor receptors (FGFRs) (FGFR1-4) are type I transmembrane receptor tyrosine kinases with three extracellular immunoglobulin-like domains and an intracellular kinase domain (Kato et al., 2024). Canonical FGFs can signal through fibroblast growth factor receptors in conjunction with heparan sulfate cofactors (Figure 1.4), while endocrine FGFs require klotho coreceptors (KLA and KLB) in addition to heparan sulfate cofactors to activate FGFRs. Binding of FGF ligands induces FGFR dimerization and downstream receptor kinase activation, leading to sequential autophosphorylation of key tyrosine residues. Activated FGFRs predominantly signal through the adaptor FRS2, triggering downstream activation of MAPK, PI3K/AKT, PLC γ , and JAK-STAT pathways (Kato et al., 2024; Ornitz and Itoh, 2015).

1.3. Rationale and aims of the study

Although the generation of highly effective targeted therapies has significantly improved patient outcomes in hormone receptor-positive, *PIK3CA* mutant luminal A-type breast cancer, the acquired resistance to these therapies remains a major clinical challenge. Drug combination therapies that concurrently target the signaling pathways that mediate the acquired resistance are an emerging strategy for patients with refractory disease progression. Concordantly, the combination of alpelisib, a p110 α -specific inhibitor targeting the PI3K pathway, with the estrogen receptor degrader fulvestrant has improved progression-free survival up to 11 months in patients with prior endocrine-resistant metastatic disease (André et al., 2019); however, ultimate relapse due to acquired resistance to this combination treatment is inevitable. Identifying actionable mechanisms that precede bypass to dual PI3K/ER α inhibition is therefore paramount for achieving durable treatment outcomes.

Fibroblast growth factor receptors (FGFRs) are receptor tyrosine kinases that play pivotal roles in cell proliferation, survival, and metabolism. Aberrant activity of FGFR receptors may promote tumorigenesis through the oncogenic activation of downstream RAS/MAPK

signaling cascades. Amplifications in FGFR2, and more prominently, FGFR1 isoforms are frequently observed in approximately 15% of breast cancer patients (Servetto, Formisano and Arteaga, 2021). Emerging evidence implicates compensatory activation of FGFR1 signaling in acquired resistance to endocrine therapy and CDK4/6 inhibitors (Formisano et al., 2019). However, the potential implications of aberrant FGFR signaling in acquired resistance to the clinically applicable fulvestrant/alpelisib combination, and whether simultaneous targeting of the FGFR pathway and p110 α could serve as a strategy to surmount this resistance, remain unknown.

Preliminary findings from our laboratory indicate that luminal A-type breast cancer cells with prior endocrine resistance and acquired resistance to fulvestrant and alpelisib exhibit elevated FGFR/MAPK signaling at the transcriptomic level (Emirmustafaoglu, 2024). These findings provide a rationale for further investigation into activated FGFR signaling as a potential mediator of resistance to fulvestrant and alpelisib combination therapy in breast cancer. Consequently, this study aims to delineate FGFR signaling-mediated resistance mechanisms that may arise from combined fulvestrant and alpelisib treatment, to evaluate the therapeutic efficacy of cotargeting the FGFR pathway and p110 α as a strategy to overcome drug resistance in both *in vitro* and *in vivo* models, and elucidate FGFR isoform-specific roles in the acquisition of drug resistance.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals and reagents

Table 2.1: List of chemicals and reagents.

	Brand	Catalog Number
β -mercaptoethanol	AppliChem	A1108.0100
β -Estradiol	Sigma-Aldrich	E2758
1kb Opti-DNA Marker	abm	G106
Acetic Acid	Sigma-Aldrich	27225
Acrylamide/Bisacrylamide	SERVA	10688.01
Agarose	PRONA	
Agarose Tablets	BIOLINE	BIO-41027
Ammonium peroxodisulfate (APS)	Carlo Erba	410627
Ampicillin	SERVA	13397.03
Bovine Serum Albumin (BSA)	CAPRICORN	BSA-DG
<i>N,N</i> -Dimethylacetamide (DMAC)	Sigma-Aldrich	185884
Dimethyl sulfoxide (DMSO)	SERVA	20385.02
Dithiothreitol (DTT)	Thermo Scientific	A39255
DMEM-w/o phenol red	Gibco	11880028
Dulbecco's Modified Eagle's Medium (DMEM)	Gibco	41966-029
Ethanol	Isolab	920.026
Ethidium bromide (EtBr)	Invitrogen	15585011
Fetal Bovine Serum (FBS)	CAPRICORN	FBS-16A
Geneticin (G418 Sulfate)	Gibco	10131-035
Glycine	neoFroxx	1154KG001
Hydrochloric Acid	Sigma-Aldrich	30721
Insulin	Diagnovum	D630
Laemmli Sample Buffer (4x)	Ecotech	LSB-4X

LB Medium Powder	SERVA	48501.01
L-Glutamine 200mM	VivaCell	C3543-0100
Lipofectamine 3000	Thermo Scientific	L3000-008
Matrigel Basement Membrane Matrix	CORNING	354234
MEM-Non-Essential Amino Acids	Sigma-Aldrich	M7145
Methanol	Isolab	947.046
(Hydroxypropyl)Methyl cellulose	Sigma-Aldrich	09963
Skimmed Milk powder	alfasol	T2592_521113
Nitrocellulose Membrane	GVS	1212632
Opti-Protein Ultra Marker	abm	G623
Penicillin/Streptomycin	CAPRICORN	PS-B
Phosphatase Inhibitor Mix	SERVA	39055.01
Polybrene	Santa Cruz	sc-134220
Ponceau S Solution	EcoTech	PS05
Potassium chloride	Merck	104936.1000
Potassium phosphate monobasic	Sigma-Aldrich	04243
Protease Inhibitor Cocktail	GlpBio	GK10014
RIPA Buffer	Intron Biotechnology	IBS-BR002a
Sodium azide	SERVA	30175.01
Sodium chloride	Isolab	969.033
Sodium dodecyl sulfate (SDS)	Merck	822050.1000
di-Sodium hydrogen phosphate dihydrate	Merck	106580.1000
Sodium orthovanadate	Sigma-Aldrich	S6508
Sulforhodamine B (SRB)	Sigma-Aldrich	S9012
N,N,N',N'-Tetramethylethylenediamine (TEMED)	SERVA	35925.01
Trichloroacetic acid (TCA)	Merck	27242
Trizma Base	Sigma Aldrich	T1503
Trypan Blue 0.4% Solution	EcoTech	TRY-B100

Trypsin-EDTA (10X)	biowest	B.X0930
Tween 20	SERVA	39796.01
WesternBright ECL HRP substrate	Advansta Inc	K-12045-D50

2.1.2. Laboratory equipment

Table 2.2: List of laboratory equipment.

	Brand	Model
Amicon Ultra Centrifugal Filter	Merck	UFC910024
Cell counting chamber		Neubauer Improved
Cell culture hood	Nuve	MN 120
Centrifuges	Eppendorf	5810R
	Eppendorf	5415R
	Hettich	UNIVERSAL 320
	Nuve	NF 800R
CO ₂ Incubator	Thermo Scientific	FORMA Direct Heat
Freezer (-80°C)	Hettich	
Gel electrophoresis system	Bio-Rad	Mini-PROTEAN Tetra Cell
Gel Imager UV	Vilber Lourmat	Quantum ST4
Horizontal electrophoresis system	Consort	EHS3200-SYS
Imaging system	Thermo Scientific	EVOS FL Auto
Inverted microscope	OLYMPUS	CKX41
Liquid nitrogen tank	Thermo Scientific	Locator 4 Plus
Microplate reader	Biotek	Synergy HT
Microvolume UV-Vis spectrophotometer	Thermo Scientific	NanoDrop One
Mr. Frosty Freezing container	Thermo Scientific	5100-0001
Scanner	Canon	CanoScan 4200F
Shaking Incubator	New Brunswick Scientific	Innova 4000
Thermal cycler	Applied Biosystems	2720

Transfer system	Bio-Rad	Trans-Blot Turbo
Vortex	IsoLab	622.02.001
Western Blot Imaging System	GE Healthcare	Amersham Imager 600

2.1.3. Buffers and solutions

Table 2.3: Recipes for buffers and solutions.

Buffer/Solution	Ingredients
0.4% SRB Staining Solution	0.4 g SRB in 100 mL 1% acetic acid
0.5% methyl cellulose, 5% DMAC Solution	0.25 g (Hydroxypropyl)methyl cellulose 2.5 mL DMAC Dissolved in 47.5 mL sterile ddH ₂ O Filtered sterile with 0.22 µM filter
1% Acetic Acid	1% acetic acid in ddH ₂ O
5% Western Blot Blocking Buffer	5 g milk powder in 100mL TBS-T
10% TCA (SRB Fixation Solution)	20 mL 50% TCA 80 mL ddH ₂ O
10 mM Tris Base (SRB Destaining Solution)	605 mg Trizma base Dissolved in 500 mL ddH ₂ O
50% TCA	50 g TCA Dissolved in 100 mL ddH ₂ O
PBS (10X)	1.37 M NaCl 27 mM KCl 18 mM KH ₂ PO ₄ 100 mM Na ₂ HPO ₄ In 1 L ddH ₂ O
PBS (1X)	100 mL 10X PBS 900 mL ddH ₂ O
Resolving Gel Buffer (pH:8.8)	187 g Trizma-Base pH adjusted to 8.8 with HCl Complete the volume to 1 L with ddH ₂ O
RIPA Lysis Buffer	1X Protease inhibitor 1X Phosphatase inhibitor mix 1 µM DTT 1 mM Sodium orthovanadate

	in RIPA buffer
Stacking Gel Buffer (pH:6.8)	60.5 g Trizma-Base pH adjusted to 6.8 with HCl Complete the volume to 1 L with ddH ₂ O
TAE Buffer (50X)	242 g Trizma-base 18.61 g EDTA 57.1 mL acetic acid Complete the volume to 1 L with ddH ₂ O
TBS (10X)	80 g NaCl 2 g KCl 30 g Trizma-Base pH adjusted to 7.4 with HCl Complete the volume to 1 L with ddH ₂ O
TBS-T	1 mL Tween-20 100 mL 10X TBS Complete the volume to 1 L with ddH ₂ O
Western Blot Running Buffer (10X)	30 g Tris-Base 144 g Glycine 10 g SDS Up to 1 L ddH ₂ O
Western Blot Running Buffer (1X)	100 mL 10X Western Blot Running Buffer Complete the volume to 1 L with ddH ₂ O
Wet Transfer Buffer (10X)	30 g Tris-Base 144 g Glycine Complete the volume to 1 L with ddH ₂ O
Wet Transfer Buffer (1X)	100 mL 10X Transfer Buffer 200 mL Methanol 700 mL ddH ₂ O

2.1.4. Antibodies

2.1.4.1. Primary Antibodies

Table 2.4: List of primary antibodies.

Antibody	WB Dilution	Source	Catalog No	
Akt	1:1000	BioLegend	680302	3% BSA & 0.04 µg/ml NaN ₃ in TBS-T
p-Akt S473	1:1000	CST	9271	3% BSA & 0.04 µg/ml NaN ₃ in TBS-T

ER α	1:1000	Santa Cruz	sc-8002	3% BSA & 0.04 μ g/ml NaN ₃ in TBS-T
Erk1/2	1:1000	CST	9102	3% BSA & 0.04 μ g/ml NaN ₃ in TBS-T
p-Erk1/2 T202/Y204	1:1000	CST	9101	3% BSA & 0.04 μ g/ml NaN ₃ in TBS-T
FGFR2	1:1000	CST	23328	3% BSA & 0.04 μ g/ml NaN ₃ in TBS-T
FGFR3	1:1000	CST	4574	3% BSA & 0.04 μ g/ml NaN ₃ in TBS-T
Flag (tag)	1:1000	GenScript	A00187-100	3% BSA & 0.04 μ g/ml NaN ₃ in TBS-T
GAPDH	1:1000	BioLegend	649202	3% BSA & 0.04 μ g/ml NaN ₃ in TBS-T
p85	1:1000	CST	4292	3% BSA & 0.04 μ g/ml NaN ₃ in TBS-T
p110 α	1:1000	CST	4249	3% BSA & 0.04 μ g/ml NaN ₃ in TBS-T
p110 β	1:1000	CST	3011	3% BSA & 0.04 μ g/ml NaN ₃ in TBS-T
PTEN	1:1000	CST	9559	3% BSA & 0.04 μ g/ml NaN ₃ in TBS-T
p-p90RSK S380	1:1000	CST	11989	3% BSA & 0.04 μ g/ml NaN ₃ in TBS-T
RSK1/2/3	1:1000	CST	9355	3% BSA & 0.04 μ g/ml NaN ₃ in TBS-T
S6	1:1000	BioLegend	691802	3% BSA & 0.04 μ g/ml NaN ₃ in TBS-T
p-S6 S235/236	1:1000	CST	4858	3% BSA & 0.04 μ g/ml NaN ₃ in TBS-T

2.1.4.2. Secondary Antibodies

Table 2.5: List of secondary antibodies.

Antibody	WB Dilution	Source	Catalog No	
Goat-anti-mouse IgG (H+L), HRP conjugate	1:3000	Advansta	R-05071	3% BSA in TBS-T
Goat-anti-rabbit IgG (H+L), HRP conjugate	1:3000	Advansta	R-05072	3% BSA in TBS-T

2.1.5. Primers

Table 2.6: List of primers.

Primer	Sequence (5'-3')
pWZL_neo_forward	GAACCTCCTCGTTTCGACC
Antisense_MGSO	TGCACCATCTGTCACTCTGTTAACCTC
Sense_GPO-3	GGGAGCAAACAGGATTAGATACCCT

2.1.6. Plasmids

Table 2.7: List of plasmids.

Plasmid	Source
pWZL_neo_Flag_C5-8_TEL (ampicillin)	J. Zhao, Dana-Farber Cancer Institute
pWZL_neo_Flag_E1-4_TEL (ampicillin)	J. Zhao, Dana-Farber Cancer Institute
pWZL_neo_Flag_FGFR1_TEL (ampicillin)	Isolated from the E1-4 Pool in this study
pBabe_neo	
pCMV_VSV-G	Addgene, #8454
gag/pol	Addgene, #14887

2.1.7. Bacterial Strains

Table 2.8: Bacterial strains.

Strain	Genotype	Source
<i>E. coli</i> (C3040)	F' <i>proA</i> ⁺ <i>B</i> ⁺ <i>lacI</i> ^q Δ(<i>lacZ</i>)M15 <i>zzf::Tn10</i> (Tet ^R)/Δ(<i>ara-leu</i>) 7697 <i>araD139 fhuA</i> Δ <i>lacX74 galK16 galE15</i> <i>e14- Φ80dlacZ</i> ΔM15 <i>recA1 relA1 endA1 nupG</i> <i>rpsL</i> (Str ^R) <i>rph spoT1</i> Δ(<i>mrr-hsdRMS-mcrBC</i>)	NEB

2.1.8. Cell Lines

Table 2.9: List of cell lines.

Cell Line	Characteristics	Medium
HEK293	Human embryonic kidney cells	DMEM; 10% FBS, 1% P/S
MCF7-tamR-fulvR	Human luminal A type breast cancer cells resistant to tamoxifen and fulvestrant treatment	DMEM-w/o phenol red; 8% FBS, 1% P/S, 2mM L-Glutamine, 1% MEM-NEAA, 4 µg/mL Insulin 1.5µM fulvestrant
MCF7-tamR-fulvR-BYLR	Human luminal A type breast cancer cells resistant to tamoxifen, fulvestrant and alpelisib treatment	DMEM-w/o phenol red; 8% FBS, 1% P/S, 2mM L-Glutamine, 1% MEM-NEAA, 4 µg/mL Insulin 1µM BYL719 and 1.5µM fulvestrant

T47D-tamR-fulvR	Human luminal A type breast cancer cells resistant to tamoxifen and fulvestrant treatment	DMEM-w/o phenol red; 8% FBS, 1% P/S, 2mM L-Glutamine, 1% MEM-NEAA, 4 µg/mL Insulin 1.5µM fulvestrant
T47D-tamR-fulvR-BYLR	Human luminal A type breast cancer cells resistant to tamoxifen, fulvestrant and alpelisib treatment	DMEM-w/o phenol red; 8% FBS, 1% P/S, 2mM L-Glutamine, 1% MEM-NEAA, 4 µg/mL Insulin 1µM BYL719 and 1.5µM fulvestrant
T47D-tamR-fulvR-C5-8	Human luminal A type breast cancer cells resistant to tamoxifen and fulvestrant treatment, transduced with NTRK1 and LYN (C5-8 pool)	DMEM-w/o phenol red; 8% FBS, 1% P/S, 2mM L-Glutamine, 1% MEM-NEAA, 4 µg/mL Insulin Maintained with 250µg/mL G418 1.5µM fulvestrant
T47D-tamR-fulvR-FGFR1	Human luminal A type breast cancer cells resistant to tamoxifen and fulvestrant treatment, transduced with FGFR1	DMEM-w/o phenol red; 8% FBS, 1% P/S, 2mM L-Glutamine, 1% MEM-NEAA, 4 µg/mL Insulin Maintained with 250µg/mL G418 1.5µM fulvestrant
T47D-tamR-fulvR-pBabe_neo	Human luminal A type breast cancer cells resistant to tamoxifen and fulvestrant treatment, transduced with empty pBabe_neo vector	DMEM-w/o phenol red; 8% FBS, 1% P/S, 2mM L-Glutamine, 1% MEM-NEAA, 4 µg/mL Insulin Maintained with 250µg/mL G418 1.5µM fulvestrant

2.1.9. Inhibitors

Table 2.10: List of inhibitors.

Inhibitor	Brand	Catalog Number
Alpelisib (BYL-719)	MedChemExpress	HY-15244
AZD6482 (KIN-193)	MedChemExpress	HY-10344
Everolimus (RAD001)	MedChemExpress	HY-10218
Fulvestrant	TargetMol	T2146
Idelalisib (CAL-101)	MedChemExpress	HY-13026
Lirafugratinib	MedChemExpress	HY-147250
Pictilisib (GDC-0941)	MedChemExpress	HY-50094

Pemigatinib	CAYMAN	33260
Pemigatinib	MedChemExpress	HY-109099

2.1.10. Kits

Table 2.11: List of commercial kits.

Kit	Source	Catalog Number
BCA Protein Assay Kit	TaKaRa	T9300A
Monarch Plasmid Miniprep Kit	NEB	T1010
Plasmid Midi Kit	Qiagen	12143

2.2. Methods

2.2.1. In vivo experiments

All animal experimentation was performed with athymic nude mice in accordance with the protocols approved by the Institutional Animal Care and Use Committee of Ihsan Dogramaci Bilkent University. Mice were housed with a 12-hour light/dark cycle and *ad libitum* access to food and water.

For xenograft studies, 12 µg/mL β-estradiol (Sigma-Aldrich) was supplemented to the drinking water 5 days prior to cell injection. β-Estradiol supplementation continued for the duration of the experiment; drinking water was replenished every week, and protected from light exposure. Five-week-old athymic female nude mice were orthotopically injected with 1×10^7 T47D-tamR-fulvR-BYLR cells in 150 µL 1:1 (v/v) PBS and Matrigel (Corning) into the right inguinal mammary fat pads. When tumor volumes reached approximately 95 mm³, animals were randomized and treated with 5% DMAC (Sigma-Aldrich) and 0.5% methyl cellulose (Sigma-Aldrich) as vehicle control, 20 mg/kg BYL719 (MedChemExpress) dissolved in 0.5% methyl cellulose, 1 mg/kg pemigatinib (MedChemExpress) dissolved in 5% DMAC and 0.5% methyl cellulose or a combination of 20 mg/kg BYL719 and 1 mg/kg pemigatinib dissolved in 5%DMAC and 0.5% methyl cellulose daily for a duration of 30 days via oral gavage. Tumors were regularly measured throughout the experiment using a caliper once every two days, and tumor volumes were calculated using the formula: (length x width²) / 2. Body weight changes were monitored

regularly, once every two days. Mice were sacrificed thirty days after the treatment initiation. Tumors were collected, weighed, and stored at -80°C as fresh frozen samples.

2.2.2. Methods in cell biology

2.2.2.1. Cell passaging

Human cell lines were cultured in a humidified atmosphere with 5% CO₂ at 37°C. T47D-tamR-fulvR, T47D-tamR-fulvR-BYLR, MCF7-tamR-fulvR, and MCF7-tamR-fulvR-BYLR cells were grown in phenol-free DMEM with 1 g/L glucose (Thermo Scientific) supplemented with 8% FBS (CAPRICORN), 1% Penicillin-Streptomycin (100 U/mL penicillin, 0,1 mg/mL streptomycin, Thermo Scientific), 1% MEM-Non-Essential Amino Acids (Sigma-Aldrich), 2 mM L-Glutamine (VivaCell), and 4µg/mL insulin (Diagnovum). Stably transduced T47D-tamR-fulvR-FGFR1, T47D-tamR-fulvR-C5-8, and T47D-tamR-fulvR-pBabe_neo cells were maintained in growth medium containing 250µg/mL G418 (Thermo Scientific). To maintain drug resistance, tamoxifen- and fulvestrant-resistant cell lines were treated with 1.5 µM fulvestrant, while tamoxifen-, fulvestrant-, and alpelisib-resistant cell lines received 1.5 µM fulvestrant and 1 µM alpelisib once every other passage. HEK293 cells were cultured in DMEM with 4.5 g/L glucose supplemented with 10% FBS and 1% Penicillin-Streptomycin.

Cells were subcultured when they reached 80-90% confluency. The growth medium was aspirated, and cells were washed once with sterile 1X PBS. For drug-resistant T47D and MCF7 cell lines, cells were incubated with 0.25% trypsin-EDTA (biowest) for 6-8 minutes at 37°C, while HEK293 cells were incubated with 0.05% trypsin-EDTA (biowest) for 3 minutes at 37°C. Detached cells were rinsed with fresh growth medium and were collected in a 15mL tube. The cells were centrifuged at 1500 rpm for 5 minutes. After the centrifugation, the supernatant was aspirated, and the cells were resuspended in fresh growth medium. Cells were subcultured into a fresh cell culture plate at a 1:3-1:5 ratio for further maintenance.

All cell culturing procedures were performed under sterile conditions, and cells were regularly checked for mycoplasma contamination as described in 2.2.3.4.

2.2.2.2. Cell thawing

Cryovials containing the cell line of interest were retrieved from the liquid nitrogen tank and rapidly thawed in a pre-warmed 37°C water bath for 2-3 minutes, ensuring the O-ring did not come into contact with the water to prevent contamination. The contents of the cryovials were transferred to a 15 mL tube using a sterile serological pipette, and 5 mL of growth medium without penicillin-streptomycin was added dropwise. The cells were centrifuged at 1500 rpm for 5 minutes. The supernatant was aspirated, the cells were resuspended in 5 mL of growth medium without penicillin-streptomycin, and seeded onto a 6 cm cell culture plate for subsequent experiments.

2.2.2.3. Cryopreservation of cells

The cell medium was aspirated, the cells were washed once with 1X sterile PBS, and were detached from the plate surface by 0.25% (drug-resistant MCF7 and T47D sublines) or 0.05% (HEK293) trypsin incubation for 6-8 minutes (drug-resistant MCF7 and T47D sublines) or 3 minutes (HEK293). Detached cells were resuspended with fresh growth medium and collected in a 15 mL tube. The cells were centrifuged at 1500 rpm for 5 minutes, and the supernatant was aspirated. The cell pellet was resuspended in cell-freezing medium composed of 50% DMEM, 40% FBS, and 10% DMSO, and the suspension was transferred to 2 mL cryogenic vials. Cryovials were placed in a Mr. Frosty freezing container (Thermo Scientific), which allows a cooling rate of -1°C/minute, and were incubated in a -80°C freezer for 12-24 hours. For long-term storage, frozen cryovials were transferred to a liquid nitrogen tank.

2.2.2.4. Cell harvesting

For subsequent western blot experiments, 6.5×10^5 drug-resistant T47D cells and 7×10^5 drug-resistant MCF7 cells were seeded in 6 cm cell culture dishes two days prior to cell harvesting. On the day of cell harvesting, the plates were placed on ice, the medium was aspirated, and the cells were washed once with 1X PBS. 1 mL PBS was added to each plate, and the cells were collected in a 1.5 mL tube by scraping the plate surface with a cell scraper. The tubes were centrifuged at 4000 rpm at 4°C for 10 minutes. The supernatants were aspirated, and the cell pellets were either used directly to prepare protein extracts as described in 2.2.4.1. or stored at -80°C after snap-freezing.

2.2.2.5. Sulforhodamine B (SRB) cell viability assay

To assess changes in cell viability upon drug treatment, the cell medium was aspirated, the cells were washed once with 1X sterile PBS, and were detached from the plate surface by incubation in 0.25% trypsin-EDTA for 6-8 minutes at 37°C. The detached cells were resuspended in fresh growth medium, collected in a 15 mL tube, and centrifuged at 1500 rpm for 5 minutes. The supernatant was removed, and the cell pellet was resuspended gently, but thoroughly, in fresh growth medium. 20 µL of cell suspension was mixed with 20 µL of trypan blue (EcoTech). In order to determine the cell quantity, 10 µL of the cell/trypan blue mix was loaded into the chamber of a hemocytometer. The viable cells in the four outer gridded squares were counted manually, and the number of cells present in 1 mL of cell suspension was calculated using the formula: mean of viable cell number per square x dilution factor x 10^4 . After completing necessary calculations, 3×10^3 cells were seeded into each well of a 96-well surface-treated cell culture plate in 100 µL of complete growth medium. The outer wells of the plate were filled with 1X PBS to prevent evaporation of the cell medium, and three replicates of wells seeded with complete growth medium without cells were included in each experiment as blank controls. The cells were incubated for two days post-seeding, a titration of drugs of interest was given to the cells in 100 µL of complete growth medium, and the cells were incubated for five days in drug-containing medium.

After the incubation period had ended, the cell medium was removed, and the cells were fixed with 50 µL of 10% TCA (Merck) at room temperature for 1 hour or at 4°C overnight. The fixative was removed, and the wells were gently rinsed once with dH₂O. Viable cells were stained with 50 µL of 0.4% SRB (Sigma-Aldrich) solution at room temperature for 45 minutes. In order to remove excess SRB stain, the wells were rinsed three times with 1% acetic acid (Sigma-Aldrich), and the plates were air-dried. The images of the cells were taken by scanning the plate with a scanner. The cell-bound SRB stain was destained with 150 µL of 10 mM Tris base (Sigma-Aldrich), and the absorbance was measured at 564 nm using a microplate reader.

2.2.2.6. 3D tumor spheroid formation with liquid overlay technique

To form 3D tumor spheroids, T47D-tamR-fulvR-BYLR cells were manually counted as described in 2.2.2.5. The wells of a sterile flat-bottom 96-well plate without surface treatment were uniformly coated with 50 μ L of sterile 1% agarose (BIOLINE) dissolved in ddH₂O. Cell suspensions were prepared with 1×10^4 cells/mL (alpelisib-pemigatinib combination spheroids) or 1.5×10^4 cells/mL (alpelisib-lirafugratinib combination spheroids) densities, and for each well 2×10^3 (alpelisib-pemigatinib combination) or 3×10^3 (alpelisib-lirafugratinib combination) cells were seeded on top of agarose-coated wells in 200 μ L complete growth medium with 10% FBS. The plates were incubated for 4 days to promote spheroid formation. After the incubation period, 100 μ L of the cell medium was removed and replaced with 100 μ L of fresh medium containing the inhibitor. The spheroid medium was regularly replenished every 3 days throughout the experiment, and the spheroids were visualized every other day.

The volumes of the spheroids were determined using the AnaSP application (Zanoni et al., 2016).

2.2.2.7. Generation of stable cell lines

For transfection of HEK293 cells, Lipofectamine 3000 (Thermo Scientific) transfection reagent was used according to the manufacturer's protocol. For each construct, two wells of a 6-well cell culture plate were seeded with 3×10^5 HEK293 cells a day before the transfection. For each well to be transfected, 625 ng VSV-G, 625 ng gag/pol, 1250 ng plasmid of interest, and 5 μ L of P3000 were added to 125 μ L of DMEM (4.5 g/L glucose, Thermo Scientific) in a 1.5 mL tube, while 3.75 μ L of Lipofectamine 3000 was added to 125 μ L of DMEM in another tube. The two tubes were mixed and incubated at room temperature for 15 minutes. The medium of HEK293 cells was gently replenished with fresh growth medium without penicillin/streptomycin, and the Lipofectamine suspension containing the plasmid of interest was given to the cells dropwise, very gently. The medium of the HEK293 cells was replenished 24 hours after transfection, and the viral particles were collected in a 15 mL tube both 48 hours and 72 hours post-transfection. The collected viral particles were stored at 4°C, protected from light exposure.

6×10^4 T47D-tamR-fulvR cells and 7×10^4 MCF7-tamR-fulvR cells were seeded on wells of a 6-well cell culture plate two days prior to transduction. Viral particles were filtered

through a 0.45 μM filter and concentrated by centrifugation with an Amicon tube (Merck). 2 mL of growth medium without penicillin/streptomycin with concentrated viral particles and 10 $\mu\text{g/mL}$ polybrene (Santa Cruz) was given to cells. 24 hours post-transduction, the medium of the cells was replenished, and 48 hours post-transduction, the selection of transduced cells was initiated. Cells were selected with 1000 $\mu\text{g/mL}$ G418 (Gibco) for 8-9 days and maintained in 250 $\mu\text{g/mL}$ G418 for subsequent experiments.

2.2.3. Methods in molecular biology

2.2.3.1. Transformation of chemically competent *E.coli*

For plasmid amplification purposes, 1 μL of plasmid DNA or 1 μL of DEPC water as a negative control was added to 50 μL of competent cells (NEB), and the contents were gently mixed by flicking the bottom of the tube. The tube was incubated on ice for 30 minutes, then heat-shocked at 42°C for 45 seconds, followed by an incubation on ice for 5 minutes. 950 μL of LB (SERVA) medium without antibiotics was added to the bacteria, and the bacteria were grown at 37°C in a shaking incubator (250 rpm) for an hour. The bacteria were centrifuged at 3000 rpm for 5 minutes, and 900 μL of the supernatant was discarded. The bacterial pellet was resuspended, and 50 μL of the transformation was evenly spread onto a 10 cm LB-Agar plate containing 100 $\mu\text{g/mL}$ ampicillin (SERVA). For colony growth, the LB-Agar plates were incubated at 37°C overnight. Single colonies of transformed *E.coli* were picked and inoculated overnight in LB medium containing 100 $\mu\text{g/mL}$ ampicillin for downstream applications.

All procedures involving bacterial culture were conducted under sterile conditions near the Bunsen burner.

2.2.3.2. Isolation of plasmid DNA from *E.coli*

Following bacterial transformation as described in 2.2.3.1., the transformed single *E.coli* colonies were grown overnight in 3 mL (for subsequent miniprep protocol) or 100 mL (for subsequent midiprep protocol) LB medium containing 100 $\mu\text{g/mL}$ ampicillin at 37°C in a shaking incubator with constant shaking at 250 rpm. The plasmid DNA was isolated from the bacteria using the Monarch Plasmid Miniprep Kit (NEB) or Plasmid Midi Kit (Qiagen) in accordance with the manufacturer's protocol. After following the midiprep protocol, the

isolated plasmid DNA was reconstituted in sterile TE buffer. The OD_{260}/OD_{280} , OD_{260}/OD_{230} ratios, and the concentration of isolated plasmid DNA were determined using the UV-Vis spectrophotometer NanoDrop One.

2.2.3.3. Isolation of a single plasmid from a plasmid pool

In order to isolate the pWZL_neo_Flag_FGFR1_TEL plasmid from the E1-4 plasmid pool, competent cells were transformed with the E1-4 plasmid pool DNA as described in 2.2.3.1. Ten single colonies were picked, and plasmid DNA was isolated from the single colonies using the miniprep protocol described in 2.2.3.2. To confirm insert sequences, isolated plasmid DNA samples were sequenced with Sanger sequencing using the pWZL_neo_forward primer (Table 2.6). The sequencing was performed by Macrogen EUROPE. The obtained blast results and chromatograms for each plasmid colony are given in Appendix B.

2.2.3.4. Polymerase chain reaction (PCR) for mycoplasma detection

To test cultured cell lines for mycoplasma contamination, a PCR protocol previously described by Young et al. (2010) was followed; cell media were collected from cultures with confluency greater than 70% and cultured in the same medium for at least 3 days. Mycoplasma contamination was tested using the MGSO and GPO-3 primer pair listed in Table 2.6. The PCR reaction components are listed in Table 2.12, and the thermocycler settings are given in Table 2.13. Cell medium collected from a cell line positive for mycoplasma contamination was used as a positive control, while DEPC water was used as a negative control.

Table 2.12: Mycoplasma PCR reaction components.

Reagent	Amount/Reaction
10X NEB Standard Taq Buffer	2.5 μ L (1X)
10 mM dNTP	0.5 μ L (200 μ M)
10 μ M antisense_MGSO	0.5 μ L (0.2 μ M)
10 μ M sense_GPO-3	0.5 μ L (0.2 μ M)
DNA Template (cell medium)	1 μ L

NEB Taq polymerase	0.125 μ L
DEPC water	Complete the volume to 25 μ L

Table 2.13: Mycoplasma PCR thermocycler settings.

Initial denaturation: 95°C	30 seconds	
Denaturation: 95°C	15 seconds	} 30 cycles
Annealing: 55°C	15 seconds	
Elongation: 68°C	15 seconds	
Final elongation: 68°C	5 minutes	
Cooling: 4°C		

When the PCR reaction had ended, the resulting amplified DNA samples were further analyzed by agarose gel electrophoresis as described in 2.2.3.5..

2.2.3.5. Agarose gel electrophoresis

To separate amplified DNA samples, agarose gel electrophoresis was performed. 1% agarose (PRONA) dissolved and boiled in 1X TAE buffer was mixed with 0.5 μ g/mL ethidium bromide (Invitrogen), and poured into a gel chamber. The polymerized gel was placed in an electrophoresis tank covered with 1X TAE buffer. The samples were mixed with 6X DNA loading dye and loaded into the gel wells along with a 1 kb DNA ladder (abm). The samples were run at 120V for 30 minutes, and DNA bands were visualized under UV light.

2.2.4. Methods in protein biochemistry

2.2.4.1. Preparation of protein extracts from mammalian cells

To prepare protein extracts, cultured cells were harvested as described in 2.2.2.4, and the cell pellets were resuspended with 60- 80 μ L (three times the volume of the pellet) of RIPA (Intron Biotechnology) lysis buffer (Table 2.3). The samples were incubated on ice for 30 minutes and vortexed once every 10 minutes. The lysates were then centrifuged at 13.000 rpm, at 4°C for 15 minutes, and the supernatants containing the protein lysates

were collected in fresh tubes. Protein concentrations were measured with BCA assay as described in 2.2.4.2.

2.2.4.2. Determination of protein concentration by BCA assay

Protein concentrations of the lysates were determined with the TaKaRa BCA Protein Assay Kit, following the manufacturer's protocol for low-concentration proteins (VI-e). 4 μ L of protein extracts (1:100) or differing amounts of BSA standard protein were diluted in sterile ddH₂O in a total volume of 400 μ L. 100 μ L of samples and standards were dispensed in a clear 96-well plate in triplicate, and 100 μ L of working solution was added to each well. The plate was incubated at 37°C for 60 minutes, and the absorbances were measured at 562 nm. The protein concentrations of the samples were calculated using the standard curve as a reference.

2.2.4.3. SDS polyacrylamide gel electrophoresis (SDS-PAGE)

To separate proteins according to their molecular weights, SDS-PAGE was performed. 4% and 20% resolving polyacrylamide gels were prepared as in Tables 2.14 and 2.15. 4%-20% gradient resolving polyacrylamide gels were cast using the Mini-Protean (Bio-Rad) gel casting system and overlaid with isopropanol. After gel polymerization, 4% stacking gel (Table 2.16) was poured on top of the resolving gels. Casted gels were transferred to the electrophoresis chamber, which was filled with 1X running buffer (Table 2.3). Protein samples were prepared in 4X Laemmli buffer (EcoTech), mixed with β -mercaptoethanol (1:9), and boiled at 95°C for 5 minutes. 20 μ g of protein samples were loaded together with a protein ladder (abm) as a reference into the gels. Samples were run at a constant voltage of 80V until the proteins had passed the stacking gel, and then at 120V.

Table 2.14: 4% Resolving Gel Preparation (10mL).

Reagent	Amount/2 Gels
Resolving gel buffer (Table 2.3)	2.5 mL
Acrylamide/Bisacrylamide (SERVA)	1.3 mL
ddH ₂ O	6.1 mL

10% APS (Carlo Erba)	50 μ L
TEMED (SERVA)	5 μ L

Table 2.15: 20% Resolving Gel Preparation (10mL).

Reagent	Amount/2 Gels
Resolving gel buffer (Table 2.3)	2.5 mL
Acrylamide/Bisacrylamide (SERVA)	6.6 mL
ddH ₂ O	0.8 mL
10% APS (Carlo Erba)	50 μ L
TEMED (SERVA)	5 μ L

Table 2.16: 4% Stacking Gel Preparation (10mL).

Reagent	Amount/2 Gels
Stacking gel buffer (Table 2.3)	2.5 mL
Acrylamide/Bisacrylamide (SERVA)	1.3 mL
ddH ₂ O	6.1 mL
10% APS (Carlo Erba)	50 μ L
TEMED (SERVA)	10 μ L

2.2.4.4. Western blot

The proteins were transferred from the polyacrylamide gels to nitrocellulose membranes (GVS) using either wet transfer technique in 1X tris-glycine transfer buffer (Table 2.3) or semi-dry transfer using the Trans-Blot Turbo (Bio-Rad). The wet transfer was performed either at a constant current of 250 mA for 150 minutes or at 90 mA overnight (16 hours). Semi-dry transfer was performed using the manufacturer's Standard SD protocol for 30 minutes.

The protein transfer was confirmed with Ponceau S staining (EcoTech), and membranes were blocked in 5% milk powder in TBS-T for 1 hour with gentle shaking. The membranes were rinsed once with TBS-T and incubated with primary antibodies against the target proteins at 4°C overnight with gentle shaking. After primary antibody incubation, the

membranes were washed three times with TBS-T for 5 minutes and incubated in HRP-conjugated secondary antibodies at room temperature for 1.5 hours. The membranes were washed three times with TBS-T for 5 minutes, and the respective signals were detected using WesternBright ECL HRP substrate (Advansta) according to the manufacturer's protocol. The protein bands were visualized using the Amersham Imager 600 imaging system.

2.2.5. Statistical analysis

Statistical analysis of the represented data was performed using GraphPad Prism 10. Unless stated otherwise, experiments were repeated three times, and the results are demonstrated as mean $\pm$ SEM for each experiment. Statistical significance was determined with one-way ANOVA or two-way ANOVA, where applicable, with Tukey's multiple comparisons test. Significance was indicated for p values of less than 0.05 (* p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001).

For the determination of the Bliss and Loewe synergy scores and their statistical significance, represented with the combinatorial treatments, the SynergyFinder application was used (Ianevski et al., 2017).

3. RESULTS

3.1. Generation and characterization of luminal A-type breast cancer cell lines with acquired resistance to tamoxifen, fulvestrant, and alpelisib treatment

In 2019, the use of fulvestrant in combination with alpelisib (BYL719) in hormone receptor (HR)-positive, human epidermal growth factor receptor 2 (*HER2*)-negative, and *PIK3CA*-mutated advanced-stage breast cancer patients who had previously shown disease progression following an endocrine therapy regimen was approved by the Food and Drug Administration (FDA) (André et al., 2019). Although the SOLAR-1 clinical trial has revealed that this combination therapy is successful in extending progression-free survival up to 11 months in patients with advanced-stage breast cancer (André et al., 2019), the emergence of drug resistance is inevitable, and therefore, the identification of

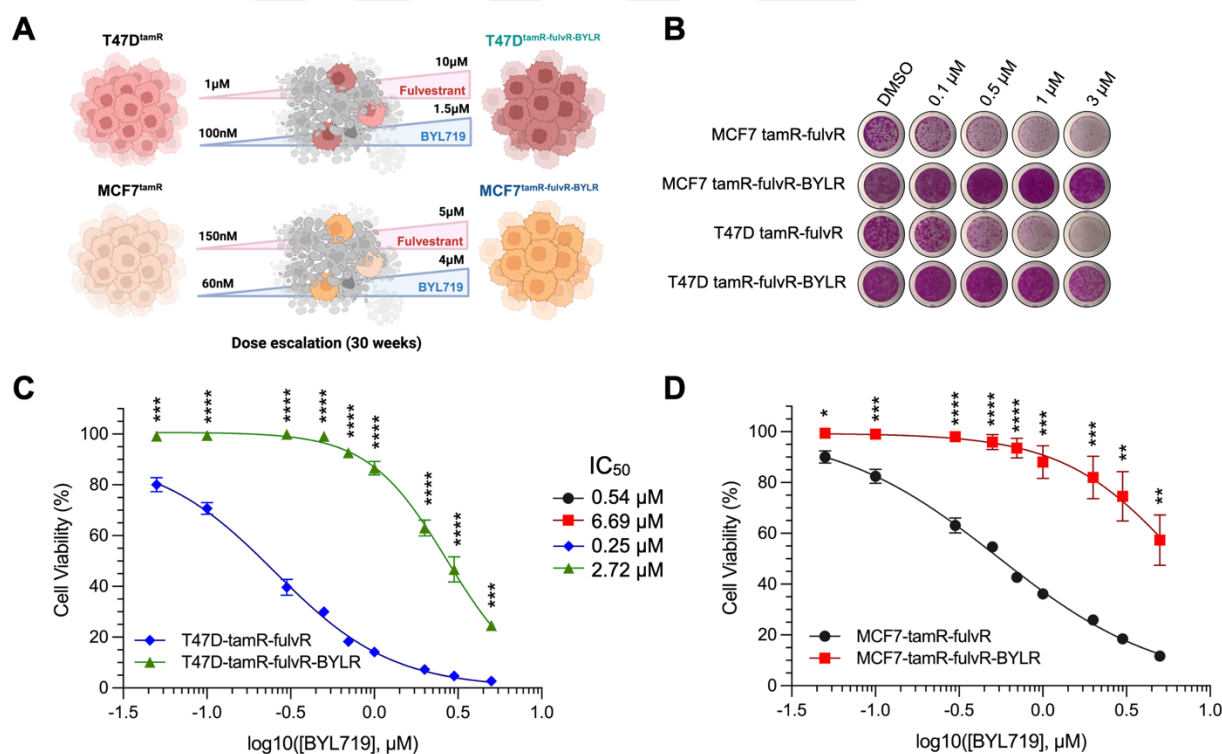


Figure 3.1: Generation and characterization of drug-resistant MCF7 and T47D cell lines. **A** Schematic of the experimental design. Tamoxifen-resistant cell lines were treated with a dose-escalated fulvestrant and alpelisib combination for 30 weeks to generate tamoxifen, fulvestrant, and alpelisib resistant (tamR-fulvR-BYLR) cell lines (Emirmustafaoglu, 2024). **B** Representative images of drug-resistant cells treated with indicated concentrations of BYL719. **C-D** Dose-dependent toxicities of BYL719 treatment in T47D-tamR-fulvR and T47D-tamR-fulvR-BYLR (**C**) or MCF7-tamR-fulvR and MCF7-tamR-fulvR-BYLR (**D**) cells. Cell viability was assessed after 5 days using Sulforhodamine B (**B-D**). Data are represented as mean ± SEM from $n=3$ independent experiments (**C-D**). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; two-way ANOVA (**C-D**). Illustrations in **A** were created with BioRender.com.

potential actionable targets that mediate drug resistance using clinically relevant models is of great importance. To generate an *in vitro* model that could recapitulate clinically relevant resistance mechanisms to the fulvestrant/alpelisib combination treatment, ER+/PR+/HER2- and *PIK3CA* mutant MCF7 and T47D cell lines, previously exposed to tamoxifen treatment and characterized to have acquired tamoxifen resistance (tamR)

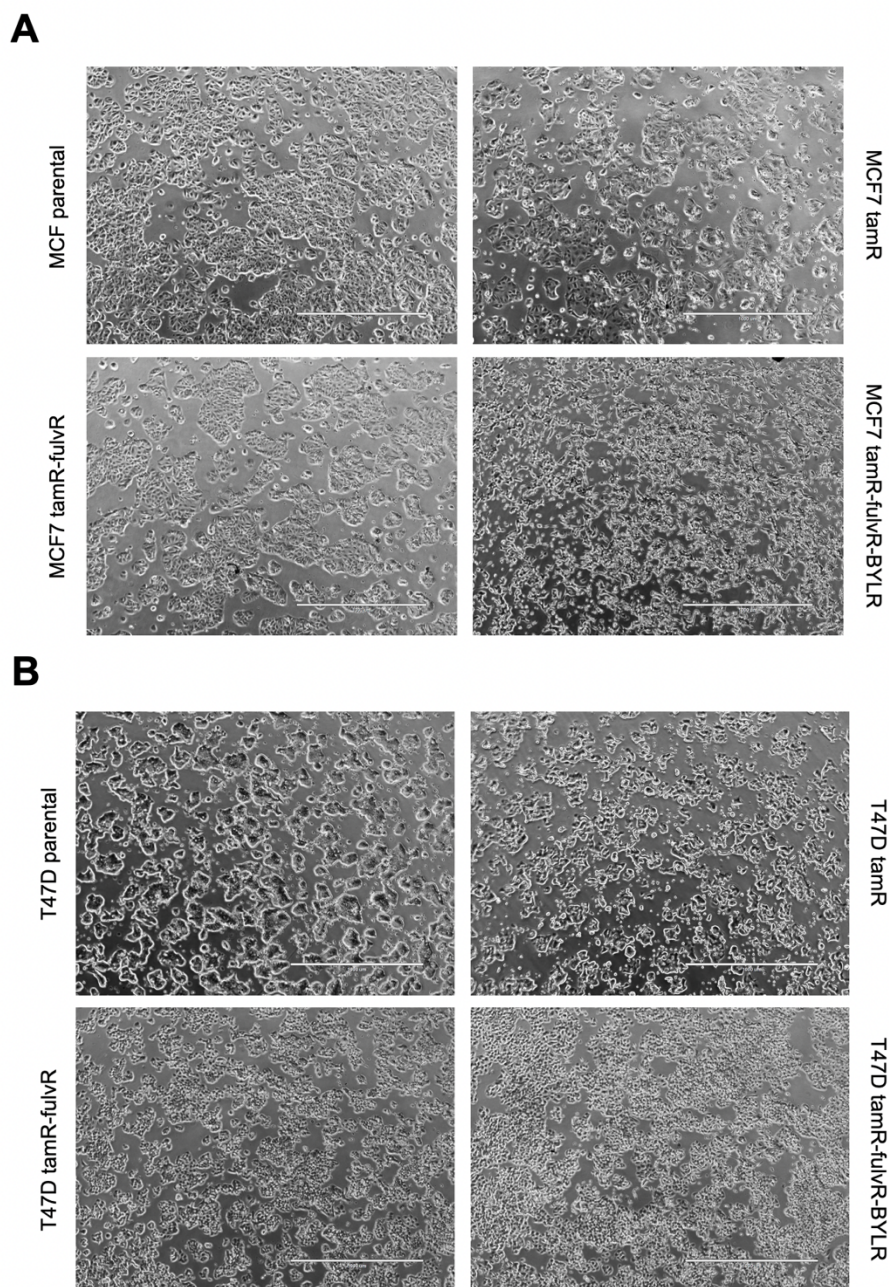


Figure 3.2: Morphologies of parental and drug-resistant MCF7 and T47D cell lines under standard growing conditions. A MCF7 cell lines with acquired resistance to the indicated drugs. **B** T47D cell lines with acquired resistance to the indicated drugs. Images were taken with EVOS FL Auto microscope; scale bar: 1000µm (A-B).

(Mishra et al., 2018), were cultured in escalated doses of fulvestrant/alpelisib combination for 30 weeks (tamR-fulvR-BYLR) (Figure 3.1.A) together with counterparts that were treated with a fulvestrant-only (tamR-fulvR) regimen and therefore remained sensitive to alpelisib treatment as detailed in Emirmustafaoglu (2024). Cell viability assays were performed to confirm the acquired alpelisib resistance in both cell lines. In line with the acquired resistance, the IC₅₀ values were elevated significantly ~11-fold in T47D-tamR-fulvR-BYLR cells in comparison to T47D-tamR-fulvR cells (Figure 3.1.C), and ~12-fold in MCF7-tamR-fulvR-BYLR cells compared to MCF7-tamR-fulvR cells (Figure 3.1.D). In addition, consistent with acquired drug resistance, drug-resistant sublines of both MCF7 and T47D cells exhibited clear, progressive morphological changes, with cells losing their typical epithelial appearance and showing decreased cohesiveness, especially in tamR-fulvR-BYLR cells (Figure 3.2.A-B). Moreover, MCF7-tamR-fulvR-BYLR cells gained a spindle-like elongated shape (Figure 3.2.A), while T47D-tamR-fulvR-BYLR cells displayed a rounder cell morphology (Figure 3.2.B) upon acquisition of drug resistance. Together, these findings indicate that the established drug-resistant tamR-fulvR-BYLR cell lines could serve as clinically relevant models for investigating resistance mechanisms that mediate acquired resistance to fulvestrant and alpelisib combination therapy in patients progressing on or after endocrine therapy.

3.2. Protein level expression changes of ER α and PI3K pathway components in alpelisib-resistant cell lines

After the generation and characterization of cell lines resistant to both tamoxifen, fulvestrant, and alpelisib treatment, to assess potential protein level expression changes of ER α and PI3K pathway components associated with acquired drug resistance in alpelisib-resistant (tamR-fulvR-BYLR) cell lines, p110 α , p110 β , p85, PTEN, and ER α levels were compared with parental, tamoxifen-resistant (tamR), and tamoxifen-fulvestrant-resistant (tamR-fulvR) MCF7 and T47D cell lines. Consistent with the endocrine therapy-resistant phenotype (Belachew and Sewasew, 2021), the results revealed a progressive decrease in ER α expression in tamR-fulvR cells compared with parental controls (Figure 3.3). In addition, a marked increase in all tested PI3K pathway components was observed in MCF7-tamR-fulvR-BYLR cells compared with tamR-fulvR

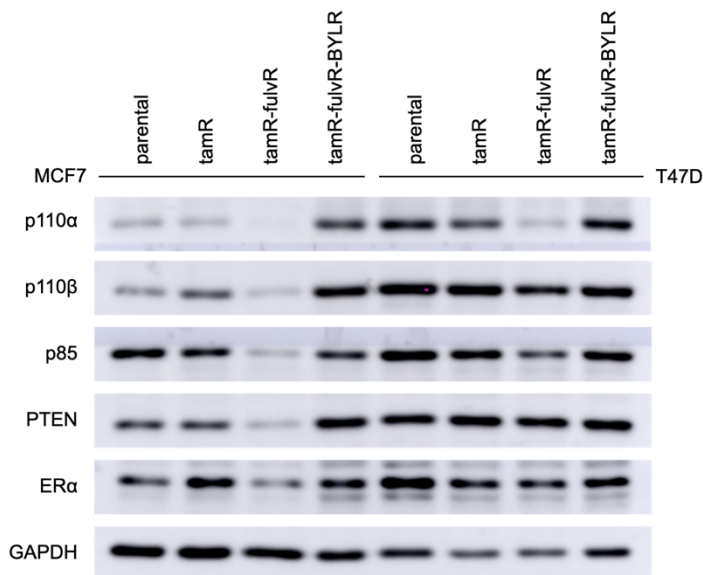


Figure 3.3: ERα and PI3K pathway component expression in drug-resistant cell lines. Immunoblot images of ERα and key PI3K pathway components, p110α, p110β, p85, and PTEN in parental, tamoxifen-resistant, tamoxifen-fulvestrant-resistant, and tamoxifen-fulvestrant-alpelisib-resistant cell lines.

counterparts, while acquired alpelisib resistance was accompanied by increases in p110α, p110β, and p85, with no qualitative changes in PTEN levels in T47D-tamR-fulvR-BYLR cells (Figure 3.3).

These results confirm adaptive rewiring of the PI3K pathway associated with acquired alpelisib resistance in tamR-fulvR-BYLR cells, which contradicts the previously proposed p110 isoform switch phenotype in alpelisib resistance, characterized by a decrease, especially in PTEN and p110α, and a reciprocal increase in p110β (Juric et al., 2015; Leroy et al., 2016; Cutano et al., 2025).

3.3. Alpelisib-resistant cell lines are cross-resistant to p110β/δ and pan-PI3K inhibitors

Following our western blot experiments, which provided no clear indication of a potential p110 isoform switching event in the acquisition of drug resistance, our aim was to functionally determine whether inhibiting p110β or p110δ alone could overcome the acquired resistance to the p110α inhibitor alpelisib in tamR-fulvR-BYLR cells. Therefore, both MCF7-tamR-fulvR-BYLR and T47D-tamR-fulvR-BYLR cells were treated with increasing doses of p110β inhibitor AZD6482 (KIN193) and p110δ inhibitor idelalisib (CAL101), along with tamR-fulvR cells for comparison. Cell viability assays showed no

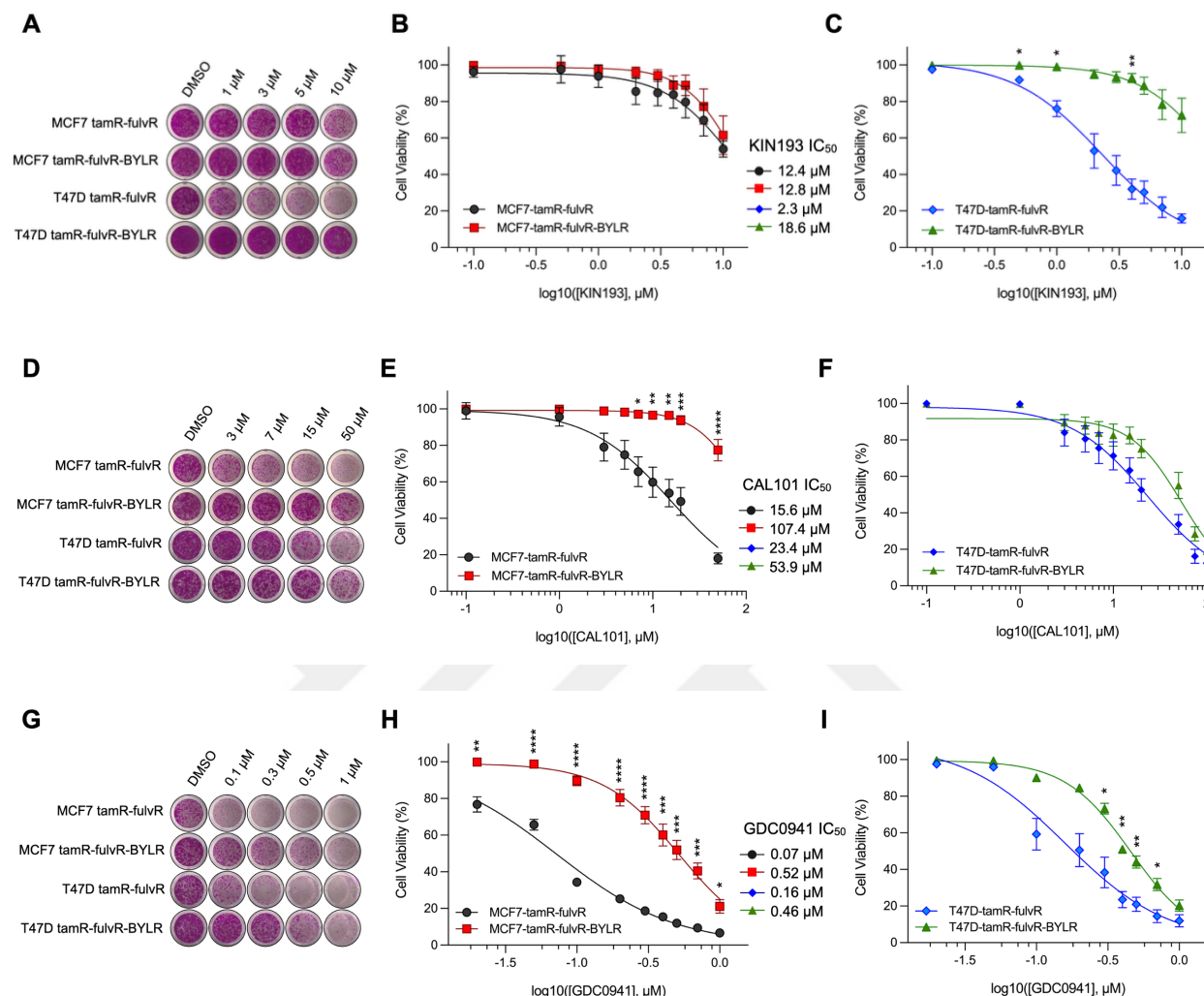


Figure 3.4: Alpelisib-resistant cell lines show cross-resistance to p110 β / δ and pan-PI3K inhibitors. **A,D,G** Representative images of drug-resistant cells treated with indicated concentrations of AZD6482 (KIN193) (**A**), idelalisib (CAL101) (**D**), or pictilisib (GDC0941) (**G**). **B-C** Dose-dependent toxicities of KIN193 treatment in MCF7-tamR-fulvR and MCF7-tamR-fulvR-BYLR (**B**) or T47D-tamR-fulvR and T47D-tamR-fulvR-BYLR (**C**) cells. **E-F** Dose-dependent toxicities of CAL101 treatment in MCF7-tamR-fulvR and MCF7-tamR-fulvR-BYLR (**E**) or T47D-tamR-fulvR and T47D-tamR-fulvR-BYLR (**F**) cells. **H-I** Dose-dependent toxicities of GDC0941 treatment in MCF7-tamR-fulvR and MCF7-tamR-fulvR-BYLR (**H**) or T47D-tamR-fulvR and T47D-tamR-fulvR-BYLR (**I**) cells. Cell viability was assessed after 5 days using Sulforhodamine B (**A-I**). Data are represented as mean $\pm$ SEM from $n=3$ independent experiments (**B-C, E-F, H-I**). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; two-way ANOVA (**B-C, E-F, H-I**).

significant difference in AZD6482 sensitivity between MCF7-tamR-fulvR-BYLR cells and MCF7-tamR-fulvR cells (Figure 3.4.A-B), while T47D-tamR-fulvR-BYLR cells were less sensitive to p110 β inhibition in comparison to T47D-tamR-fulvR cells, with an IC₅₀ value $\sim$ 8-fold higher (Figure 3.4.A, C). Additionally, both MCF7 and T47D tamR-fulvR-BYLR cells exhibited reduced sensitivity to p110 δ inhibition, with IC₅₀ values $\sim$ 7-fold (Figure 3.4.D-E) and $\sim$ 2-fold (Figure 3.4.D, F) higher than those of their tamR-fulvR counterparts,

respectively. Consistent with the acquired resistance to alpelisib phenotype and decreased sensitivity to p110 β and p110 δ inhibition, the tamR-fulvR-BYLR cells also demonstrated cross-resistance to the pan-PI3K inhibitor pictilisib (GDC0941), with ~7-fold and ~3-fold increases in IC₅₀ for MCF7 (Figure 3.4.G-H) and T47D (Figure 3.4.G, I) cells, respectively.

The simultaneous upregulation of p110 α , p110 β , p85, and PTEN proteins (Figure 3.3), in addition to the represented cross-resistance to p110 β / δ and pan-PI3K inhibitors in tamoxifen, fulvestrant, and alpelisib-resistant MCF7 and T47D breast cancer cells (Figure 3.4), suggests that the acquired resistance to fulvestrant-alpelisib combination therapy occurs due to extensive remodeling of alternative signaling networks rather than p110 isoform switching that predisposes the observed resistance.

3.4. Transcriptomic analysis reveals activated FGFR signaling in tamR-fulvR-BYLR cell lines

To investigate signaling pathways that could potentially mediate acquired resistance to the fulvestrant-alpelisib combination therapy, RNA sequencing was performed on both MCF7 and T47D tamR-fulvR-BYLR cells, along with tamR-fulvR cells for comparison, as detailed by Emirmustafaoglu (2024). The results showed enrichment of FGFR2 (Figure 3.5.A-B), FGFR3 (Figure 3.5.A, C), FGFR1 (Figure 3.5.A, D), and NTRK2 (Figure 3.5.A, E) pathways in alpelisib-resistant (tamR-fulvR-BYLR) T47D cell lines. Although the reciprocal activation of receptor tyrosine kinases (RTKs), especially HER2 and EGFR, in endocrine therapy resistance has been well characterized in previous studies (Will et al., 2023), only recently have reports highlighted co-amplifications of FGFR1 and associated factors in endocrine resistance (Uemoto et al., 2025; Drago et al., 2019), while the roles of FGFR2 (Mao et al., 2020) and FGFR3 isoforms, particularly in the context of acquired resistance to fulvestrant-alpelisib combination treatment, remain less understood. Consequently, we further examined the expression patterns of individual differentially expressed genes involved in the PI3K/Akt/mTOR, FGFR, and downstream MAPK pathways. Concordant with acquired alpelisib resistance, transcriptomic levels of *PIK3CA*, *PIK3CB*, *AKT1*, *AKT2*, *mTOR*, and *RICTOR* genes were observed to be

elevated (Figure 3.6). In addition, the two FGFR isoforms, *FGFR1* and *FGFR4*, were

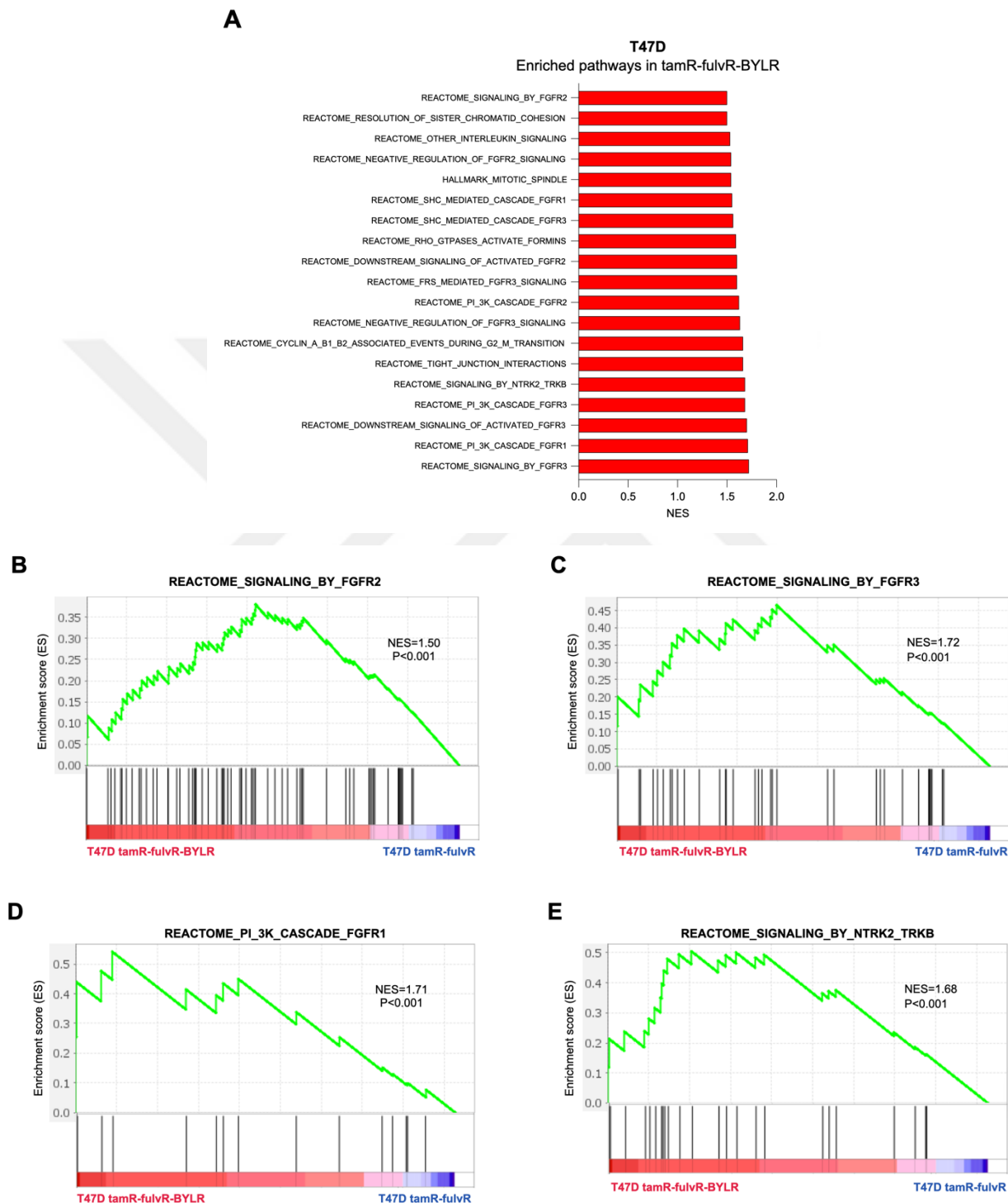


Figure 3.5: Transcriptomic analysis indicates enrichment of FGFR and NTRK2 signaling in tamR-fulvR-BYLR cell lines. A Enriched pathways in T47D-tamR-fulvR-BYLR cells. **B-E** GSEA analysis representing FGFR2 (**B**), FGFR3 (**C**), FGFR1 (**D**), and NTRK2 (**E**) signaling-related gene enrichment in T47D-tamR-fulvR-BYLR cells. GSEA analysis was performed by Dr. Özge Saatçi, MUSC, and included in this study with her kind permission.

transcriptionally reduced, whereas *FGFR2*, *FGFR3*, *MAP2K2*, *MAPK1*, *MAPK3*, and *FOS* genes were notably upregulated in T47D-tamR-fulvR-BYLR cells, while the MAPK phosphatase *DUSP4* was downregulated (Figure 3.6). This pattern indicates increased MAPK activity downstream of FGFR pathway activation in alpelisib-resistant cell lines. Following the identification of activated FGFR/MAPK signaling in tamR-fulvR-BYLR cells

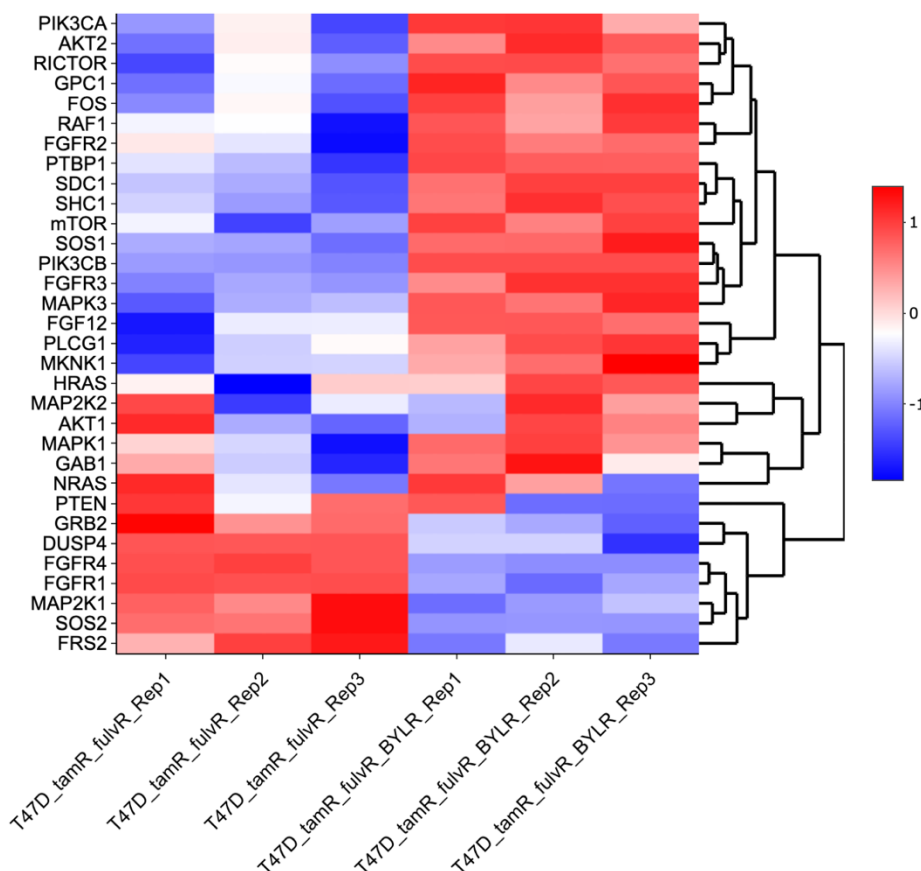


Figure 3.6: Heatmap representing key differentially expressed genes involved in PI3K, FGFR, and MAPK pathways. The gene panel comprises key differentially expressed components of the PI3K/Akt/mTOR, FGFR, and MAPK signaling pathways, including, but not limited to, receptor tyrosine kinases, adaptor proteins, and downstream kinases. Differentially expressed genes are analyzed using the DESeq2 package in R.

through transcriptomic analysis, we then aimed to investigate whether this activation is also evident at the protein level. Remarkably, immunoblots revealed increased total FGFR2 protein in both T47D and MCF7 cells with resistance to tamoxifen, fulvestrant, and alpelisib, more prominently in T47D cells, compared to their tamR-fulvR counterparts (Figure 3.7. A-B). However, a similar pattern was not observed for FGFR3 in T47D-tamR-

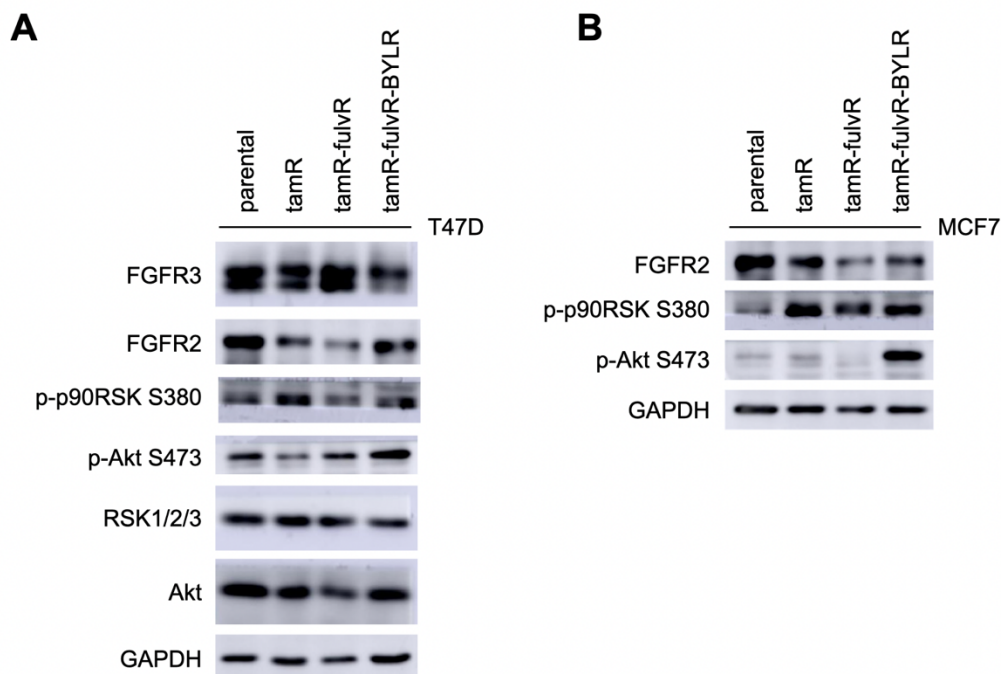


Figure 3.7: FGFR and downstream p90RSK expression changes in drug-resistant cell lines. A-B Immunoblot images of FGFR2, FGFR3, p-p90RSK (S380), RSK1/2/3, p-Akt (S473) and Akt protein levels in parental, tamoxifen-resistant (tamR), tamoxifen-fulvestrant-resistant (tamR-fulvR), and tamoxifen-fulvestrant-alpelisib-resistant (tamR-fulvR-BYLR) T47D (A) and MCF7 (B) cell lines.

fulvR-BYLR cells (Figure 3.7.A). Previously, increased activity of the MAPK pathway in tamR-fulvR-BYLR cells was demonstrated by elevated p-Erk1/2 T204/Y202 levels in Emirmustaoglu (2024). Additionally, we qualitatively observed increased p-p90RSK S380 levels in T47D-tamR-fulvR-BYLR cells compared to the T47D-tamR-fulvR counterparts (Figure 3.7.A). However, this observation remains to be confirmed by quantitative analysis. Potential changes in FGFR1 protein levels in alpelisib-resistant sublines and whether our endocrine therapy-resistant tamR-fulvR cell lines convey the previously reported FGFR1-amplified phenotype (Turner et al., 2010; Drago et al., 2019; Mao et al., 2020) are yet to be determined.

Overall, the transcriptomic analysis, in addition to the represented immunoblot experiments, implicates an association between activated FGFR/MAPK signaling, particularly the FGFR2 isoform, and the acquired resistance to the fulvestrant-alpelisib combination treatment and provides a basis for further experimentation.

3.5. FGFR inhibition can restore alpelisib sensitivity in alpelisib-resistant *in vitro* models

Pemigatinib is a selective pan FGFR1/2/3 inhibitor, which has been approved for advanced-stage cholangiocarcinoma patients with *FGFR2* fusions and/or rearrangements (Abou-Alfa et al., 2020) and, more recently, for myeloid/lymphoid neoplasms with *FGFR1* fusions (Gotlib et al., 2021). Following the results of our transcriptomic analysis implicating a potential association between activated FGFR signaling and the acquired resistance to fulvestrant/alpelisib combinatory treatment, we aimed to investigate whether targeting the FGFR pathway with the pan-FGFR inhibitor pemigatinib could overcome the acquired resistance to alpelisib in our fulvestrant/alpelisib-resistant cell models. Therefore, cells were treated with either single

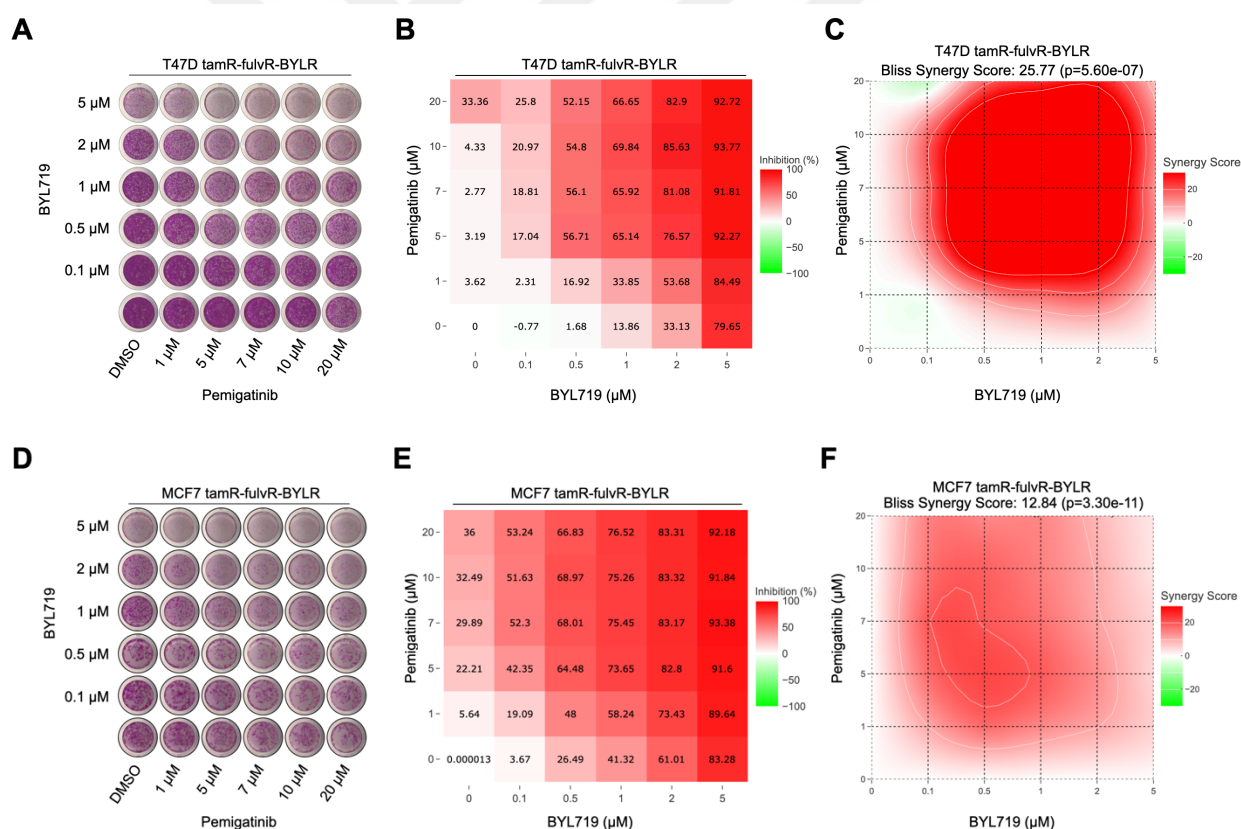


Figure 3.8: Pemigatinib resensitizes fulvestrant/alpelisib-resistant breast cancer cells to alpelisib treatment. **A, D** Representative images of drug-resistant T47D (**A**) or MCF7 (**D**) cells treated with indicated concentrations of alpelisib (BYL719) in combination with pemigatinib. **B-C** Dose-response matrix (**B**) and Bliss synergy score surface plot (**C**) for T47D-tamR-fulvR-BYLR cells treated with combined doses of alpelisib and pemigatinib. **E-F** Dose response matrix (**E**) and Bliss synergy score surface plot (**F**) for MCF7-tamR-fulvR-BYLR cells treated with combined doses of alpelisib and pemigatinib. Cell viability was assessed after 5 days of drug treatment using Sulforhodamine B (**A-F**). Dose-response matrix and synergy score plots were generated with SynergyFinder.

doses or combinations of alpelisib (BYL719) and pemigatinib. The results revealed that the combined pemigatinib and alpelisib treatment restored alpelisib sensitivity and provided significant synergistic growth inhibition at drug concentrations where single

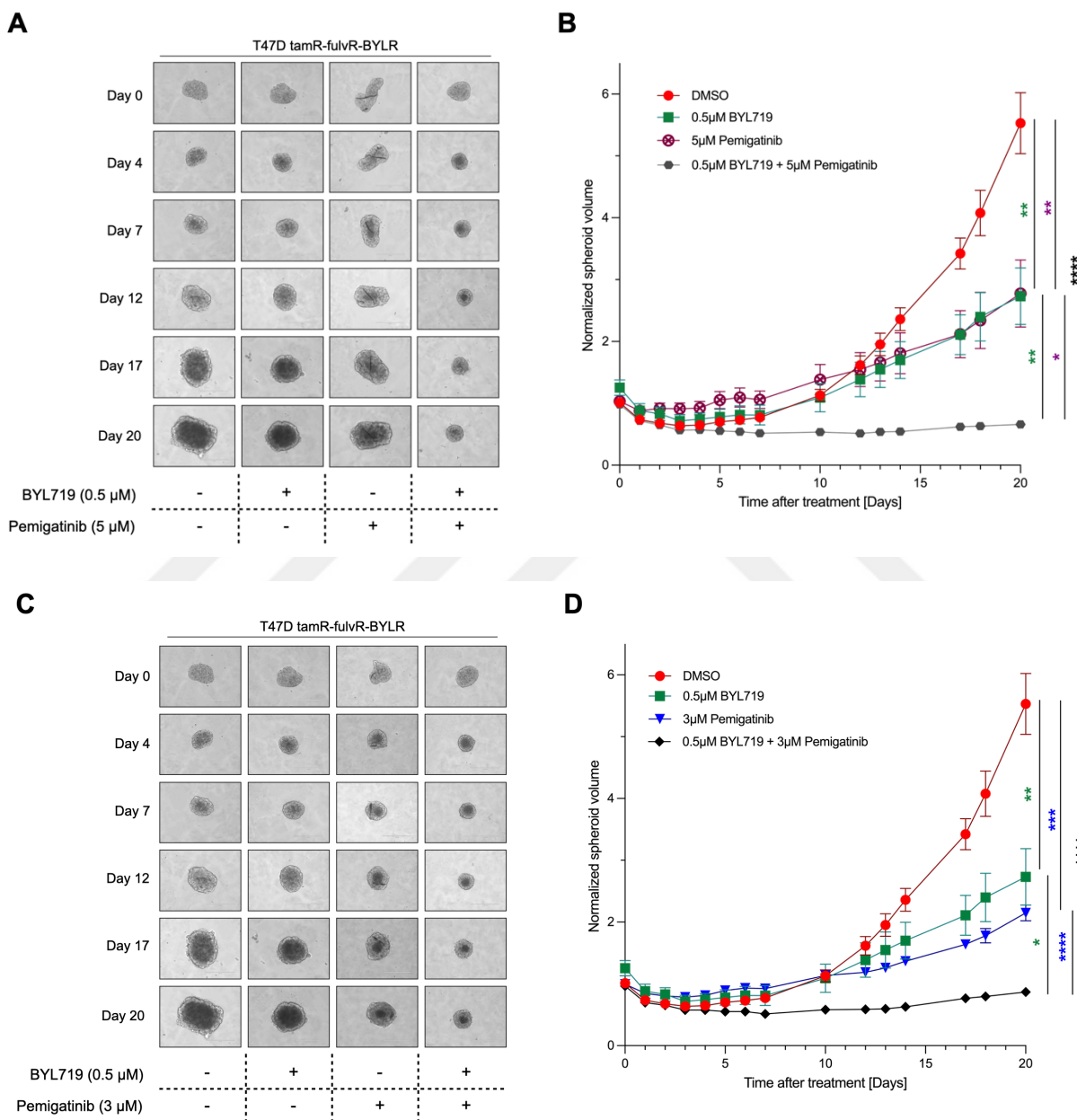


Figure 3.9: Pemigatinib-alpelisib combination enables sustained inhibition of cellular growth in drug-resistant 3D tumor spheroids. **A, C** Representative images of drug-resistant T47D tumor spheroids treated with indicated concentrations of alpelisib (BYL719) and pemigatinib, either alone or in combination. **B, D** Quantitative depiction of spheroid volume changes after treatment initiation. Each tumor spheroid was generated with 2000 cells using the liquid overlay technique (**A-D**). Data are represented as mean $\pm$ SEM from $n=12$ individual spheroids (**B, D**). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; two-way ANOVA (**B-D**). Images were taken with EVOS FL Auto microscope; scale bar: 400 μ m (**A, C**). Spheroid volumes were determined with AnaSP.

treatments of either drug were ineffective for both T47D-tamR-fulvR-BYLR (Figure 3.8.A-C) and MCF7-tamR-fulvR-BYLR (Figure 3.8.D-F) cells. To further assess the efficacy of the alpelisib-pemigatinib combination in drug-resistant breast cancer cells under more physiologically relevant conditions, 3D spheroids were generated from the T47D-tamR-fulvR-BYLR cells using the liquid overlay technique described in 2.2.2.6. Notably, the combination of pemigatinib and alpelisib treatment enabled sustained growth inhibition of alpelisib-resistant T47D tumor spheroids for up to 20 days, surpassing the effects of either treatment alone (Figure 3.9). Although single treatments resulted in slower growth, they were insufficient to suppress spheroid expansion in 3D culture (Figure 3.9).

3.5.1. Biochemical analysis of pemigatinib-alpelisib combinatory treatments

Guided by the previous transcriptomic analysis implying activated MAPK signaling downstream of FGFR (Figure 3.6), next, we sought to biochemically determine the potential mechanism that may result in the observed growth suppression and temporally assess the rewiring of the downstream MAPK and PI3K/Akt/mTOR pathways upon combined treatment of alpelisib and pemigatinib. MCF7-tamR-fulvR-BYLR and T47D-tamR-fulvR-BYLR cells were treated with 1 μ M BYL719, 5 μ M Pemigatinib, or the combination of the two for 0.5-36 hours. This time-dependent assessment allowed us to compare early drug responses and late adaptive signaling changes. At early stages (0.5-4 hours), the combination treatment rapidly suppressed ERK1/2 phosphorylation (Figure 3.10.A-B), more potently than either alpelisib or pemigatinib treatment alone, followed by a transient rebound (8-12 hours) (Figure 3.10.B-C) and a recurring reduction at later stages (24-36 hours) (Figure 3.10.C) in MCF7-tamR-fulvR-BYLR cells. The similar pattern of an acute inhibition followed by a rebound and a subsequent reduction of phosphorylation in ERK1/2 was also recapitulated in T47D-tamR-fulvR-BYLR cells (Figure 3.10.D). Of note, except for the increased phosphorylation observed at 2 hours (Figure 3.10.A), p90RSK, a downstream effector of ERK1/2, phosphorylation mirrored ERK1/2 phosphorylation dynamics (Figure 3.10.A-C), suggesting an adaptive regulation of the MAPK/ERK pathway downstream of FGFR signaling upon treatment with pemigatinib/alpelisib combination in drug-resistant cell lines. Although the combination treatment was also more effective at suppressing S6 phosphorylation when compared to

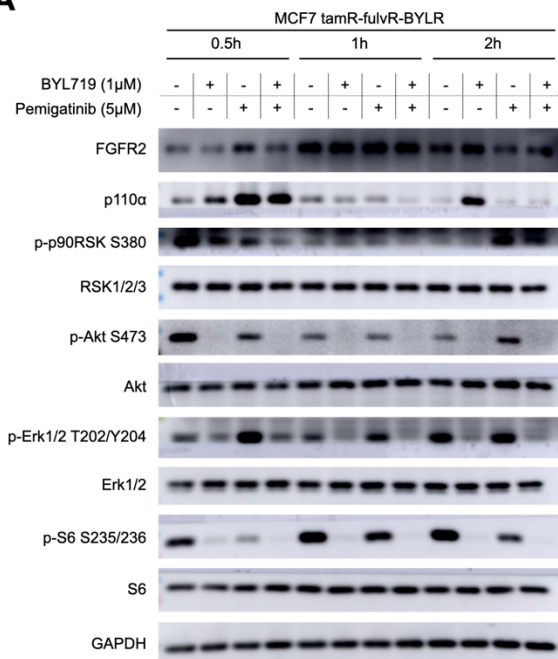
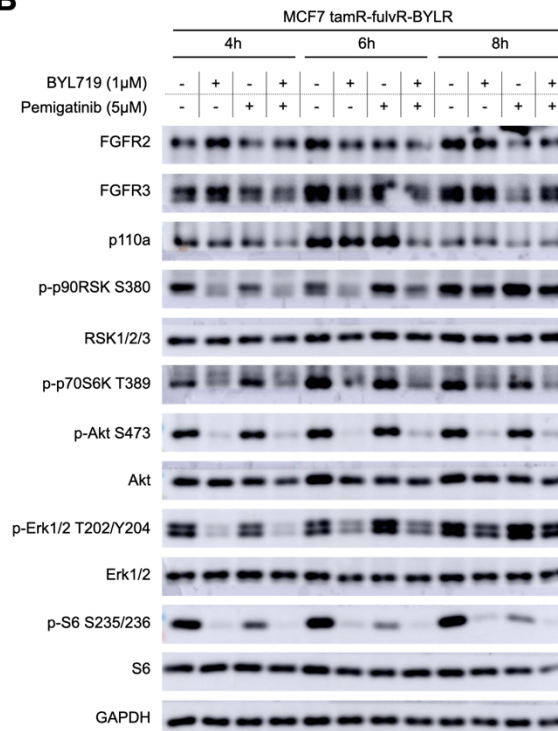
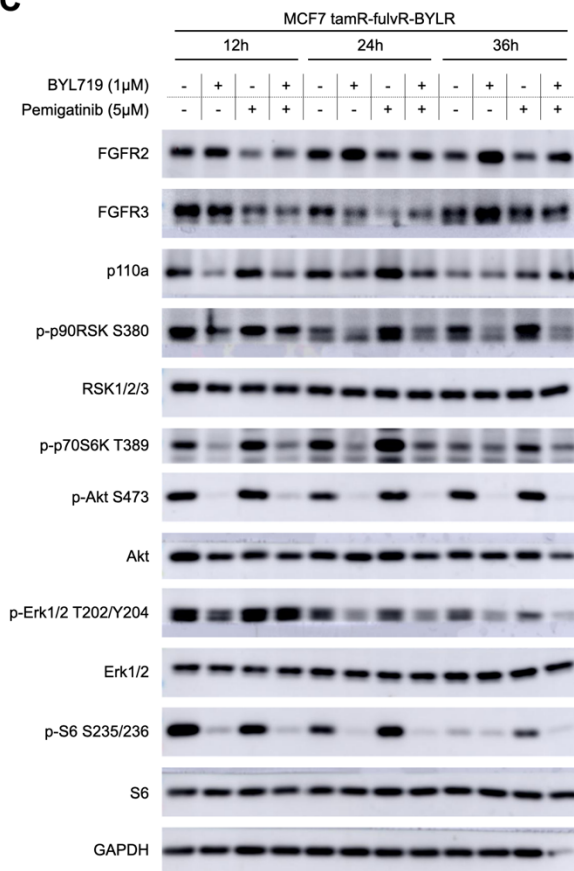
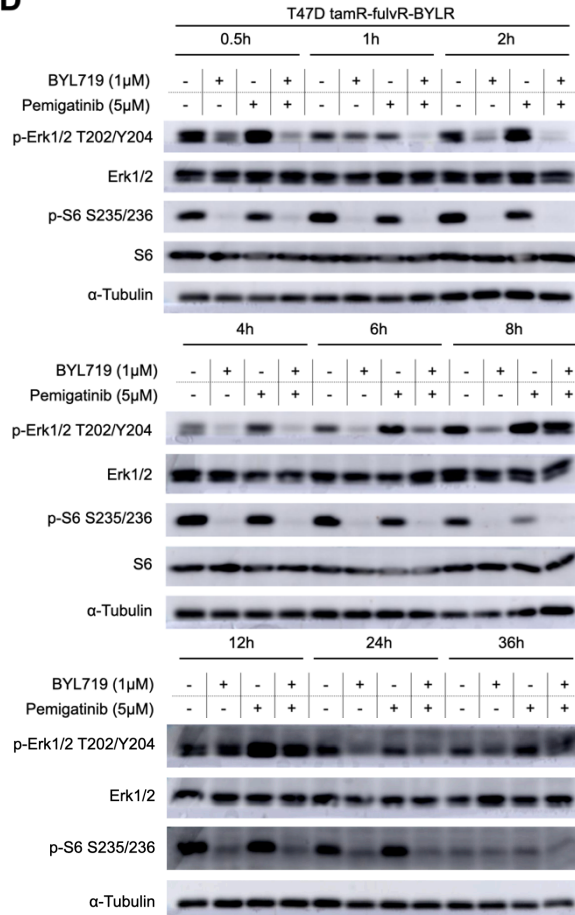
A**B****C****D**

Figure 3.10: Combinatory treatment of pemigatinib and alpelisib targets MAPK pathway for growth inhibition. A-D Immunoblot images of MCF7-tamR-fulvR-BYLR (A-C) and T47D-tamR-fulvR-BYLR (D) cells treated with 1 μ M alpelisib (BYL719), 5 μ M pemigatinib, or a combination of the two for the indicated time periods. The cells were seeded two days prior to initiation of treatments in standard growth medium.

single-agent treatments, the adaptive signaling regulation was not present. Interestingly, alpelisib treatment alone resulted in increased FGFR2 levels at late-adaptive stages (24-36 hours) (Figure 3.10.C). Although downstream phosphorylation of the receptor and, alternatively, the adaptor FRS2 is surely needed to confirm signaling activity, this observation may indicate compensatory FGFR2 activity upon alpelisib treatment.

3.5.2. FGFR2-specific inhibition synergizes with alpelisib treatment in 2D and halts 3D growth in tumor spheroid models

Lirafugratinib (RLY4008) is a recently developed covalent FGFR2-selective inhibitor (Subbiah et al., 2023), currently under clinical trials for its efficacy in cholangiocarcinoma and other solid tumors harboring *FGFR2* alterations (NCT04526106) (Schram et al., 2023). Our previous findings demonstrated overexpression of FGFR2 at both transcript and protein levels upon the acquisition of fulvestrant/alpelisib therapy resistance, while, although elevated at the transcriptomic level, total FGFR3 protein was not increased in our tamR-fulvR-BYLR cell lines (Figure 3.7). Therefore, we aimed to examine the particular role of the FGFR2 isoform in acquired drug resistance and to determine whether inhibition of the FGFR2 isoform alone with the isoform-specific inhibitor lirafugratinib could recapitulate the restored alpelisib sensitivity and synergistic growth inhibition observed with the pan-FGFR1/2/3 inhibitor pemigatinib (Figure 3.8). Markedly, FGFR2 inhibition alone could also resensitize resistant cells to alpelisib treatment and, when combined with alpelisib, yielded synergistic growth inhibition in our fulvestrant/alpelisib-resistant breast cancer cells (Figure 3.11). Experiments testing the combined effect of lirafugratinib and alpelisib in suppressing cellular growth in 3D tumor spheroids further demonstrated that lirafugratinib/alpelisib combination treatment enables sustained growth inhibition of drug-resistant spheroids (Figure 3.12).

Together, these findings suggest that the enhanced activity of the FGFR/MAPK signaling, particularly overexpression of the FGFR2 isoform, promotes resistance to the combined fulvestrant/alpelisib therapy in *PIK3CA*-mutant ER⁺ advanced-stage breast cancer cells,

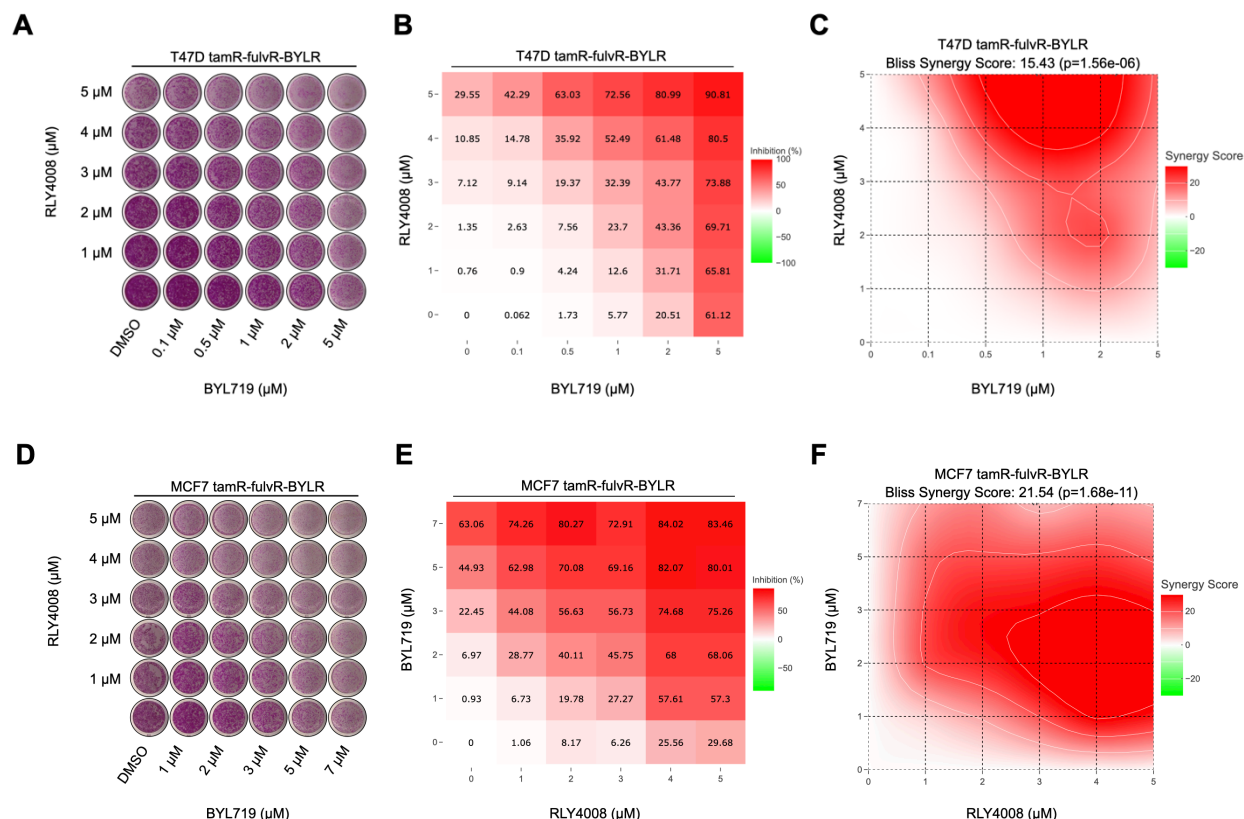


Figure 3.11: Lirafugratinib allows synergistic growth inhibition in fulvestrant/altamiprisib-resistant breast cancer cells. **A, D** Representative images of drug-resistant T47D (**A**) or MCF7 (**D**) cells treated with indicated concentrations of altamiprisib (BYL719) in combination with lirafugratinib. **B-C** Dose-response matrix (**B**) and Bliss synergy score surface plot (**C**) for T47D-tamR-fulvR-BYLR cells treated with combined doses of altamiprisib and lirafugratinib. **E-F** Dose response matrix (**E**) and Bliss synergy score surface plot (**F**) for MCF7-tamR-fulvR-BYLR cells treated with combined doses of altamiprisib and lirafugratinib. Cell viability was assessed after 5 days of drug treatment using Sulforhodamine B (**A-F**). Dose-response matrix and synergy score plots were generated with SynergyFinder.

and combined inhibition of p110α and FGFR could serve as a potential strategy to overcome the acquired resistance.

3.6. FGFR/PI3K and mTOR/PI3K combination treatments in comparison

Multiple prior studies proposed that the acquired resistance to p110α inhibition is derived chiefly from overactivation of the downstream mTOR pathway (Gremke et al., 2025), and dual inhibition of p110α and mTORC1 could reverse resistance in breast cancer (Elkabets et al., 2013). In concordance with these findings, our prior DAVID pathway enrichment analysis revealed an enrichment in PI3K cascade upon acquisition of altamiprisib resistance in MCF7-tamR-fulvR-BYLR cells (Emirmustafaoglu, 2024), while transcript

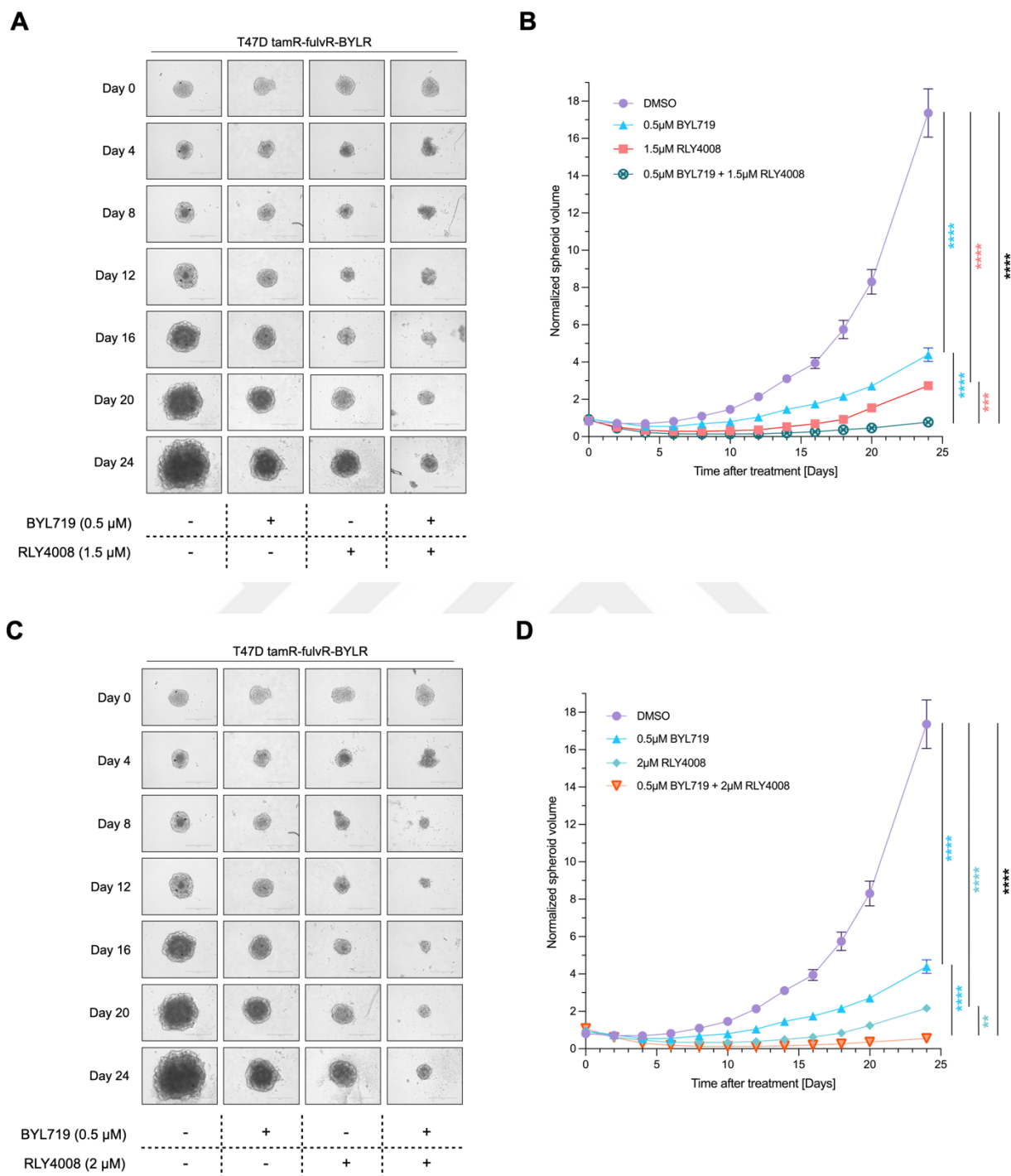


Figure 3.12: FGFR2-specific inhibition combined with alpelisib halts cellular growth in drug-resistant 3D tumor spheroids. **A, C** Representative images of drug-resistant T47D tumor spheroids treated with indicated concentrations of alpelisib (BYL719) and lirafugratinib (RLY4008), either alone or in combination. **B, D** Quantitative depiction of spheroid volume changes after treatment initiation. Each tumor spheroid was generated with 3000 cells using the liquid overlay technique (**A-D**). Data are represented as mean $\pm$ SEM from $n=6$ or $n=18$ individual spheroids (**B, D**). ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; two-way ANOVA (**B-D**). Images were taken with EVOS FL Auto microscope; scale bar: 400 μ m (**A,C**). Spheroid volumes were determined with AnaSP.

levels of the mTOR gene were observed to be elevated in T47D-tamR-fulvR-BYLR cells

(Figure 3.6). Everolimus (RAD001) is an mTORC1 inhibitor approved by the FDA for its use in combination with exemestane in advanced-stage HR+ breast cancer patients (Baselga et al., 2012). Guided by preceding reports and our transcriptomic analysis, in order to understand whether the observed FGFR activation is a primary bypass or a secondary adaptation emerging upon acquired resistance to the fulvestrant/alpelisib combination, we evaluated the efficacy of everolimus (RAD001) in combination with alpelisib in our tamR-fulvR-BYLR cell lines. As expected, everolimus provided synergistic growth inhibition when combined with alpelisib in both T47D (Figure 3.13.A-C) and MCF7 (Figure 3.13. D-F) cell lines, although the synergy was higher in MCF7-tamR-fulvR-BYLR cells. However, the Loewe synergy score for RAD001-BYL719 combination (12.56) (Figure 3.13.C) was relatively lower than both pemigatinib-BYL719 (25.77) (Figure 3.8.C)

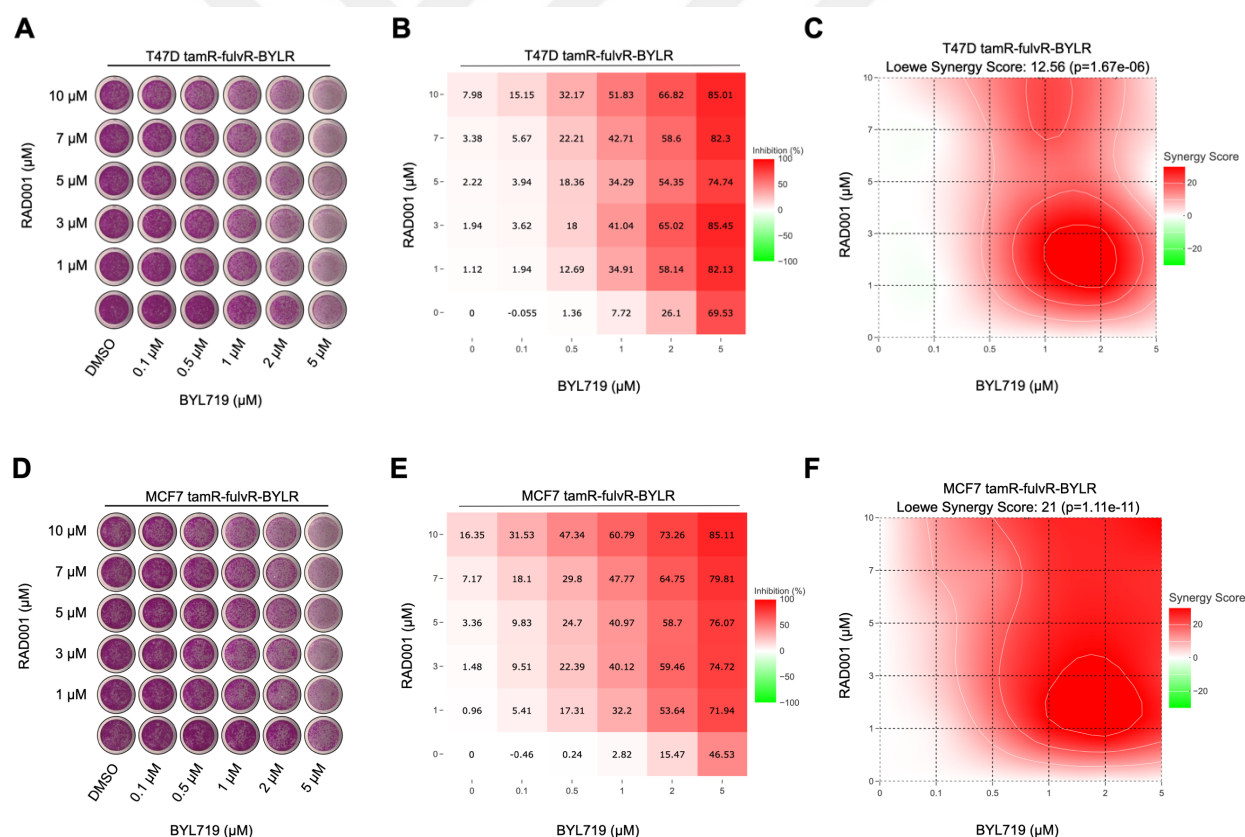


Figure 3.13: Synergistic growth inhibition occurs when everolimus is combined with alpelisib in drug-resistant cells. **A, D** Representative images of drug-resistant T47D (**A**) or MCF7 (**D**) cells treated with indicated concentrations of alpelisib (BYL719) in combination with everolimus (RAD001). **B-C** Dose-response matrix (**B**) and Bliss synergy score surface plot (**C**) for T47D-tamR-fulvR-BYLR cells treated with combined doses of alpelisib and everolimus. **E-F** Dose response matrix (**E**) and Bliss synergy score surface plot (**F**) for MCF7-tamR-fulvR-BYLR cells treated with combined doses of alpelisib and everolimus. Cell viability was assessed after 5 days of drug treatment using Sulforhodamine B (**A-F**). Dose-response matrix and synergy score plots were generated with SynergyFinder.

and liralumab-BYL719 (15.43) (Figure 3.11.C) combinations in drug-resistant T47D cells, suggesting that FGFR activation could be a more dominant mediator of drug resistance than mTOR activation in these cell lines. Parallel comparisons in drug-resistant MCF7 cells revealed that the synergy for RAD001-BYL719 (21) (Figure 3.13.F) was comparable with liralumab-BYL719 combination (21.54) (Figure 3.11.F), while higher than pemigatinib-BYL719 (12.84) (Figure 3.8.F). However, caution is warranted in interpreting this observation, as synergy is observed at high RAD001 concentrations, where off-target effects may have been present.

3.7. Pemigatinib-alpelisib combination inhibits tumor growth in drug-resistant tumor xenografts *in vivo*

To determine if FGFR inhibition combined with alpelisib could overcome the acquired resistance proposed to be mediated by FGFR signaling activation *in vivo*, T47D-tamR-fulvR-BYLR cells were orthotopically implanted into athymic nude female mice supplemented with 12 µg/mL β -Estradiol in drinking water five days prior to injection. After 20 days post tumor implantation, when tumors reached approximately 95 mm³ in size, mice were randomly divided into four groups: vehicle, alpelisib (BYL719), pemigatinib, and a combination of alpelisib and pemigatinib. Results revealed that after 30 days of treatment, the pemigatinib and alpelisib combination was more effective at inhibiting tumor growth than either treatment alone in tumors harboring fulvestrant/alpelisib resistance, whereas single treatments with alpelisib or pemigatinib resulted in modestly delayed tumor growth (Figure 3.14.B). Additionally, only the combined treatment of pemigatinib and alpelisib significantly reduced tumor weight when compared with vehicle control (Figure 3.14.C). During the course of the treatment, no significant weight change was observed in mice treated with pemigatinib and alpelisib, indicating that the combination therapy is well-tolerated (Figure 3.14.D).

These results served as an *in vivo* validation of previously reported findings in 3D tumor spheroid models (Figure 3.9) and further indicated that dual inhibition of p110 α and FGFR could represent a promising actionable strategy to overcome acquired resistance to the combined fulvestrant/alpelisib therapy in *PIK3CA*-mutant ER⁺ advanced-stage breast cancer.

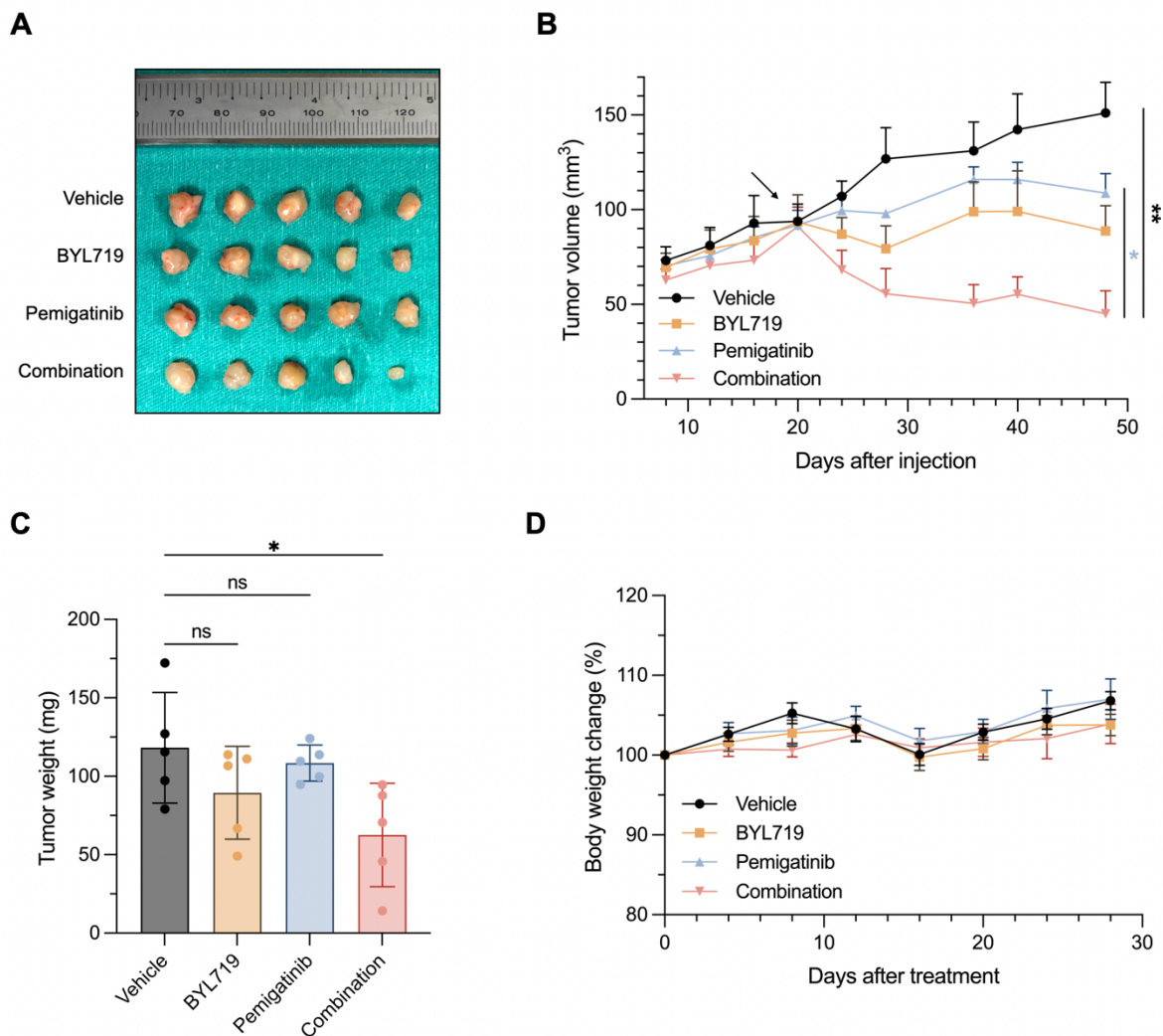


Figure 3.14: pan-FGFR inhibitor pemigatinib in combination with alpelisib enables reduction of tumor size in alpelisib-resistant xenografts *in vivo*. **A** Images of extracted tumors after treatment with 5%DMAC and 0.5% methylcellulose (vehicle), 20 mg/kg BYL719, 1 mg/kg pemigatinib, or a combination of 20 mg/kg BYL719 and 1 mg/kg pemigatinib for 30 days. **B** Growth curves of tumor xenografts derived from T47D-tamR-fulvR-BYLR cells. The arrowhead indicates initiation of drug treatment. **C** Weights of extracted tumors after treatment for 30 days. **D** Body weight changes in mice treated with 5%DMAC and 0.5% methylcellulose (vehicle), 20 mg/kg BYL719, 1 mg/kg pemigatinib, or a combination of 20 mg/kg BYL719 and 1 mg/kg pemigatinib for 30 days via oral gavage. Data are represented as mean $\pm$ SEM from $n=5$ biologically independent samples (**A-D**). ns: non significant, * $p < 0.05$, ** $p < 0.01$; two-way ANOVA (**B-D**).

3.8. FGFR1 overexpression alone cannot promote resistance to alpelisib in endocrine therapy-resistant luminal A cells

Earlier studies have shown that overexpression of FGFR1 alone can confer resistance to fulvestrant treatment (Drago et al., 2019; Turner et al., 2010). Additionally, a recent report postulates that alpelisib resistance can also be conferred by FGFR1 overexpression in

luminal A-type breast cancer cells (Shi et al., 2025). Based on these findings and our transcriptomic data indicating an enrichment of activated FGFR signaling in breast cancer cells resistant to tamoxifen, fulvestrant, and alpelisib, we aimed to determine whether we could replicate the alpelisib-resistant phenotype conferred by FGFR1 overexpression in our breast cancer cell models. Since patients currently receiving alpelisib in combination with fulvestrant are known to have refractory disease after prior endocrine therapies, we reasoned it would be more clinically relevant to assess whether FGFR1 overexpression could confer resistance to alpelisib in our tamoxifen and fulvestrant-resistant MCF7-tamR-fulvR cells. The cells were transduced with a plasmid pool (E1-4) containing flag-tagged

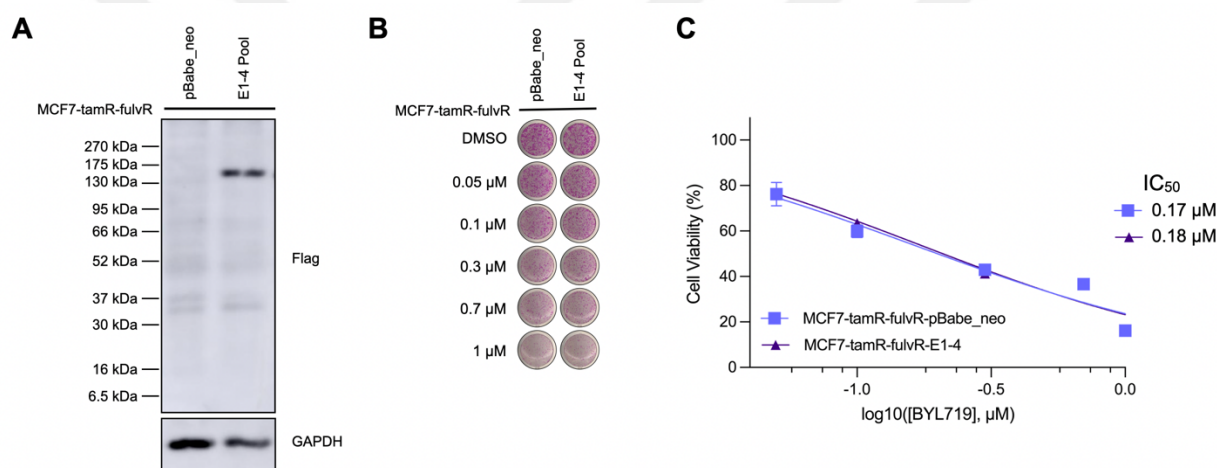


Figure 3.15: *FGFR1* overexpression cannot confer resistance to alpelisib in fulvestrant-resistant MCF7 cells. **A** Immunoblot images showing flag expression after transduction of MCF7-tamR-fulvR cells with the FGFR1-containing plasmid pool (E1-4) in comparison to the empty vector (pBabe_neo). **B** Representative images of MCF7-tamR-fulvR cells transduced with either empty vector (pBabe_neo) or FGFR1 containing E1-4 plasmid pool and treated with indicated concentrations of alpelisib (BYL719). **C** Dose-dependent toxicities of BYL719 treatment in transduced MCF7-tamR-fulvR cells. Cell viability was assessed after 5 days using Sulforhodamine B (**B-C**). Data are represented as mean $\pm$ SEM (**C**).

FGFR1 with a TEL sequence at its C-terminus. The TEL sequence facilitates receptor dimerization and, therefore, the constitutive activation of the transduced FGFR1 receptor. Consistent with successful transduction, immunoblot results demonstrated flag expression within the FGFR1 molecular weight range when cells were transduced with the FGFR1-containing E1-4 plasmid pool (Figure 3.15.A). Contrary to the aforementioned study (Shi et al., 2025), subsequent cell viability assays assessing changes in alpelisib sensitivity upon *FGFR1* overexpression in MCF7-tamR-fulvR cells revealed no significant differences (Figure 3.15.B-C), suggesting FGFR1 overexpression may not confer alpelisib resistance in endocrine-resistant breast cancer cells.

3.9. *NTRK1* overexpression confers resistance to alpelisib treatment

TRK receptors, encoded by *NTRK1*, *NTRK2*, and *NTRK3* genes, are receptor tyrosine kinases with pivotal roles in neural development. Through activation of downstream MAPK and PI3K pathways, *NTRK* gene fusions enhance tumor proliferation across a variety of tumor types (Hechtman, 2022). In addition to activated FGFR signaling, our GSEA analysis also showed activated *NTRK2* signaling in T47D-tamR-fulvR-BYLR cells (Figure 3.5.A, E). Consistent with the GSEA results, DAVID pathway enrichment analysis presented in Emirmustafaoglu (2024) indicated enrichment of the *NTRK1* pathway in drug-resistant T47D cells. Guided by these findings, we sought to test whether overexpression of *NTRK1* could impart resistance to alpelisib in endocrine-resistant T47D cells. Therefore, we transduced T47D-tamR-fulvR cells with a plasmid pool (C5-8) that contains flag-tagged *NTRK1* with a TEL sequence on its C-terminus. Transduction was confirmed by flag expression (Figure 3.16.A), and, notably, cell viability assays revealed a 3-fold increase in BYL719 IC_{50} values when cells were transduced with *NTRK1* (Figure 3.16.B-C), suggesting that overexpression of *NTRK1* can confer resistance to alpelisib in endocrine-resistant luminal A-type breast cancer cells.

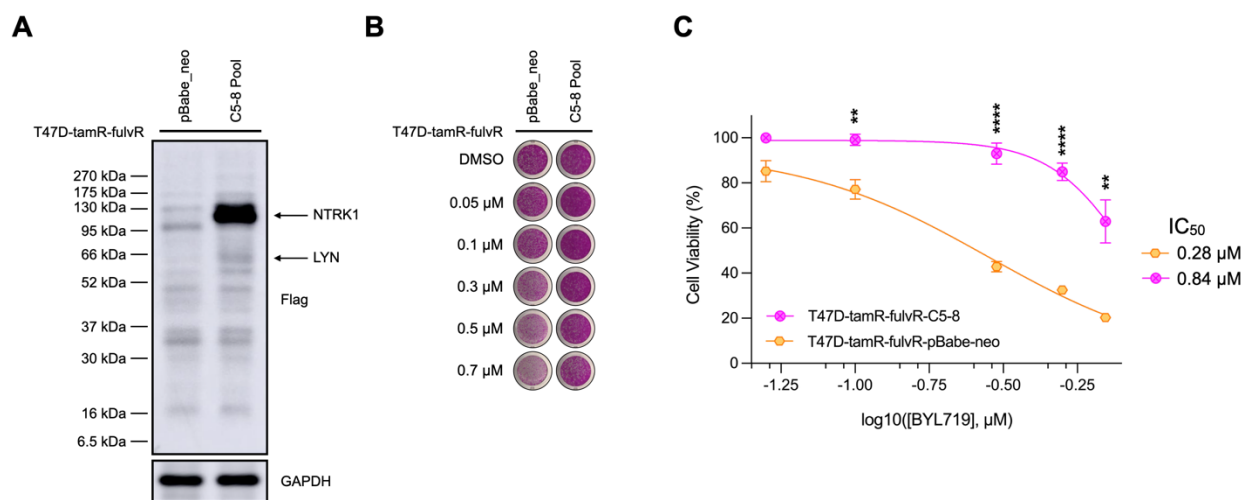


Figure 3.16: *NTRK1* overexpression confers partial resistance to alpelisib in fulvestrant-resistant T47D cells. **A** Immunoblot images showing flag expression after transduction of T47D-tamR-fulvR cells with the NTRK1-containing plasmid pool (C5-8) in comparison to the empty vector (pBabe_neo). **B** Representative images of T47D-tamR-fulvR cells transduced with either empty vector (pBabe_neo) or NTRK1 containing C5-8 plasmid pool and treated with indicated concentrations of alpelisib (BYL719). **C** Dose-dependent toxicities of BYL719 treatment in transduced T47D-tamR-fulvR cells. Cell viability was assessed after 5 days using Sulforhodamine B (**B-C**). Data are represented as mean $\pm$ SEM from $n=3$ independent experiments (**C**). ** $p < 0.01$, **** $p < 0.0001$; two-way ANOVA.

4. DISCUSSION

Breast cancer remains a major health concern globally, accounting for an estimated 2.3 million new diagnoses and 666,000 cancer-related deaths each year, making it the most commonly diagnosed cancer type in women (Bray et al., 2024). It is a heterogeneous disease with multiple molecular subtypes, each with its distinct biological features and therapeutic vulnerabilities. The first-line treatment strategy for HR+/HER2- breast cancer, the most prevalent subtype, involves endocrine therapy with aromatase inhibitors, selective estrogen receptor modulators (SERMs) such as tamoxifen, or selective estrogen receptor degraders (SERDs) like fulvestrant; however, exposure to endocrine therapy can eventually lead to acquired resistance (Xiong et al., 2025). These acquired resistances may arise through various distinct molecular bypass mechanisms and prompted the development of many treatment strategies in clinical settings combining endocrine therapy with CDK4/6 (Rastogi et al., 2024; Slamon et al., 2024), mTOR (Baselga et al., 2012), AKT (Turner et al., 2023), and PI3K (Turner et al., 2025; André et al., 2019) inhibitors. However, therapeutic resistance still remains inevitable, and identifying actionable mechanisms that mediate drug resistance is critical for improving long-term clinical outcomes in advanced-stage breast cancer patients.

The FDA approved the combined treatment of the SERD fulvestrant and p110 α inhibitor alpelisib (BYL719) in patients with ER+ and *PIK3CA* mutant breast cancer, showing refractory disease either on or after endocrine therapy in 2019. The combinatory treatment led to clinically meaningful improvements in progression-free survival with a median of approximately 11 months (André et al., 2019). To investigate resistance mechanisms that enable the bypass of combined fulvestrant/alpelisib therapy, we have generated luminal A-type breast cancer cell models with acquired drug resistance to tamoxifen, fulvestrant, and alpelisib. Concordant with the therapeutic trajectory in clinics, ER+ and *PIK3CA* mutant MCF7 and T47D cells previously exposed to tamoxifen treatment and characterized to have tamoxifen resistance (Mishra et al., 2018) were subjected to escalated doses of fulvestrant/alpelisib dual therapy for 30 weeks. The ~11-fold (T47D-tamR-fulvR-BYLR) and ~12-fold (MCF7-tamR-fulvR-BYLR) increase in IC₅₀ values for BYL719 following dose-escalated treatments (Figure 3.1) was sufficient to

classify cells as resistant. Acquired resistance was further supported by the observed morphological changes (Figure 3.2) and immunoblot experiments (Figure 3.3), which demonstrated resistance-related protein-level changes in both PI3K and ER α pathway components. Given the tight regulation between the ER and PI3K pathways in breast cancer (Bosch et al., 2015), unlike previously published studies that investigated alpelisib resistance using cell models with acquired resistance to alpelisib alone (Gremke et al., 2025), our cell models enable the investigation of resistance mechanisms that may arise from simultaneous inhibition of both ER and PI3K pathways.

Prior studies have proposed reciprocal activation of the PI3K pathway, either through PTEN loss (Juric et al., 2015), p110 β isoform switch (Leroy et al., 2016), or mTOR pathway overactivation (Elkabets et al., 2013) as the main bypass mechanisms in acquired alpelisib resistance. However, therapeutically targeting both p110 α and p110 β isoforms with pan-PI3K inhibitors (Krop et al., 2016) or concurrently inhibiting p110 α and mTORC1 (Makker et al., 2016) has been associated with limited tolerability in patients due to severe toxicities. To investigate whether the adaptive isoform switch phenotype emerged in our fulvestrant/alpelisib-resistant cell lines and resulted in sensitization of our cells to p110 β and/or p110 δ inhibition, we treated our tamR-fulvR-BYLR cells, in comparison to their tamR-fulvR counterparts, with p110 β (AZD6482) and p110 δ specific (CAL101) inhibitors. On the contrary, the tamR-fulvR-BYLR cells, with resistance to tamoxifen, fulvestrant, and alpelisib, displayed cross-resistance to both inhibitors, suggesting no acquired sensitization to inhibition of either isoform (Figure 3.4). Further validating these results, our tamR-fulvR-BYLR cells were also cross-resistant to the pan-PI3K inhibitor GDC0941, which inhibits both p110 α , p110 β , and p110 δ isoforms (Figure 3.4).

Identification of potential actionable targets that drive the acquired resistance is essential to ensure the highest patient benefit. Therefore, we performed RNA sequencing on our triple-resistant tamR-fulvR-BYLR cells compared with their endocrine-resistant counterparts (tamR-fulvR) to determine enriched pathways that may contribute to the bypass of the fulvestrant/alpelisib combination treatment. Our gene set enrichment

analysis (GSEA) revealed enrichment of both FGFR1, FGFR2, and FGFR3 signaling components in alpelisib-resistant cells (Figure 3.5). Fibroblast growth factor receptors (FGFRs) are receptor tyrosine kinases with pivotal roles in cellular proliferation and homeostasis, and whose activation engages multiple downstream pathways, including PLC γ , JAK/STAT, PI3K, and, most potently, the MAPK cascade. At the receptor level, we observed upregulation of FGFR2 and FGFR3, accompanied by increases in expression levels of multiple downstream MAPK pathway components, whereas FGFR1 was downregulated (Figure 3.6). These findings led to the postulation that activated FGFR/MAPK signaling may serve as an escape route for the acquired resistance in drug-resistant cells. The observed elevation in total FGFR2 protein and in activatory phosphorylations of downstream MAPK effectors ERK1/2 (Emirmustafaoglu, 2024) and p90RSK in tamR-fulvR-BYLR cells, compared to tamR-fulvR cells, further supported our hypothesis (Figure 3.7). Although these results provide sufficient evidence of MAPK pathway activation upon the acquisition of drug resistance, the mechanistic link could be further supported by assessing changes in phosphorylated levels of FGFR receptors and/or the adaptor protein FRS2, which mediates MAPK activation downstream of FGFR activation.

Guided by our findings indicating increased FGFR activity at both the transcript and protein levels, we aimed to evaluate whether targeting FGFR could exert functional effects in our drug-resistant cells. Strikingly, the combination of the pan-FGFR inhibitor pemigatinib with alpelisib restored alpelisib sensitivity in resistant cells and resulted in synergistic growth inhibition (Figure 3.8). Of note, this effect was evident even under experimental conditions in which cells were not exposed to additional exogenous FGF stimulation, in contrast to previous breast cancer studies where FGFR inhibition was evaluated following ligand stimulation (Formisano et al., 2019; Drago et al., 2019). To explore this observation further in a more physiologically relevant setting, we generated 3D tumor spheroids from our fulvestrant/alpelisib-resistant T47D cells and treated the spheroids with either alpelisib and pemigatinib alone, or with the combination of both. While single-agent treatments initially slowed or partially inhibited spheroid growth, the spheroids eventually resumed growth over time. On the contrary, the combined treatment

of pemigatinib and alpelisib resulted in sustained suppression of spheroid growth for at least 20 days (Figure 3.9). These findings suggested that FGFR pathway activation has functional implications in acquired fulvestrant/alpelisib resistance and revealed FGFR inhibition in combination with alpelisib as a potential strategy to overcome drug resistance.

Due to the high degree of homology among FGFR isoforms, previous efforts to generate isoform-specific FGFR inhibitors that target a single isoform while sparing the others had mainly remained unsuccessful. However, several isoform-specific inhibitors, currently under clinical evaluation, have recently emerged, including FGFR2-selective lirafugratinib (Subbiah et al., 2023), FGFR3-selective LOXO-435 (Ballard et al., 2021) and TYRA-300 (Hudkins et al., 2024), and FGFR4-selective roblitinib (Weiss et al., 2019) and fisogatinib (Hatlen et al., 2019). Since our previous transcriptomic and protein-level investigations suggested a more pronounced involvement of the FGFR2 isoform in acquired drug resistance, we sought to test whether selective inhibition of the FGFR2 isoform alone could recapitulate the effects we have observed with the pan-FGFR inhibitor pemigatinib. Markedly, combined treatment of lirafugratinib with alpelisib also resensitized alpelisib-resistant cells to alpelisib, and the combination led to synergistic growth inhibition (Figure 3.11). Consistent with findings in 2D settings, 3D tumor spheroid assays demonstrated that single-agent treatments provided only transient growth suppression, whereas the lirafugratinib/alpelisib combination yielded sustained growth inhibition for up to 24 days (Figure 3.12). These findings suggest that selectively targeting the FGFR2 isoform alone may offer a promising approach to overcoming fulvestrant/alpelisib resistance.

To evaluate our in vitro findings in an in vivo model and determine whether the dual inhibition of the FGFR receptor and p110 α could suppress tumor growth in drug-resistant tumors in mice, we generated orthotopic xenograft models using our fulvestrant/alpelisib-resistant T47D cells. Notably, in line with the represented spheroid results, the combined pemigatinib/alpelisib treatment significantly reduced tumor size compared to both vehicle control and pemigatinib treatment alone, whereas single treatments, although slowed tumor growth, did not induce tumor regression (Figure 3.14.B). Although the combination

treatment showed significantly greater efficacy than both the vehicle control and single-agent pemigatinib, the difference between alpelisib monotherapy and the combination did not reach statistical significance. In addition, when compared, the extracted tumor weights were significantly reduced in the combination group relative to vehicle; however, differences between the combination and single-agent groups were not statistically significant (Figure 3.14.C). Therefore, these findings can be further improved by increasing the number of biological replicates and optimizing the given drug concentrations. No significant changes in body weight or overt signs of toxicity were observed in mice throughout the experiment, suggesting that the combination treatment is well-tolerated *in vivo* (3.14.D).

To further delineate the contribution of each individual FGFR isoform to the acquired fulvestrant/alpelisib resistance, we investigated whether FGFR1 overexpression in our endocrine-resistant tamR-fulvR cells could confer resistance to alpelisib. Several previous studies have implicated FGFR1 overexpression in endocrine resistance (Mao et al., 2020; Drago et al., 2019) and, more recently, in alpelisib resistance (Shi et al., 2025), suggesting that FGFR1 overexpression alone may be sufficient to drive resistance to fulvestrant or alpelisib. However, we could not replicate these findings, as in our experimental system, overexpression of FGFR1 did not reduce alpelisib sensitivity in endocrine-resistant MCF7-tamR-fulvR cells when compared with empty-vector transduced controls (Figure 3.15). This discrepancy may be due to differences in cellular context, since the aforementioned study utilized parental cells (Shi et al., 2025) for forced FGFR1 overexpression, whereas our model represents an endocrine-resistant background in which PI3K signaling may already be rewired and partial co-resistance to alpelisib may be present (Appendix A-Figure A.1). These experiments could be further improved by examining whether our transduction resulted in increased downstream activity of the FGFR/MAPK signaling, for example, by assessing total FGFR1, phosphorylated FGFR1, and ERK1/2 protein levels. Moreover, because the transduction was performed with a pool of constructs that included other genes in addition to FGFR1, repetition of the experiments with an FGFR1 construct alone would facilitate eliminating any potential off-target effects.

NTRK1 was one of the enriched pathways in our previous transcriptomic analysis (Emirmustafaoglu, 2024). To investigate whether overexpression of NTRK1 could confer alpelisib resistance in our endocrine-resistant cell lines, we transduced T47D-tamR-fulvR cells with a pool of constructs containing NTRK1 and LYN. Flag expression confirmed the overexpression of NTRK1 and, to a lesser extent, LYN. Interestingly, overexpression of NTRK1 resulted in partial resistance to alpelisib, suggesting that this RTK may also play a supporting role in acquired alpelisib resistance. These findings could be further refined by examining downstream pathway activity and isolating the single NTRK1 plasmid from the pool to eliminate any effects attributed to LYN overexpression.

4.1. Conclusion and future perspectives

Our research identifies activated FGFR signaling as a key mediator of resistance to the clinically approved combination of alpelisib and fulvestrant in *PIK3CA*-mutant luminal A-type advanced-stage breast cancer. These findings suggest that incorporation of FGFR pathway inhibition to p110 α inhibition may constitute a promising approach to overcome acquired drug resistance in advanced-stage luminal A breast cancer.

The presented results of this study may have broader implications for the resistance mechanisms that may take part in other treatment strategies in ER+ breast cancer involving recently approved mutant-specific p110 α inhibitor inavolisib, in combination with fulvestrant and palbociclib (Turner et al., 2025) as well as distinct malignancies such as endometrial cancer, where combined fulvestrant and alpelisib treatment is currently under clinical investigations (Gaillard et al., 2024).

Collectively, our findings support a rationale for proposing dual inhibition of FGFR and p110 α as a potential strategy for the treatment of advanced-stage refractory breast cancer. However, further investigations comparing the efficacy of the pemigatinib/alpelisib or lirafugratinib/alpelisib combinations in FGFR-overactivated (tamR-fulvR-BYLR) and baseline (tamR-fulvR) conditions could substantiate the

hypothesis that resistance is primarily driven by alterations in FGFR signaling, thereby enhancing the sensitivity of drug-resistant cells to the combination treatment.

Several distinct FGFR alterations, including gene amplifications, oncogenic activating mutations, and gene fusions, have been reported in approximately 12.6-18% of all breast cancer patients (Zhang et al., 2024), with FGFR1 amplification representing the most frequent event occurring in 15% of ER+ cases (Servetto, Formisano and Arteaga, 2021). Although the incidence of oncogenic FGFR mutations is relatively rare in therapy-naïve tumors, the rate is reported to increase with metastatic disease progression, suggesting a potential association with acquired drug resistance and FGFR mutations (Uemoto et al., 2025). In addition, clinical benefit from non-covalent ATP-competitive inhibitors such as pemigatinib has been shown to be limited in some FGFR kinase domain mutations (Rodón et al., 2024), likely due to the mutation interfering with the inhibitor's interaction with the drug-binding pocket (Kato et al., 2024). Therefore, to further strengthen the physiological relevance of our research, future experiments could evaluate the efficacy of the pemigatinib/alpelisib combination in cells harboring FGFR kinase-domain mutations.

Several previous studies have established the role of FGFR1 overexpression in endocrine resistance or resistance to CDK4/6 therapy (Formisano et al., 2019). Concordantly, there are a number of clinical studies investigating the role of pan-FGFR inhibitors, erdafitinib (Mayer et al., 2021; Gonzalez-Ericsson et al., 2025) and rogaratinib (Martinez-Jañez et al., 2025), in combination with palbociclib and fulvestrant for breast cancer patients with prior drug resistance. However, adverse dose-limiting toxicities, such as hyperphosphatemia due to FGFR1 inhibition and diarrhea due to FGFR4 inhibition, are observed in most patients (55%-85%) receiving pan-FGFR inhibitor treatment, leading to treatment discontinuation and limiting clinical benefit (Kato et al., 2024). Importantly, early-phase clinical trials with FGFR2 isoform-specific inhibitor lirafugratinib (Schram et al., 2023) and preclinical studies with FGFR3-specific inhibitor LOXO-435 (Ballard et al., 2021) indicated improved tolerability, avoiding hyperphosphatemia caused by FGFR1 inhibition, which may lead to durable clinical outcomes. These findings highlight the importance of understanding isoform-specific FGFR alterations in acquired drug

resistance, and therefore, future experiments determining the specific roles of FGFR2 and FGFR3 isoforms in acquired fulvestrant/alpelisib resistance could be highly valuable in enhancing the impact of the current study.

Finally, to further understand the clinical relevance of our findings, establishing the efficacy of combined FGFR receptor and p110 α inhibition in patient-derived xenograft (PDX) models with prior exposure to endocrine therapy and PI3K inhibition would substantially strengthen our hypothesis.



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APPENDICES

APPENDIX A – Supplementary Figures

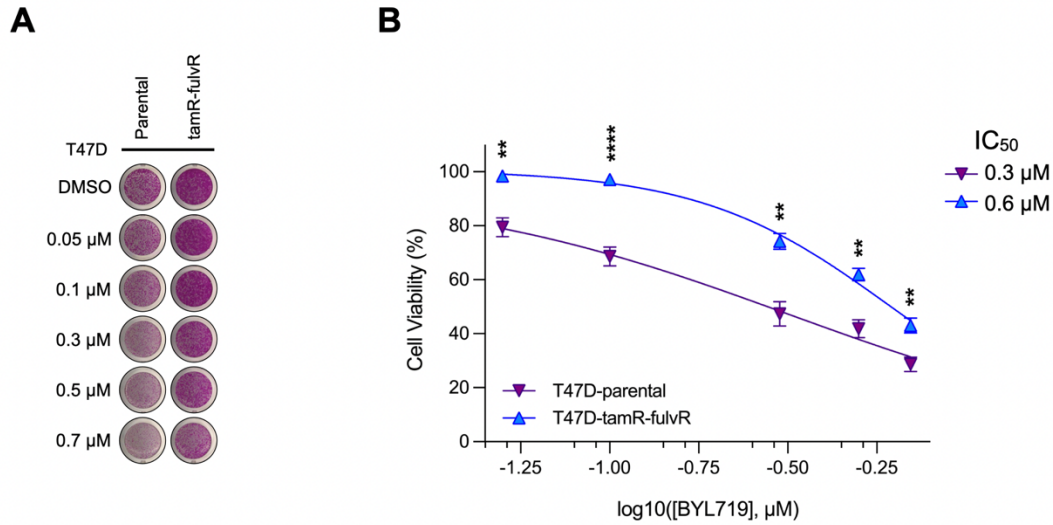


Figure A.1: Acquired endocrine resistance reduces sensitivity to alpelisib. **A** Representative images of T47D parental and tamR-fulvR treated with indicated concentrations of alpelisib (BYL719). **B** Dose-dependent toxicities of BYL719 treatment in T47D parental and tamR-fulvR cells. Cell viability was assessed after 5 days using Sulforhodamine B (**A-B**). Data are represented as mean $\pm$ SEM from $n=3$ independent experiments (**B**). ** $p < 0.01$, **** $p < 0.0001$; two-way ANOVA.

APPENDIX B – Sanger Sequencing Results

Table B.1: Sanger sequencing results.

Sample	Insert
C5-8_Colony_1_pWZL_Forward	<i>LYN</i>
C5-8_Colony_2_pWZL_Forward	<i>LYN</i>
C5-8_Colony_3_pWZL_Forward	<i>LYN</i>
C5-8_Colony_4_pWZL_Forward	<i>LYN</i>
C5-8_Colony_5_pWZL_Forward	-
C5-8_Colony_6_pWZL_Forward	<i>LYN</i>
C5-8_Colony_7_pWZL_Forward	<i>LYN</i>
C5-8_Colony_8_pWZL_Forward	<i>LYN</i>
C5-8_Colony_9_pWZL_Forward	<i>LYN</i>
C5-8_Colony_10_pWZL_Forward	<i>LYN</i>
E1-4_Colony_1_pWZL_Forward	<i>INSRR</i>
E1-4_Colony_2_pWZL_Forward	<i>FGFR1</i>
E1-4_Colony_3_pWZL_Forward	<i>INSRR</i>
E1-4_Colony_4_pWZL_Forward	<i>FGFR1</i>
E1-4_Colony_5_pWZL_Forward	<i>INSRR</i>
E1-4_Colony_6_pWZL_Forward	<i>FGFR1</i>
E1-4_Colony_7_pWZL_Forward	<i>INSRR</i>
E1-4_Colony_8_pWZL_Forward	<i>FGFR1</i>
E1-4_Colony_9_pWZL_Forward	-
E1-4_Colony_10_pWZL_Forward	<i>FGFR1</i>
E9-12_Colony_1_pWZL_Forward	<i>EPHB6</i>
E9-12_Colony_2_pWZL_Forward	<i>EPHB6</i>
E9-12_Colony_3_pWZL_Forward	<i>JAK1</i>
E9-12_Colony_4_pWZL_Forward	<i>EPHB6</i>
E9-12_Colony_5_pWZL_Forward	<i>EPHB6</i>

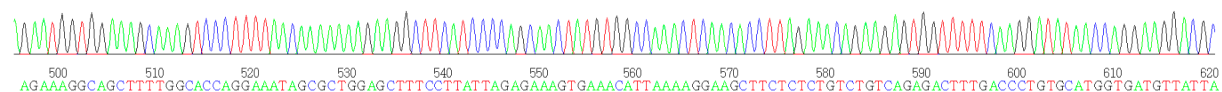
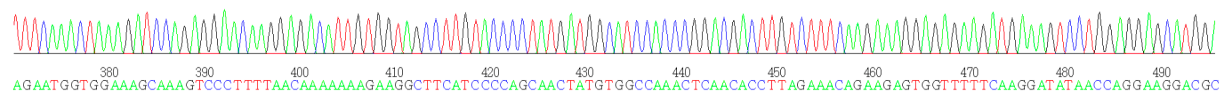
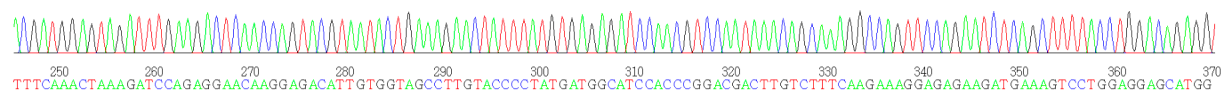
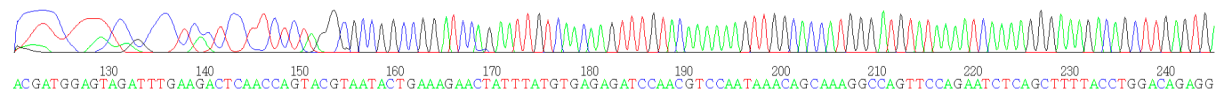
E9-12_Colony_6_pWZL_Forward	<i>EPHB6</i>
E9-12_Colony_7_pWZL_Forward	<i>EPHB6</i>
E9-12_Colony_8_pWZL_Forward	<i>EPHB6</i>
E9-12_Colony_9_pWZL_Forward	<i>JAK1</i>
E9-12_Colony_10_pWZL_Forward	<i>EPHB6</i>





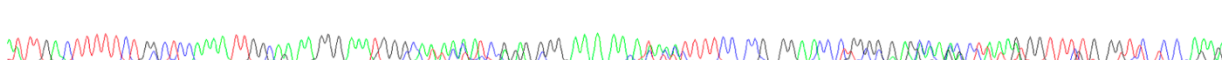
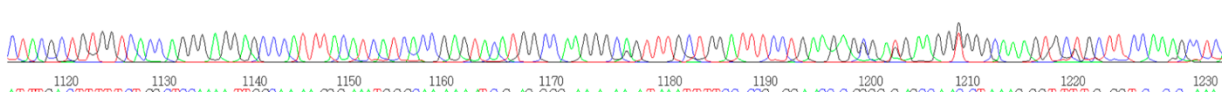
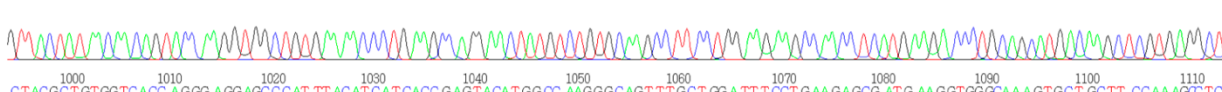
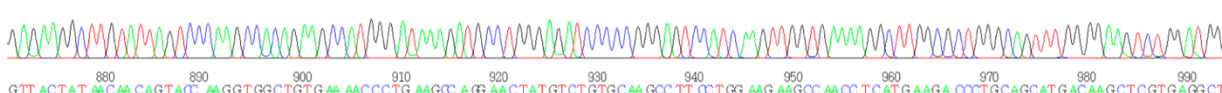
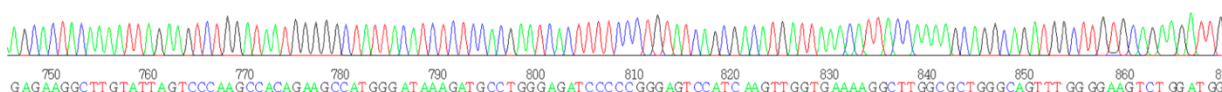
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Sample: C5-8_Colony_1_pWZL_Forward Lane: 12 Base spacing: 14.569293 null bases in 16150 scans Page 1 of 2

G C A C G T T A C A G A T C T C T C T C T A G G C G C C G G C C G G A T C C A G A A T T G T C A A C A A G T T T G T A C A A A A A A G T T G C A C C A T G G G A T G T A T A A A T C A A A A G G G A A A G A C A G C T T G A G T G

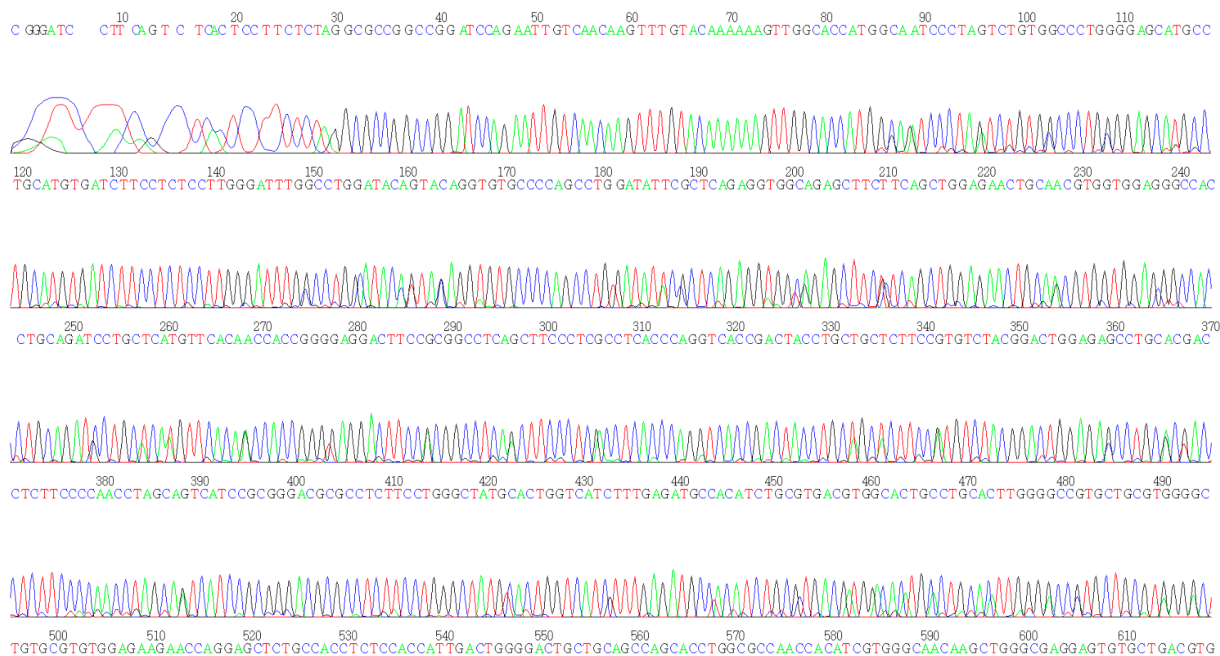


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Sample: C5-8_Colony_1_pWZL_Forward Lane: 12 Base spacing: 14.569293 null bases in 16150 scans Page 2 of 2

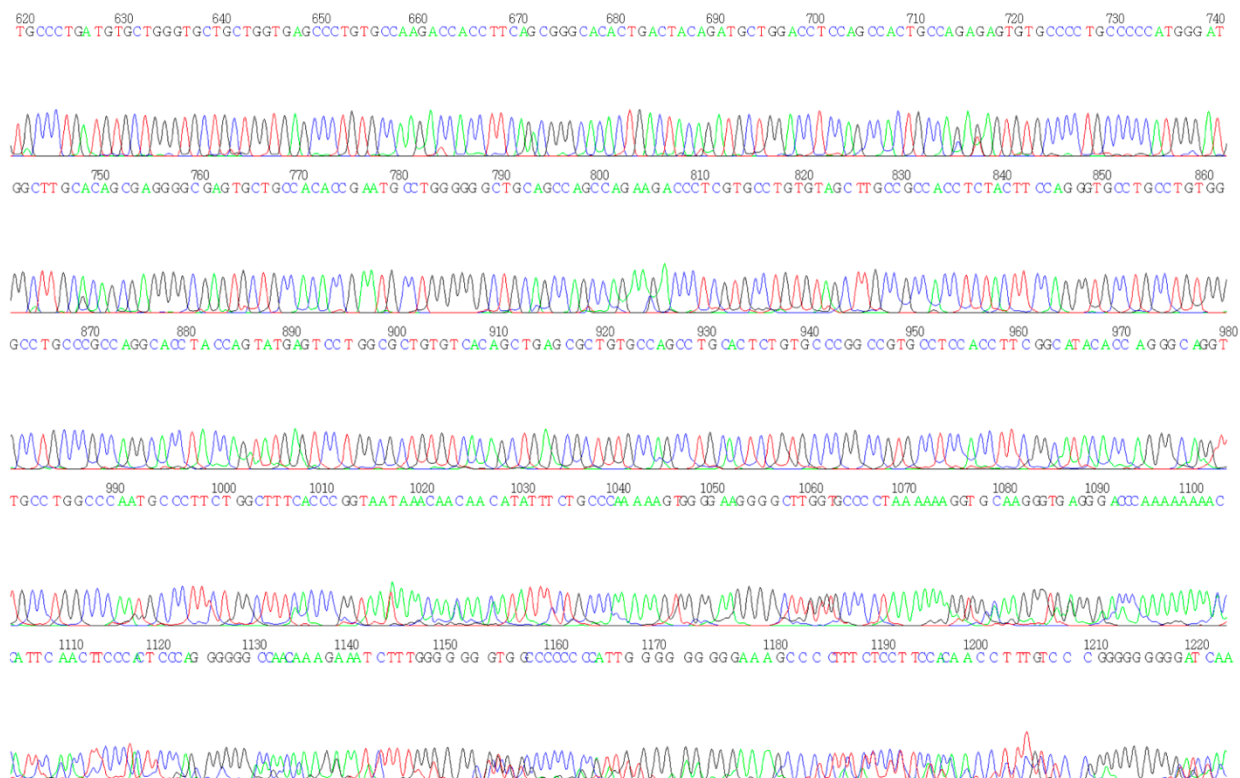
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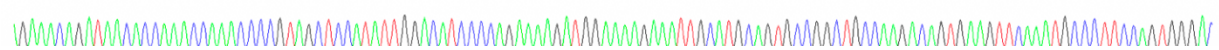
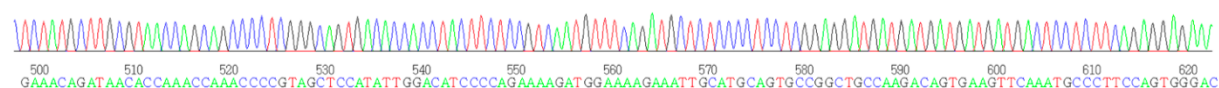
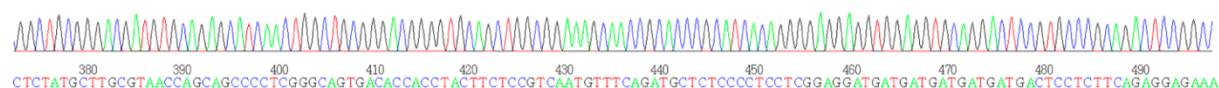
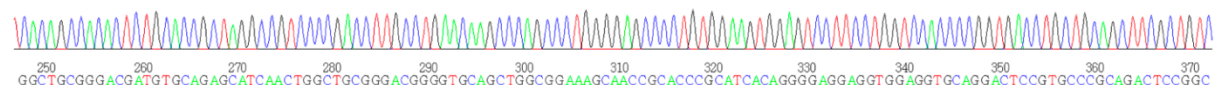
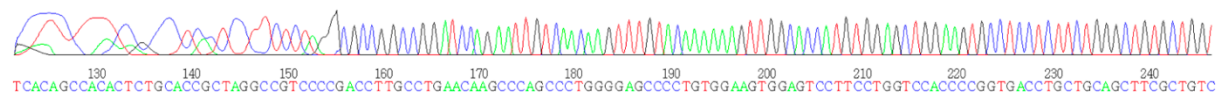
Order No: EC00198606 File: E1-4_Colony_1_pWZL_Forward.ab1 Run Ended: 2025/10/22 18:8:16 Signal G:1037 A:1228 C:2121 T:1841
Sample: E1-4_Colony_1_pWZL_Forward Lane: 15 Base spacing: 15.278925 null bases in 16175 scans Page 1 of 2



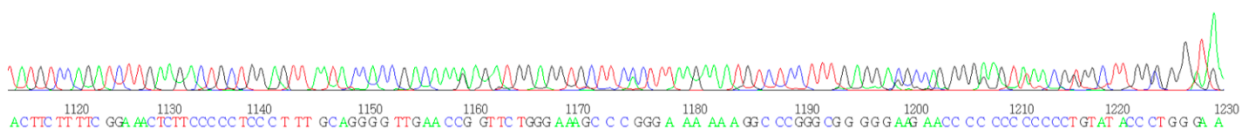
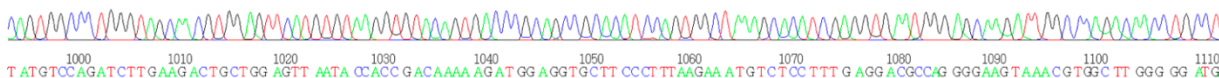
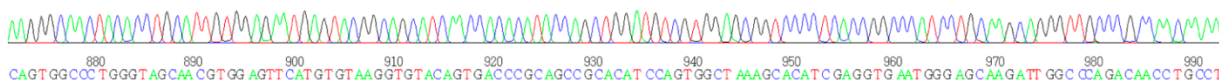
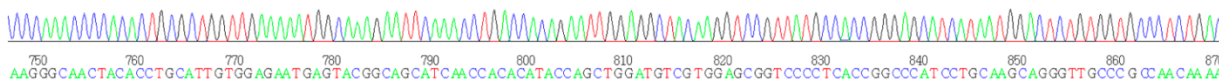
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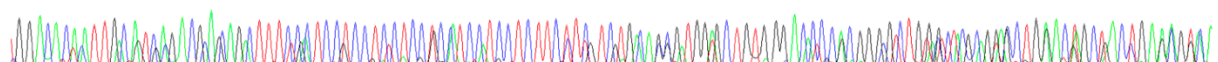
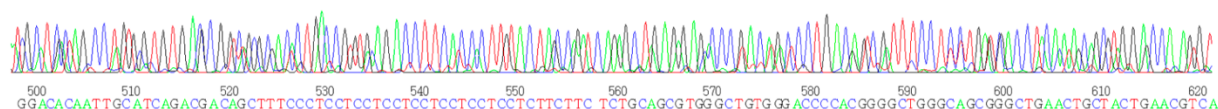
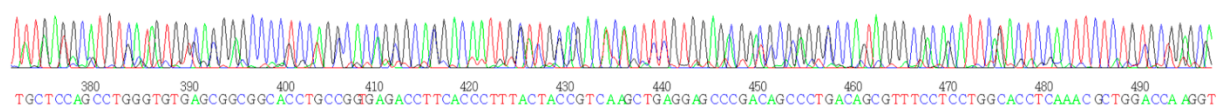
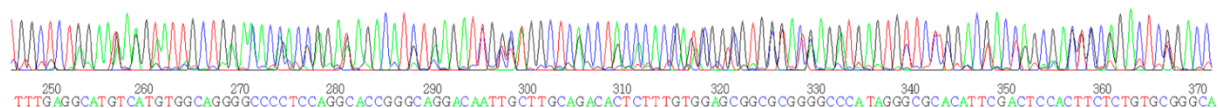
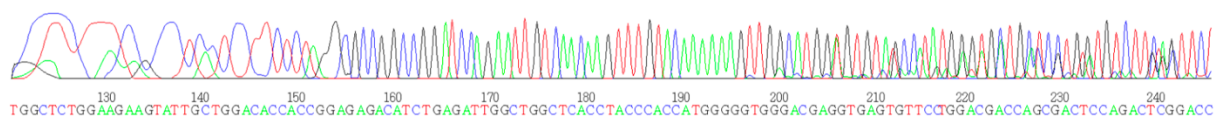
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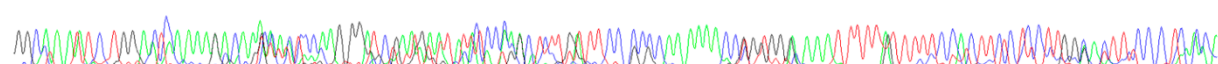
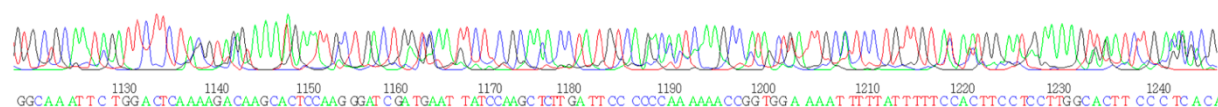
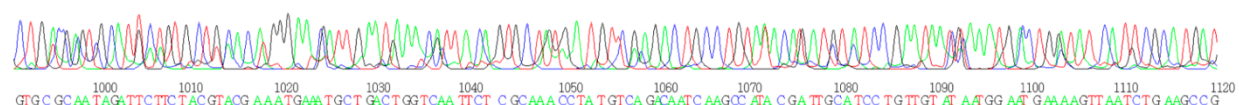
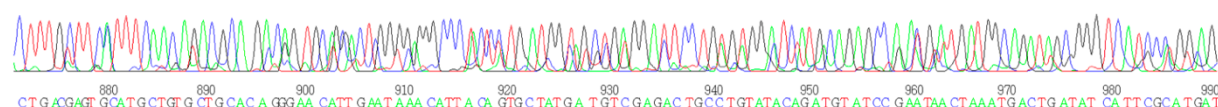
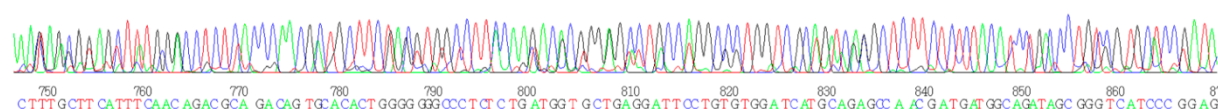
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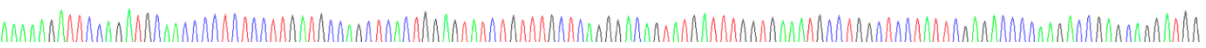
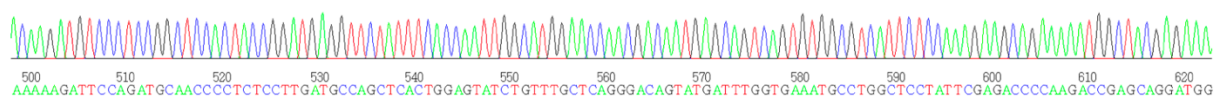
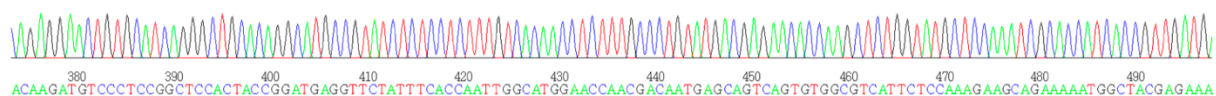
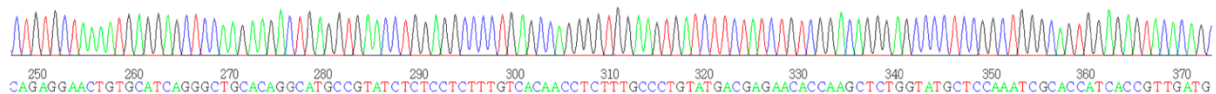
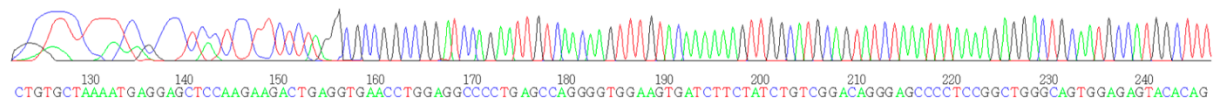
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Order No: EC00198606 File: E9-12_Colony_3_pWZL_Forward.ab1 Run Ended: 2025/10/22 18:8:16 Signal G:1311 A:1748 C:1687 T:1859
Sample: E9-12_Colony_3_pWZL_Forward Lane: 19 Base spacing: 14.654696 null bases in 16201 scans Page 1 of 2

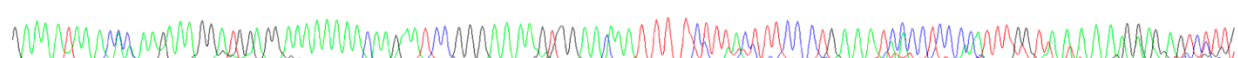
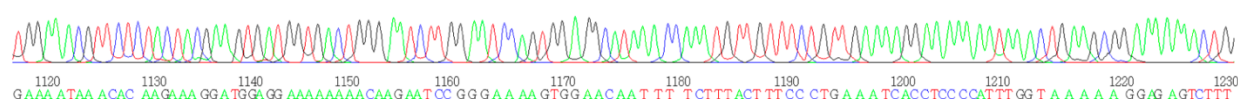
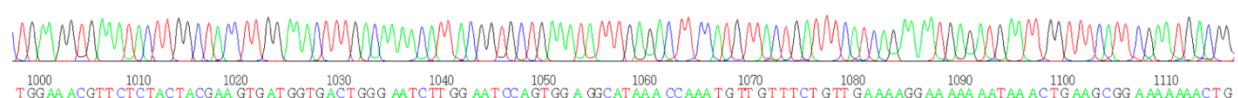
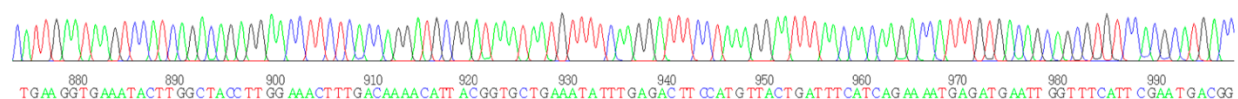
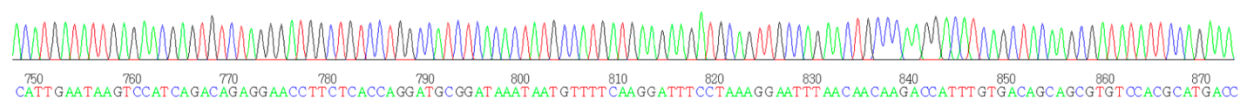


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Order No: EC00198606 File: E9-12_Colony_3_pWZL_Forward.ab1 Run Ended: 2025/10/22 18:8:16 Signal G:1311 A:1748 C:1687 T:1859
Sample: E9-12_Colony_3_pWZL_Forward Lane: 19 Base spacing: 14.654696 null bases in 16201 scans Page 2 of 2

ACATGATATTGAGAACGAGTGTCTAGGGATGGCTGTCTCTGCCATCTCACACTATGCCATGATGAAGAAAGATGCAGTTGCCAGAACTGCCCAAGACATCAGCTACAAAGCGATATATTCCAGAAA



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