

**CO-TARGETING OF PI3K PATHWAY WITH SPHINGOLIPID METABOLISM
CONSTITUTES A PROMISING THERAPEUTIC STRATEGY AGAINST ADVANCED
STAGE PROSTATE CANCER**

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by

Şevval Yılmaz Ürgen

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May 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

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M.S. in Molecular Biology and Genetics

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Prostate cancer is the second most diagnosed malignancy and the fifth leading cause of cancer-driven death in men worldwide. The identification of driver mutations and genomic alterations, therefore, is crucially important to develop targeted strategies. Phosphatase and Tensin Homolog (PTEN) deletion has been shown among early and frequent alterations. Moreover, the PTEN loss-of-function has been reported to be enriched in metastatic and high stage prostate cancers highlighting the role of PTEN deletion in metastatic growth. In this regard, our research group focuses on understanding the impact of PTEN loss in prostate epithelia from multiple aspects including metabolic reprogramming and oncogenic signaling. In this project, we revisit the influence of PTEN loss on PI3K Class IA isoform dependency shifts by using non-transformed prostate epithelial cells to model early PTEN deletion. Our findings show that PTEN loss results in a partial dependency shift to p110 β and p110 δ isoforms for growth and survival while p110 α still remains as the predominant isoform. In parallel, we conduct targeted metabolomics to identify PTEN-regulated sphingolipid metabolites under the light of our previous work showing sphingolipid metabolism as a PTEN status regulated node. Our screening reveals that the ceramide levels are upregulated upon PTEN re-expression in all cell lines used in our screening whilst sphingosine-1-phosphate (S1P) metabolites are downregulated upon PTEN expression selectively in androgen deprivation therapy (ADT) resistant cells. We hypothesize that co-targeting PTEN loss-driven alterations synergistically inhibits the growth of high stage prostate cancers while

sparing the PTEN-WT cells. To this end, we develop a combinatory treatment against p110 β /p110 δ and S1PR/SPHK1 to test our hypothesis. Our results conclude that co-inhibition of either p110 β or p110 δ along with S1P production or signaling acts synergistically to eliminate PTEN-null and metastatic prostate cancers.



Keywords: Prostate cancer, PI3K, PTEN, sphingolipid metabolism, S1PR, isoform-dependency, metabolomics

ÖZET

PI3K SİNYAL YOL AĞI İLE SFİNGOLİPİD METABOLİZMASININ BİRLİKTE HEDEFLENMESİ İLERİ EVRE PROSTAT KANSERİNE KARŞI ETKİLİ BİR TEDAVİ STRATEJİSİ OLUŞTURMAKTADIR

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Prostat kanseri, dünya genelinde erkeklerden sık teşhis edilen ikinci kanser türüdür ve kanser kaynaklı ölümlerde beşinci sırada yer alır. Bu nedenle, sürücü mutasyonların ve genomik değişimlerin tespit edilmesi hedefleyici tedavi geliştirilebilmesi için hayati önem taşımaktadır. Fosfataz ve Tensin Homoloğu (PTEN) genomik kaybı erken ve yaygın değişimler arasındadır. Ayrıca, PTEN görev kaybı metastatik ve ileri evre prostat kanserlerinde daha da yaygın görünür ki bu durum PTEN'in metastatik büyümedeki rolünü vurgulamaktadır. Bu bağlamda, bizim araştırma grubumuz PTEN kaybının prostat epiteli üzerindeki etkisini metabolik ve onkogenik sinyal yol ağları dahil olmak üzere birçok açıdan anlamaya odaklanır. Bizler bu projede erken PTEN kaybını modellemek amacıyla neoplastik dönüşüme uğramamış prostat epitel hücrelerini kullanarak PTEN delesyonunun PI3K SINIF IA izoform bağımlılığı üzerindeki etkisini yeniden değerlendirdik. Sonuçlarımız, PTEN kaybının hücre büyümesi ve hayatta kalımı için p110 β ve p110 δ izoformlarına kısmi bir bağımlılık kaymasına yol açtığını ancak p110 α 'nın baskın izoform olmaya devam ettiğini göstermektedir. Paralel olarak, sfingolipid metabolizmasını PTEN statüsü tarafından regüle edildiğini gösteren önceki sonuçlarımızdan yola çıkarak hedefe yönelik metabolomiks taraması gerçekleştirdik. Sonuçlarımız, taramamızda kullandığımız tüm hücre hatlarında seramit seviyelerini PTEN'in yeniden ifade ettirilmesi durumunda arttığını gösterirken sfingozin-1-fosfat (S1P) metabolitlerinin sadece androjen yoksunluğu tedavisi (ADT) direncine sahip hücrelerde PTEN'in yeniden ifade edilmesi durumunda azaldığını gösterdi. PTEN kaybına bağlı değişikliklerin birlikte hedeflenmesinin, ileri evre prostat kanserlerinin büyümesini

sinerjistik biçimde baskılayabileceğini ve aynı zamanda PTEN ifadesi olan hücrelerin zarar görmeden korunabileceğini hipotez ettik. Bu amaçla hipotezimizi test etmek için p110 β / p110 δ ve S1PR/SPHK1'e karşı kombinasyon tedavileri geliştirdik. Sonuçlarımız, p110 β veya p110 δ izoformlarının S1P üretimi veya sinyal iletimiyle birlikte inhibisyonunun, PTEN eksikliği bulunan ve metastatik prostat kanser hücrelerini sinerjistik şekilde yok ettiğini ortaya koymaktadır.



Anahtar Kelimeler: Prostat kanseri, PI3K, PTEN, sfingolipid metabolizması, S1PR, izoform-bağımlılığı, metabolomiks

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ABBREVIATIONS

ADT: androgen deprivation therapy
apoM: HDL-associated apolipoprotein
AR: androgen receptor
ATG9A: autophagy-related protein 9A
CDases: ceramidases
CERK: ceramide kinase
CERS: ceramide synthases
CREB1: cAMP responsive element binding protein 1
CRPC: castration resistant prostate cancer
DEGS1: Δ 4-desaturase DES1
DGAT: diacylglycerol O-acyltransferases
ERG: Ets related gene
FAK: focal adhesion kinase
FASN: fatty acid synthase
FDA: Food and Drug Administration
FOXO: forkhead box protein
IRS1: insulin receptor substrate 1
JAK: janus kinase
KDSR: 3-ketodihydrosphingosine reductase
MFSD2B: major facilitator superfamily domain containing 2B
MOMP: mitochondrial outer-membrane permeabilization
mTORC1: mammalian target of rapamycin complex 1
PCa: prostate cancer
PDK1: phosphoinositide-dependent protein kinase 1
PH: pleckstrin homology
PI3K: phosphatidylinositol 3-kinase
PLPP: phospholipid phosphatase
PTEN: phosphatase and tensin homolog
PX: Phox-homology
S1P: sphingosine-1-phosphate

S1PR: sphingosine-1-phosphate receptor

SERBP: sterol regulatory element-binding protein

SGPP: S1P phosphatase

SPHK: sphingosine kinase

SPL: sphingolipid

SPNS2: protein spinster homolog 2

SPT: serine palmitoyl-transferase

TCA: tricarboxylic acid

UGCG: UDP-glucose ceramide glucosyltransferase

VEGF-VEGFR: vascular endothelial growth factor - vascular endothelial growth factor recept

1. INTRODUCTION

1.1. Prostate Cancer

Prostate cancer has been reported as the second most prevalent cancer type and the fifth cause of cancer-driven death in men. The worldwide annual number of incidents are estimated to increase to 2.63 million by 2045. (The Global Cancer Observatory, 2024) In this regard, it is crucial to develop therapeutic strategies and identify the potential vulnerabilities of prostate cancer.

1.1.1. Genomic Alterations in Prostate Cancer

Genomic alterations in the early onset and high-grade prostate cancer have been studied extensively to characterize the genes contributing to the progression of the disease. The loss-of-function mutations that mediate the early progression of prostate cancer includes PTEN, TP53 and Rb gene loss. (Abate-Shen and Shen, 2000) In addition to these, TMPRSS2-ERG fusions which results in androgen-dependent activation of ERG (Ets related gene) gene expression, are frequently observed in primary prostate cancers. (John et al., 2012) Lastly, primary prostate cancers harbor CHD1 deletion and SPOP mutations to a considerable extend. (Barbieri et al., 2013)

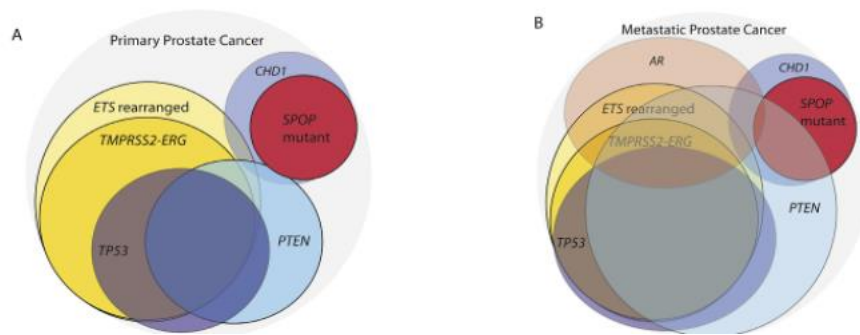


Figure 1. 1. Classification and relative quantification of commonly observed genomic alterations in primary and metastatic prostate cancers taken from Barbieri et al, 2013. When the alterations in the primary prostate cancer are compared to those in metastatic prostate cancers, the main differences are enriched mutations in AR and higher occurrence of tumor suppressor PTEN loss.

The occurrence of PTEN gene locus alterations is correlated with the Gleason score, which means that PTEN function loss may be one of the contributing factors for metastatic stage

transition. (Krohn et al, 2012) AR alteration emergence in metastatic stage, on the other hand, is primarily due to the fact that the patients are administered anti-androgen therapies early in the disease. The tumors with gain-of-function mutations in AR gene, thus, might relapse and give rise to metastatic tumors.

Since PTEN loss is the most common alteration in high stage PCa and also can co-occur with other well-defined alterations (**Figure 1.1**), developing targeted strategies against PTEN function loss might benefit higher fraction of patients.

1.1.2. Metabolic Reprogramming in Prostate Cancer

In order to comprehend the metabolic changes during prostate cancer progression, it is important to initially understand metabolic dependencies of healthy prostate gland. The cells located in the peripheral zone of prostate, where most of the tumors emerges (Rebello et al., 2021), are reported to produce high amount of citrate and selectively not to oxidize it. The accumulated citrate is eventually secreted as a key component of semen. (Costello et al., 2004) This unique feature is accompanied by zinc accumulation, which is responsible for the inhibition of an enzyme called m-aconitase that catalyzes citrate oxidation during TCA cycle. (Costello et al., 2005)

Although the majority of solid tumors comply with the Warburg Effect, prostate cancer is an exception. Since prostate epithelia has a dependency on citrate production as described, the early cancer cells shift their metabolism towards citrate oxidizing stage. (Franz et al., 2013) As a result, they depend more on TCA cycle and oxidative phosphorylation when compared to healthy epithelia. (Costello et al., 2004) Nevertheless, it has been described that CRPC relies more on anaerobic glycolysis by increasing GLUT1 expression. (Wang et al., 2020) This suggests that Warburg effect becomes prominent in the advanced stage of PCa.

The uptake and utilization of amino acids are also reprogrammed in prostate cancer. The uptake of particular amino acids such as glutamine, phenylalanine, tryptophan, leucine and arginine is increased by enriching their transporters on cell membrane through AR-regulated mechanisms. (Wang et al., 2013) The elevated amino acid levels are crucial to upregulate cell growth and anabolic pathways.

The reprogramming of lipid metabolism has been identified as an indispensable metabolic node in advanced prostate cancer. Our lab has shown that the PI3K-mediated upregulation of *de*

novo steroidogenesis drives ADT-resistance in advanced stage PTEN-null prostate cancer. (Kaysudu et al., 2023) Not only steroids, but also other lipid species including fatty acids are enriched in prostate cancer through the upregulation of SREBP family and FASN to serve as building blocks and energy source. (Guo et al., 2014 and Yoshii et al., 2013)

1.2. Phosphatidylinositol 3-kinases (PI3Ks)

Phosphatidylinositol 3-kinases (PI3Ks) are dedicated lipid kinases for the phosphorylation of the third carbon on the inositol ring of phosphatidylinositol species. Based on their substrates, structures and regulatory domains, there are three different classes. (Yilmaz and Cizmecioglu, 2024)

1.2.1. Class I PI3Ks

The class I PI3Ks are responsible for the production of PtdIns(3,4,5)P₃ from PtdIns(4,5)P₂, the resulting PIP₃ species are potent secondary signaling molecules. The class I PI3Ks consist of a heterodimeric structure containing a regulatory and a catalytic domain. The downstream signaling of class I family is highly associated with glucose homeostasis, cell cycle regulation, cytoskeletal remodeling and hallmarks of cancer including migration, proliferation and resistance to apoptosis. (Yilmaz and Cizmecioglu, 2024)

The class I PI3Ks are further divided into Class IA and Class IB subclasses. The catalytic domains of class IA are p110 α , p110 β , and p110 δ which are coupled with p85 α (p55 α and p50 α), p85 β , and p55 γ regulatory domains. On the other hand, class IB catalytic domain is p110 γ and the regulatory subunits are p84 and p101. (Jean and Kiger, 2014)

The downstream applications and the activatory mechanisms of these proteins significantly differ from one another. (**Figure 1.2**) The enriched PIP₃ pool upon PI3K class I activity selectively recruits the proteins containing pleckstrin homology (PH), Phox-homology (PX), and FYVE domains to the cell membrane. (Lemmon, 2008) The main effector of PI3K signaling is protein kinase B (also known as AKT) which receives two activatory phosphorylations on the positions S473 by mTORC2 and T308 by PDK1 after being recruited to cell membrane. (Sugiyama et al., 2019) AKT, then, phosphorylates and activates multiple effector proteins and complexes such as mTORC1 serving as the master regulator of peptide anabolism and also activator of SREBP family for lipid biogenesis (Palm and Thompson, 2017)

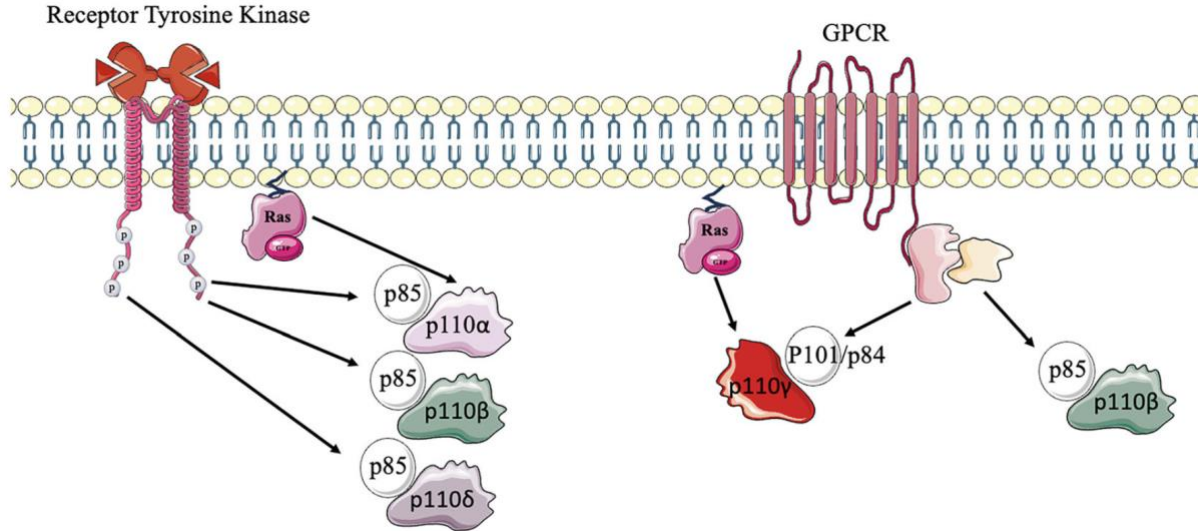


Figure 1. 2. The mechanistic overview of PI3K Class I activation taken from Yilmaz and Cizmecioglu, 2024. Class IA PI3Ks are activated by RTKs and p110 α can be also stimulated by small GTPase Ras. p85 regulatory subunit harbor a SH2 domain which can interact with phospho-Y residues in the RTK tails. Upon this binding, p110 subunits become catalytically active. Additional to RTKs, p110 β functions in downstream of GPCRs as well as Rho GTPases. Class IB PI3K does not possess a SH2 domain and it is activated by GPCRs. (Yilmaz and Cizmecioglu, 2024)

1.2.1.1. Class IA Isoform Dependency

Mammalian cells express all four members of class I PI3Ks, yet p110 δ and p110 γ are enriched in leukocytes while p110 α and p110 β are ubiquitously expressed. (Kok et al., 2019) Utilizing pan-PI3K inhibitors, nevertheless, have been reported with severe side effects including hyperglycemia, rash, mood swings, etc. (Nunnery and Mayer, 2019) For this reason, the identification of PI3K mutations which result in dependency on particular isoforms has become the center of attention for researchers since the activation and signaling of these isoforms follow different paths (**Figure 1.2**). It has been identified that PTEN loss is highly associated with p110 β reliance for growth and survival (Ni et al., 2012) while p110 α activatory mutations lead to the dependency on p110 α (Liu et al., 2011). In this study, we focus on Class IA isoform shifts in prostate cancer as a result of PTEN loss, which is a frequent alteration (**Figure 1.1**).

1.2.2. Class II and Class III PI3Ks

Class II PI3Ks are responsible for the conversion of PtdIns(4)P into PtdIns(3,4)P₂ and PtdIns into PtdIns(3)P. There are three members of this family: PI3KC2 α , PI3KC2 β and PI3KC2 γ . Unlike other classes, class II PI3Ks do not possess a regulatory domain and they function as monomeric structures. Class II PI3K activity is associated with vesicular trafficking, clathrin-mediated endocytosis, primary cilium formation and cell migration. PI3KC2 β isoform also serves as a negative regulator of mTORC1 upon starvation while PI3KC2 γ is crucial for glucose metabolism and mainly functions in liver and pancreas. (Gulluni et al., 2019)

Class III PI3K catalytic subunit is Vps34 and coupled with the regulatory subunit Vps15. It is responsible for generation of PtdIns(3)P species. Vps34 is crucial for autophagosome formation, endocytosis and protein sorting. (Jean and Kiger, 2014)

1.3. Phosphatase and Tensin Homolog (PTEN)

Phosphatase and Tensin Homolog (PTEN) found on human chromosome 10q23 was discovered in 1917 by three independent research groups as a tumor suppressor and protein phosphatase. (Li et al., 1997; Steck et al., 1997; Li and Sun, 1997) Further research on PTEN identified that it functions as a haploinsufficient tumor suppressor (Cristofano et al., 1999) and the reduction of PTEN levels more than 50% accelerates tumor progression in multiple models (Alimonti et al., 2010). PTEN has multiple tumor suppressor functions each of which requires attention.

1.3.1. PTEN Protein Structure and Function

PTEN was initially identified as a protein tyrosine phosphatase due to its high sequence homology with PTP family proteins. (Li et al., 1997; Steck et al., 1997; Li and Sun, 1997) Nevertheless, PTEN has two more important functions which are lipid phosphatase function and phosphatase-independent (scaffolding) function. (Lee et al., 2018) The crystal structure of PTEN showed that PTEN has five distinct domains. (**Figure 1.3**)

PTEN consists of 403 amino acids and five distinct functional domains from N-terminus to C-terminus: a short N-terminal PtdIns(4,5) P₂ (PIP₂)-binding domain (PBD), a catalytic phosphatase domain, a C2 membrane binding domain and a C-terminal tail containing PEST (Pro, Glu, Ser, Thr) sequences and a class I PDZ domain-binding motif. (Lee et al., 1999)

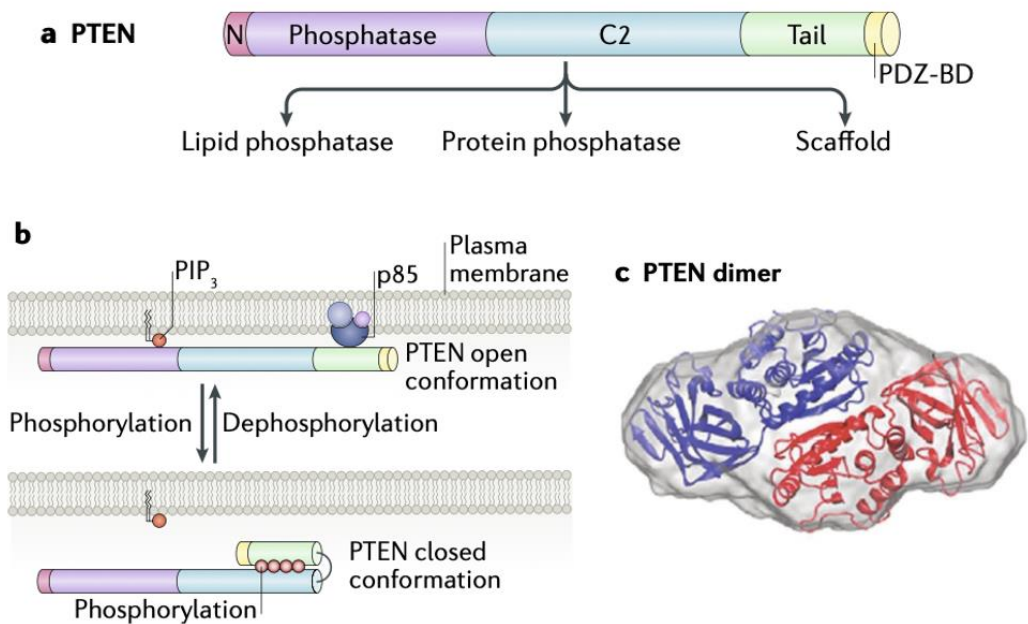


Figure 1. 3. The animated representation of functional PTEN structure and activatory mechanism taken from Lee et al., 2018. A) The functional PTEN domains depicted from N-terminal to C-terminal end. **B)** The regulatory mechanism of open-closed confirmation transition of PTEN. **C)** The crystal structure of PTEN homodimer which is crucial for its full functionality.

PBD acts as a platform to mediate PTEN's membrane localization, which is critical for its phosphatase function. (Walker et al., 2004) The phosphatase domain is responsible for both lipid and protein phosphatase functions. PTEN is a dual protein phosphatase which dephosphorylates phospho-serine, -threonine and -tyrosine residues. (Myers et al., 1997) The well-characterized protein substrates of PTEN are FAK, SRC, CREB1 and IRS1 and PTEN's dephosphorylation of these substrates yield tumor suppressive outcomes. (Gu et al., 2011; Shi et al., 2014; Tamura et al., 1998; Zhang et al., 2018) The most studied PTEN catalytic activity is its lipid phosphatase function through which it counteracts class I PI3K and converts PIP₃ back to PIP₂. (Maheama and Dixon, 1998) Lipid phosphatase function of PTEN is indispensable to tune down PI3K activity and keep it at a physiologically balanced level. C2 domain of PTEN serves as a binding site to phospholipid membranes through which interaction PTEN inhibits cell migration. (Lee et al., 1999; Raftopoulou et al., 2004) C-tail domain receives post translational modifications which govern PTEN's stability,

cellular localization and activity. (Mingo et al., 2019) Lastly, PTEN has PDZ-BD domain which acts as a protein-protein interaction site for PDZ-domain containing proteins. (Yan et al., 2018)

The lipid phosphatase activity of PTEN is regulated by its conformational changes. When the C-tail is phosphorylated, PTEN assumes a closed conformation in which C-tail interacts with C2 domain. As a result, PTEN becomes more stable but its interaction with membrane lipid is lost. (Rahdar et al., 2009; Odriezola et al., 2007) (**Figure 1.3 – panel B**) The open conformation alone, nevertheless, is insufficient to achieve PTEN's full lipid phosphatase capacity. The homodimer formation of PTEN is crucial for its efficient lipid phosphatase activity. (Papa et al., 2014) (**Figure 1.3- panel C**)

1.3.2. PTEN's Role in Cellular Metabolism

PTEN affects cell metabolism mainly due to its counteracting function against PI3K pathway, which is a core pathway for growth factor and metabolism coordination. PI3K pathway plays a crucial role in glucose hemostasis as a response to insulin secretion. The binding of IGF to IGFR leads to the activation of an adaptor protein called IRS1 which recruits and activates PI3K. (Metz and Houghton, 2011) Activated AKT, in return, phosphorylates and inactivates a protein substrate AS160 which is a GTPase-activating protein (GAP) for Rab and the function of Rab is to facilitate GLUT4 vesicle transport to cell membrane. As a result of AKT activity, AS160 activity drops and thus, Rab remains in its GTP-bound active state which leads to increase in GLUT4 representation on cell membrane. Additionally, AKT phosphorylates and inactivates GSK3 which inactivates glycogen synthase enzyme. The overactivation of PI3K pathway, therefore, increases glucose uptake and elevates cellular glycogen stores. (Chen et al., 2018)

PTEN loss-mediated aberrant PI3K signaling has impact also on lipid metabolism. The master transcriptional regulator of *de novo lipogenesis* SREBP activation is controlled by PI3K/AKT/mTORC1 axis. AKT inhibits FOXO transcription factors, which represses the transcription of SREBP genes. AKT, thus, indirectly activates the SREBP transcription. Both AKT and mTORC1 boost the cleavage of SREBP proteins with separate mechanisms, which is necessary for SREBP nuclear import and function. (Yilmaz and Cizmecioglu, 2024) Our lab previously showed that cholesteryl ester accumulation in ADT-resistant PTEN null prostate cancer is regulated by PTEN status and used as a mechanism to acquire resistance against anti-hormone therapies. (Kaysudu et al., 2023)

1.4. Sphingolipid Metabolism

Sphingolipids are the class of membrane lipids which have a sphingosine backbone and serve as bioactive signaling molecules. Ceramide is the core species in their metabolic pathway that can be converted into each sphingolipid species. A ceramide molecule consists of an 18-carbon sphingoid base amide-linked to a fatty acyl group with different carbon length possibilities. The carbon number of the second acyl group defines the biological characteristics of the ceramide moiety and distinguishes it from other ceramide species. (Ogretmen, 2017)

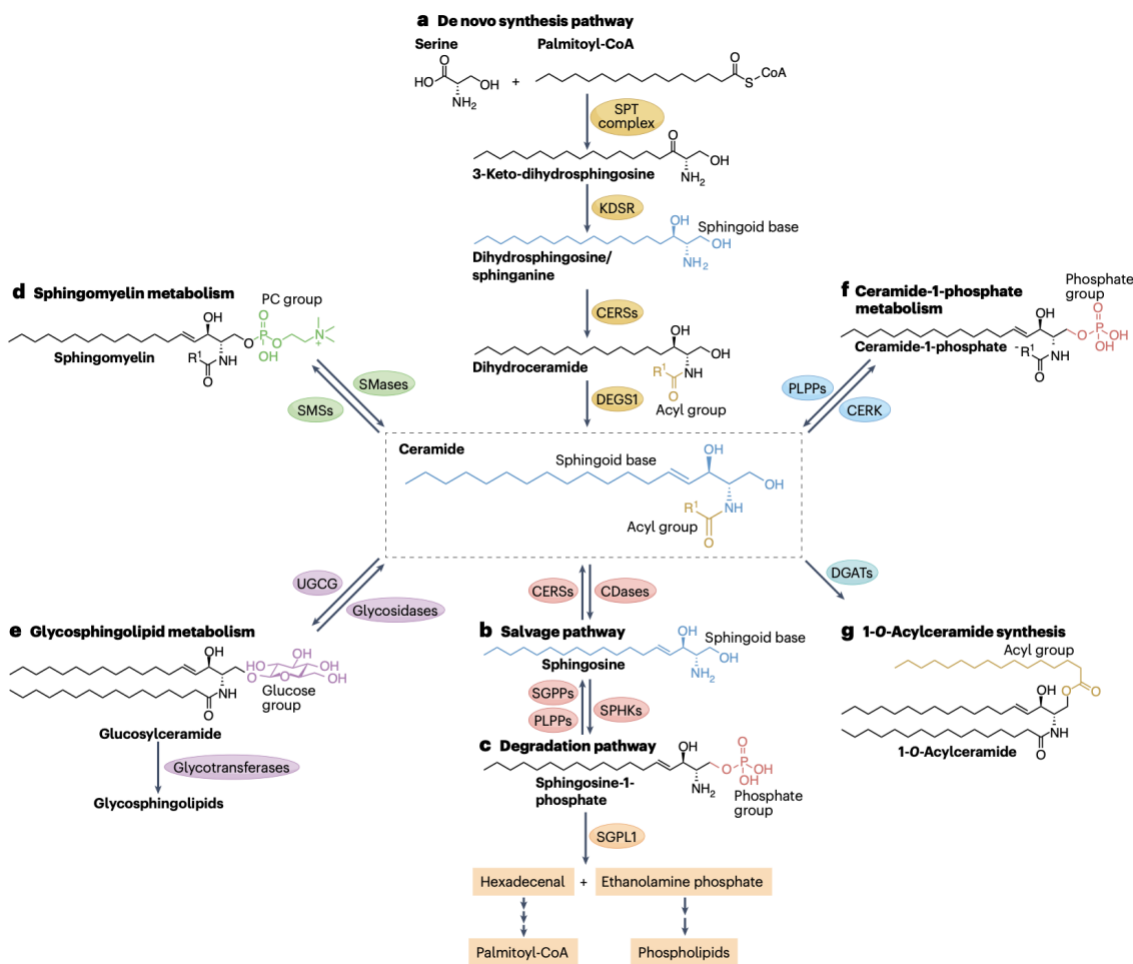


Figure 1. 4. The metabolic pathways of sphingolipid metabolism depicted in a schematic representation taken from Kuo and Hla, 2024. The ceramides, the core species in sphingolipid metabolism that can be converted into complex sphingolipids, are mainly generated either by **A)** de novo synthesis pathway or by **B, C)** the hydrolysis of complex sphingolipids called salvage pathway. The produced ceramides can be further used to form sphingosine-1-phosphates, **F)** ceramide-1-phosphates, **D)** sphingomyelins, **E)** glycosphingolipids and **G)** 1-O-Acylceramides.

De novo ceramide synthesis pathway starts with the synthesis of 3-keto-dihydrosphingone from a serine and a palmitoyl-CoA by serine palmitoyl-transferase (SPT) enzyme. Then, 3-ketodihydrosphingosine is converted into dihydrosphingosine, also called sphinganine, by 3-ketodihydrosphingosine reductase (KDSR). The next step is catalyzed by ceramide synthases (CERS) which link the second fatty acyl chain and determine the acyl chain length and type of ceramide. Lastly, dihydroceramide sphingolipid Δ^4 -desaturase DES1 converts dihydroceramide to ceramide (DEGS1). (**Figure 1.4 – panel A**) (Kuo and Hla, 2024)

The second pathway of ceramide synthesis is called salvage pathway. In salvage pathway, the hydrolysis of ceramides by ceramidases (CDases) gives rise to sphingosines which can be recycled back to ceramides by ceramide synthases. The enrichment of ceramides by recycling is called salvage pathway. (**Figure 1.4 – panel B**) The sphingosines generated through ceramide hydrolysis, can be also converted to sphingosine-1-phosphates (S1P) by sphingosine kinases (SPHKs) to form sphingosine-1-phosphates. S1P can be dephosphorylated back to sphingosine by S1P phosphatases (SGPPs) and phospholipid phosphatases (PLPPs). The irreversible turnover of S1P is catalyzed by S1P lyase 1 enzyme and the formed products are hexadecenal and ethanolamine phosphate. (**Figure 1.4 – panel C**) (Kuo and Hla, 2024)

Ceramide molecules can give rise to four different metabolites as well. The ceramides can be phosphorylated to form ceramide-1-phosphate by ceramide kinase (CERK) enzyme. (**Figure 1.4 – panel F**) Ceramide also forms the backbone of complex sphingolipids such as sphingomyelins formed by sphingomyelin synthases and glycosphingolipids produced by UDP-glucose ceramide glucosyltransferase (UGCG). (**Figure 1.4 – panel D and E**) Nevertheless, 1-O-acylceramides join lipid droplets for energy storage and they are produced from ceramides by the activity of diacylglycerol O-acyltransferases (DGATs) enzyme. (**Figure 1.4 – panel G**) (Kuo and Hla, 2024)

Sphingolipids, furthermore, have been identified as bioactive signaling molecules. The most well-characterized classes are ceramides and S1Ps associated with tumor suppressive and promoting outcomes, respectively.

1.4.1. Sphingosine-1-Phosphate Signaling

Sphingosine-1-phosphate (S1P) functions as a paracrine and/or an autocrine ligand by activating cognate G protein-coupled receptors which are known as S1P receptors (S1PR1–

S1PR5). (Blaho and Hla, 2014). After being produced, S1Ps are transported to extracellular space by the transporters called the protein spinster homolog 2 (SPNS2) and major facilitator superfamily domain containing 2B (MFSD2B). (Hisano et al., 2012; Vu et al., 2017) Secreted S1P moieties need to be attached to a carrier protein due to their amphipathic structure. The primary carrier proteins mediating S1P transport is albumin and HDL-associated apolipoprotein (apoM). (Yilmaz and Cizmecioglu, 2024)

The signaling mediated by S1P has been characterized to have a tumor promoting outcome. Upon activation, S1P receptors modulate the activity of its downstream effectors such as JAK, AKT and Rho. (Yilmaz and Cizmecioglu, 2024) For example, S1PR1 signaling has been reported to potentiate VEGF-VEGFR signaling to boost angiogenesis. (Balaji Ragunathrao et al., 2019) Moreover, S1PRs except for S1PR4 are reported to regulate PI3K pathway activity. S1PR1/3 signaling, as an example, is known to enable apoptosis evasion by aberrantly activating PI3K. (Wang et al. 2018)

1.4.2. Bioactive Ceramide Signaling

Ceramide levels have been reported to increase as a response to stress inducers such as chemotherapy, radiotherapy and ER stress. (Fekry et al., 2016; Yura et al., 2020) Moreover, the enrichment of ceramides on organelle membranes is associated with numerous tumor suppressive outcomes. For example, accumulated ceramides generate pores on mitochondrial membrane thereby increasing the mitochondrial outer-membrane permeabilization (MOMP), which is the primary driver of cytochrome C release and initiation of intrinsic apoptotic pathway (Colombini, 2013; Chang et al., 2015).

The ceramides are also involved in starvation responses which enables cells to thrive under harsh environments. It has been suggested that ceramide accumulation on endosomal membrane enhances the recruitment of autophagy-related protein 9A (ATG9A) thereby potentiating the autophagosome elongation and maturation (Corcelle-Termeau et al., 2016).

1.5. Aim of the Project

It has been discussed in the introduction part that PTEN loss-of-function is a very common and early event in metastatic prostate cancers. For this reason, the impact of PTEN loss on cell metabolism and signaling has been studied extensively. Our lab focuses on identifying the metabolic vulnerabilities resulting from PTEN loss and developing novel co-targeting strategies which would act only on PTEN-deficient cells. We, therefore, aimed to identify metabolic reprogramming and PI3K signaling alterations in PTEN-null prostate cancer cells.

Even though PTEN-deficient prostate cancers are characterized by a reliance on p110 β catalytic isoform for growth and survival, these cells harbor various mutations that may collectively influence PI3K isoform dependency, which makes it difficult to directly associate PTEN loss with p110 β dependency. In this regard, we aimed to reassess the effect of PTEN status on PI3K class IA isoform dependency by using non-transformed prostate epithelial cells. To do so, we knocked out PTEN gene locus through CRISPR-Cas9 strategy in this cell line and generated a corresponding control cell line with a non-targeting RNA. We analyzed the isoform dependency of this cells by using western blot and cell viability approaches.

We employed a targeted metabolomics approach to spot altered metabolites in PTEN-null PCa cells when compared to their PTEN-WT re-expressing counterparts. Since our lab identified sphingolipid metabolism as the most altered metabolic pathway upon PTEN loss, our screening focused only on sphingolipids. As a result, we identified that both ceramide and sphingosine-1-phosphate metabolism seem to be regulated by PTEN status.

We combined p110 isoform specific inhibitors alongside S1P metabolism and tested the efficacy of our strategy on PTEN-null prostate cancer cells. Our combinations yielded significant synergy indicating that co-targeting PI3K and S1P metabolism confirms a promising strategy to treat PTEN-null metastatic prostate cancers.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Cell Lines and Maintenance Media Formulations

Cell Line Name	Full Medium
LNCaP Parental	10% FBS + 1% P/S in RPMI w/stable L-glutamine
C4-2 Parental	10% FBS + 1% P/S in RPMI w/stable L-glutamine
PC3 Parental	10% FBS + 1% P/S in RPMI w/stable L-glutamine
LNCaP-rtTA-pRXTN (Mock and PTEN-WT)	10% Tet-Free FBS + 1% P/S + 50 µg/ml G418 + 50 µg/ml NAT in RPMI w/stable L-glutamine
C4-2-rtTA-pRXTN (Mock and PTEN-WT)	10% Tet-Free FBS + 1% P/S + 50 µg/ml G418 + 50 µg/ml NAT in RPMI w/stable L-glutamine
PC3-rtTA-pRXTN (Mock and PTEN-WT)	10% Tet-Free FBS + 1% P/S + 50 µg/ml G418 + 60 µg/ml NAT in RPMI w/stable L-glutamine
PNT1A Parental	10% FBS + 1% P/S in RPMI + 2mM L-Glu in w/stable L-glutamine
PNT1A pLCV2-puro-sgSCRAMBLED	10% FBS + 1% P/S in RPMI + 2mM L-Glu in w/stable L-glutamine
PNT1A pLCV2-puro-sgPTEN4	10% FBS + 1% P/S in RPMI + 2mM L-Glu in w/stable L-glutamine
DU145 Parental	10% FBS + 1% P/S in RPMI w/stable L-glutamine
HEK293	10% FBS + 1% P/S in High Glucose DMEM

2.1.2. Cell Culture Reagents

Product Name	Brand	Catalog Number
RPMI1640 w/stable L-glutamine	Capricorn	RPMI-A
High Glucose DMEM	Gibco	11965092
Fetal Bovine Serum (FBS) - Advanced	Capricorn	FBS-11A
Tet- Free Fetal Bovine Serum (tFBS)	Capricorn	FBS-TET-12A
Penicillin-Streptomycin (P/S)	Gibco	15140122
Nourseothricin (NAT)	Thermo Scientific Chemicals	H37154.MC
Geneticin (G418 Sulfate)	Gibco	10131035
Puromycin	InvivoGen	ant-pr-1
Doxycycline hydrochloride	Thermo Scientific Chemicals	J60422.06
BYL719	MedChemExpress	HY-15244
GSK2636771	Cayman Chemical	17380
KIN193 (AZD6482)	MedChemExpress	HY-10344
CAL101	MedChemExpress	HY-13026
GDC0941	MedChemExpress	HY-50094
Fingolimod (hydrochloride)	Cayman Chemical	10006292
ACT128800	MedChemExpress	HY-10569
PF-543 (hydrochloride)	Cayman Chemical	17034
ABC294640	MedChemExpress	HY-16015
Trypsin-EDTA (0.25%)	Gibco	25200056
Trypsin-EDTA (0.05%)	Gibco	25300054
PBS, pH 7.4	Gibco	10010023
Lipofectamine™ 3000 Transfection Reagent	Invitrogen	L3000015
Dimethyl sulfoxide	Serva	20385.02
Hexadimethrine bromide	MedChemExpress	HY-112735

2.1.3. Reagents and Materials for Cell Culture Experiments

Reagent	Brand	Catalog Number
Crystal Violet	Thermo Scientific Chemicals	022866.14
Paraformaldehyde	Thermo Scientific Chemicals	416780010
Ethanol	Isolab	LB.IS.920.026.2500
Sodium dodecyl sulfate	Sigma	436143
Sulforhodamine B	Sigma	230162
Trichloroacetic Acid	Sigma	100807
Acetic Acid	Isolab	901.013
Trizma Base	Sigma	T1503
Mr. Frosty™ Freezing Container	Thermo Scientific™	5100-0001
Amicon® Ultra-15 Centrifugal Filter Unit	Merck	UFC910024

2.1.4. Solutions for Cell Culture Experiments

Solution	Ingredients
SRB Assay Cell Fixation Solution – 50% Trichloroacetic acid (Diluted to 10% before use)	50 g TCA in 100 ml ddH ₂ O
1% Acetic Acid	10 ml 99.9% Acetic Acid in 1 L ddH ₂ O
0.4% SRB Staining Solution	400 mg SRB in 100 ml 1% Acetic Acid
SRB Destaining Solution (Non-buffered 10 mM Tris-Base)	1.21 g Trizma-Base in 1 L ddH ₂ O
Crystal Violet Cell Fixation Solution – 4% Paraformaldehyde	4 g paraformaldehyde in 100 ml PBS
0.4% Crystal Violet Staining Solution	400 mg crystal violet 20 ml (20%) Ethanol (99.99%)

	in 80 ml ddH ₂ O
Crystal Violet Destaining Solution	50 ml (10%) Acetic Acid 2.5 g (0.5%) SDS in 500 ml ddH ₂ O

2.1.5. Reagents and Kits for sgRNA Cloning into the pLCV2 Backbone

Product Name	Brand	Catalog Number
T4 Polynucleotide Kinase	Neb	M0201S
Adenosine 5'-Triphosphate (ATP)	Neb	P0756S
BsmBI-v2	Neb	R0739S
GeneJET Gel Extraction Kit	Thermo Scientific™	K0691
T4 DNA Ligase	Neb	M0202T
Taq DNA Polymerase with Standard Taq Buffer	Neb	M0273L
lentiCRISPR v2	Addgene	#52961
pCMV-dR8.2	Addgene	#8455
pCMV-VSV-G	Addgene	#8454
Plasmid Midi Kit	Qiagen	12143
Plasmid Mini Kit	Qiagen	12123
NEB® Stable Competent E. coli (High Efficiency)	Neb	C3040I
LB Medium (Lennox), powder	Serva	48501.01

Ampicillin trihydrate	Serva	13397.03
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2.1.6. Sequences of sgRNAs

Name	Forward (5' to 3')	Reverse (5' to 3')
Scrambled	caccCACCGACGGAGGCTAAGCGTC GCAA	aaacTTGCGACGCTTAGCCTCCGTC GGTG
sgPTEN ₁	caccgAAACAAAAGGAGATATCAAG	aaacCTTGATATCTCCTTTTGTTC
sgPTEN ₂	caccgAAAGACTTGAAGGCGTATAC	aaacGTATACGCCTTCAAGTCTTC
sgPTEN ₃	caccgACTTTGATATCACACACAC	aaacGTGTGTGGTGATATCAAAGTC
sgPTEN ₄	caccGATGTGCATATTATTACAT	aaacATGTAATAAATATGCACATC

2.1.7. Solutions for Cloning Experiments

Solution	Ingredients
50X TAE	242 g Trizma-base 57.1 mL acetic acid 100 mL 0.5M EDTA

2.1.8. Reagents, Kits and Antibodies for Western Blot Analysis

Product Name	Brand	Catalog Number
Trizma Base	Sigma	T1503
Glycine	Sigma	G7126
Sodium dodecyl sulfate	Sigma	436143
RIPA Buffer	Thermo Scientific™	89900
Protease Inhibitor Cocktail	Sigma	P8340

Phosphatase-Inhibitor-Mix II, solution	Serva	39055.02
Pierce™ DTT (Dithiothreitol), No-Weigh™ Format	Thermo Scientific™	A39255
Sodium Orthovanadate	Neb	P0758S
4x Laemmli Sample Buffer	BioRad	1610747
2-Mercaptoethanol	Serva	28625.01
Acrylamide/ Bisacrylamide (30% w/v)	Serva	10688.01
Ammonium Persulfate	Thermo Scientific™	17874
N,N,N',N'-Tetramethyl-ethylenediamine	Serva	35930.02
Precision Plus Protein Dual Color Standards	BioRad	1610374
Pierce™ BCA Protein Assay Kits	Thermo Scientific™	A55864
Bovine Serum Albumin (BSA)	Capricorn	BSA-1U
Pierce™ ECL Western Blotting Substrate	Thermo Scientific™	32106
Nitrocellulose Blotting Membrane	Merck	GE10600003
ClearBand Ponceau S	EcoTech	PS05
Tween 20	Serva	37470.01
Skim Milk Powder for Biotechnology and Microbiology	Merck	70166
Sodium azide	Serva	30175.01
GAPDH Antibody	Santa Cruz	sc-47724
PTEN (138G6) Rabbit mAb	Cell Signaling Technology	9559S
Phospho-Akt1 (Ser473) Rabbit mAb	Cell Signaling Technology	9271S

Phospho-Akt (Thr308) Rabbit mAb	Cell Signaling Technology	9275S
AKT Antibody	Cell Signaling Technology	9272S
PI3 Kinase p110 α Antibody	Cell Signaling Technology	4255S
PI 3-kinase p110 β Antibody	Santa Cruz	sc-7175
anti-mTOR Phospho (Ser2448) Antibody	BioLegend	610302
Phospho-p70 S6 Kinase (Thr389) Antibody	Cell Signaling Technology	9205S
Phospho-S6 Ribosomal Protein (Ser235/236) Antibody	Cell Signaling Technology	2211S
4E-BP1 Antibody	Cell Signaling Technology	9452S
Phospho-PRAS40 (Thr246) (C77D7) Rabbit mAb	Cell Signaling Technology	2997S
SPHK1 (D1H1L) Rabbit mAb	Cell Signaling Technology	12071S
Goat anti-rabbit IgG (H+L), HRP conjugate	Advansta	R-05072-500
Goat anti-mouse IgG (H+L), HRP conjugate	Advansta	R-05071-500

2.1.9. Buffers and Solutions for Western Blotting

Solution	Ingredients
10X TBS	80 g NaCl 2 g KCl 30 g Trizma-Base pH adjusted to 7.4 with HCl Complete with ddH ₂ O to 1L
1X TBS-T	1 mL Tween-20 100 ml 10X TBS

	Complete with ddH ₂ O to 1L
Cell Lysis Buffer	1X RIPA 1X Protease inhibitor 1X Phosphatase inhibitor mix 1 μ M DTT 1 mM Na ₃ VO ₄
Stacking Gel Buffer (pH=6.8)	60.5 g Trizma-Base pH adjusted to 6.8 with HCl Complete with ddH ₂ O to 1L
Resolving Gel Buffer (pH=8.8)	187 g Trizma-Base pH adjusted to 8.8 with HCl Complete with ddH ₂ O to 1L
10X Running Buffer	30 g Trizma-Base 144 g Glycine 10 g SDS in 1 L ddH ₂ O
10X Transfer Buffer	30.3 g Trizma-Base 144.1 Glycine in 1 L ddH ₂ O
5% Milk Blocking Solution	5 mg milk powder 100 ml 1X TBS-T
Primary Antibody Solution	3% BSA + 0.04 μ g/ml NaN ₃ + 1:1000 primary Ab
Secondary Antibody Solution	3% Milk powder + 1:10000 secondary Ab

2.1.10. Reagents and Kits Used for RNA Isolation, cDNA Synthesis and RT-qPCR

Product Name	Brand	Catalog Number
Aurum™ Total RNA Mini Kit	BioRad	7326820

iScript™ cDNA Synthesis Kit	BioRad	1708891
SsoAdvanced Universal SYBR Green Supermix	BioRad	1725275
qPCR Plates	Nest	402601
Optical qPCR Seals	Nest	410011
Applied Biosystems 7500 Real-Time PCR Machine	Applied Biosystems	4351105

2.1.11. Sequences of qPCR Primers

Gene Name	Forward (5' to 3')	Reverse (5' to 3')
CERK	CACCTTAGCCTCCATCACCCTG	AACATACCATCTCCGCCGACAC
SPHK1	TATGAATGCCCTACTTGGTATTG	GCCTCGCTAACCATCAATTCC
SPHK2	ATGGCATCGTCACGGTCTC	CTCCCAGTCAGGGCGATCTA
SGPL1	TCGAGGGAAGGAGACTGGAA	AGAAGGTCTGTGCTAGGCATC
S1PR1	CCACCTACAAGCTCACTCCC	ATGGCGAGGAGACTGAACAC
S1PR2	ACCAAGGAGACGCTGGAAAC	CAGGTACATTGCCGAGTGGA
S1PR3	TCTGGCATTTCGAGCGCA	GCTTCATTAGCAAGGCGTG
S1PR4	CTCTCTGAGGCCAAGGGACA	ACCATCCACACGCAAGACTG
S1PR5	ACGCTTGCTCCACTGTCTTG	CACAGATAGCGGCCAGGATG
GAPDH	GCCCAATACGACCAAATCC	AGCCACATCGCTCAGACAC
RPL19	CGGAAGGGCAGGCACAT	GGCGCAAATCCTCATTCTC

2.2. Methods

2.2.1. Cell Culturing, Freezing and Thawing

The cells were subcultured into 10-cm dish when they reached 80-90% of confluency. In the passaging protocol, cells were initially washed with 1X sterile PBS and incubated with 1 ml

trypsin at 37°C and 5% CO₂ incubator for 5 minutes. Of note, LNCaP, C4-2 and HEK293 cells were trypsinized with 0.05% trypsin while 0.25% trypsin was used for PC3, DU145 and PNT1A cells. During trypsinization, new dishes with fresh media (recipes at material part) were prepared. The detached cells were collected in fresh medium and transferred to the new dish by the dilution ratio. The dilution ratios were 1:6 for LNCaP cells, C4-2 cells; 1:5 for PNT1A cells and 1:8 for PC3, HEK293 and DU145 cells. The cells were returned to the incubator adjusted at 37°C and 5% CO₂.

For freezing protocol, after trypsinization, cells were collected in a centrifuge tube and centrifuged at 180 x g for 5 minutes. Then, cells were resuspended in freezing medium formulated as 10% DMSO + 50% FBS + 40% RPMI (DMEM for HEK293). The resuspended cells were transferred to cryovials and placed into Mr. Frosty. Mr. Frosty was stored at -80°C for at least five hours. The vials were transferred to liquid nitrogen for longer storage.

For thawing protocol, the thawing medium formulated as 10% FBS in RPMI (DMEM for HEK293) was prepared in advance and heated to 37°C. The cryovials were immersed in a 37°C water bath and incubated until fully thawed. Then, the defrosted cells were transferred to centrifuge tubes and pre-heated thawing media was added on top drop-by-drop. The cells were centrifuged at 180 x g for 5 minutes. The DMSO containing medium was aspirated and the cells were resuspended in thawing medium. The resuspended cells were plated to a new dish and incubated at 37°C and 5% CO₂ incubator.

2.2.2. Generation of Cell Lines

Lentiviral delivery was used to generate PNT1A cells containing CRISPR constructs. For this reason, the experimental procedure was conducted in the isolated lentiviral cell culture. To generate lentiviral particles encapsulating the construct of interest, HEK293 cells were seeded at 5×10^5 density to 6-well plate. The next day, the HEK293 cells were co-transfected with 1.25 µg/ml pLCV2-puro CRISPR vector, 0.625 µg/ml VSV-G and 0.625 µg/ml D8.9 packaging plasmids accordingly to the lipofectamine 3000 manufacturer's protocol. The next day, HEK293 media was replenished. The media were collected for the following two days and stored in dark at 4°C. The PNT1A cells at the second day were seeded to 6-well plates at 0.6×10^5 density. The collected media were concentrated using an amicon ultra centrifugal unit. The tubes were centrifuged at max speed for 10 minutes at 4°C. The concentrated viral media then, administered

to PNT1A cells with 10 µg/ml polybrene. The PNT1A cells were incubated at dark for 48 hours at 37°C and 5% CO₂ and then, were selected using 0.4-0.45 µg/ml puromycin.

2.2.3. sgRNA Cloning into pLCV2 Backbone

Prior to the cloning protocol, the ordered sgRNA pairs were annealed and phosphorylated on 5' end. To this end, T4 polynucleotide kinase by Neb was used. The arrived oligos were reconstituted in 1X TE buffer at the 100 µM concentration.

Table 2. 1. Components of the oligo annealing reaction for pLCV2 cloning.

Reaction Component	Amount
Forward Oligo (100µM)	1 µL
Reverse Oligo (100µM)	1 µL
10X PNK Buffer (or T4 DNA Ligase Buffer)	1 µL
10 mM ATP	1 µL
PNK	0.5 µL
Nuclease-free water	5.5 µL
TOTAL	10 µL

The reaction was incubated at 37°C for 60 minutes followed by a heat inactivation at 95°C for 5 minutes. The oligos were then annealed by gradually cooling the temperature to 25°C at a rate of 5°C per minute. pLCV2-puro plasmid (2.5 µg) was digested with BsmBI-v2 enzyme in r3.1 buffer at 55°C for 150 minutes followed by a heat inactivation at 80°C for 20 minutes. The product then was loaded to an 0.8% TAE agarose gel and the band at 13kB was extracted and purified according to the manufacturer's protocol of Thermo GeneJet Extraction kit.

The gel extraction product was used in the following ligation reaction.

Table 2. 2. Ligation reaction setup for pLCV2 cloning.

Ligation Reaction	Amount
50 ng pLCV2 Gel Extraction Product	Variable
1:200 Diluted Annealed Oligo	1 µL

10X T4 DNA Ligase Buffer	1 μ L
10 mM ATP*	1 μ L
DNA Ligase	0.5 μ L
Nuclease-free water	Complete up to 10 μ L
TOTAL	10 μ L

The reaction described in **Table 2.2** was incubated at 16°C overnight.

*: ATP addition is not compulsory since T4 DNA Ligase has 1mM ATP content, but known to increase the efficiency of ligation.

Then, the ligation product along with ligation negative control – DEPC water was used instead of oligo- were transformed to NebStable E.Coli strain. The colonies formed, next day, were further expanded according to Qiagen Mini Prep protocol and were pelleted to be stored at -20°C.

A portion of the pellet were taken by using a 10 μ L pipette tip and suspended in 10 μ L DEPC water to be used in the following colony PCR reaction. As for the negative control, the colonies derived from the negative ligation control were used and no DNA amplification was detected.

Table 2. 3. The reaction conditions for colony PCR

PCR Reaction	Amount
10X Standard Taq Buffer	2.5 μ L
10 mM dNTP	0.5 μ L
10 μ M forward primer*	0.5 μ L
10 μ M reverse primer	0.5 μ L
Template DNA	5 μ L
Taq Polymerase	0.125 μ L
Nuclease-free water	Complete up to 25 μ L
TOTAL	25 μ L

Table 2. 4. The thermal cycling conditions for colony PCR

	In Thermocycler	Cycle
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Initial Denaturation	95°C for 8 minutes	1
Denaturation	95 °C for 30 seconds	X 30 cycle
Annealing	<i>Annealing temp**</i> for 60 seconds	
Elongation	68 °C for 1 minutes	
Final Extension	68 °C for 5 minutes	1
Hold	4°C for hold	1

*: Forward primer is the forward primer of sgRNA. In this way, we also verify if the correct oligo is cloned. Reverse primer is available in lab.

** : For the annealing temperature, please use NEB's Tm Calculator.

Sequence of reverse primer: GTCCACGATGTTGCCGAAGA

After PCR reaction, PCR product was run on 1% TAE agarose and the band was expected at 770 bp. After the verification of cloning, the plasmids were further amplified using Qiagen Midi Kit Protocol.

2.2.4. Western Blotting

2.2.4.1. Cell Lysis and Sample Prep

Western blot protocol was initiated with the extraction of cellular proteins. To this end, the lysis buffer was prepared as indicated in materials part. Then, the cells were placed on ice, the media were aspirated. The cells were washed once with ice cold 1X PBS to get rid of remnant proteins from FBS and detached/ dead cells. Subsequent to PBS aspiration, 60 µL of the lysis buffer was directly added to a well of 6-well plate. The cells were scraped in lysis buffer and collected in sterile Eppendorf tubes. The tubes were incubated on ice for 15 minutes. At the end of incubation, the lysis reactions were vortexed vigorously. Then, they were centrifuged at 13.000 x g and 4°C. The supernatant containing proteins were transferred to a fresh Eppendorf tube.

The protein concentration measurement was conducted accordingly to the manufacturer's protocol for Pierce™ BCA Protein Assay Kit. Based on the quantification results, western blot samples were prepared to achieve 1 µg/µl final concentration in 4X Laemmli buffer containing β-

mercaptoethanol and Laemmli at a 1:9 ratio. The prepared samples were heat-inactivated at 95°C for 5 minutes.

2.2.4.2. Western Blot Gel Casting

According to the size of the analyzed proteins resolving gel was casted as 10% or 15%, while the stacking gel was prepared as 4%. Firstly, resolving gel was casted and 200-300 μ L isopropanol was placed on top to ensure the formation of a straight line. The gel was incubated for 40 minutes. Then, the isopropanol was discarded. Subsequently, the stacking gel was casted and the comb was placed. After 20 minutes, the comb was removed and the gel was placed in western blot running buffer.

Table 2. 5. The recipe for western blot gels

	Resolving Gel		Stacking Gel
	10%	15%	4%
Acrylamide 30% (w/v)	3.3 ml	5 ml	0.55 ml
Stacking Buffer (pH = 6.8)	-	-	1.05 ml
Resolving Buffer (pH =8.8)	2.5 ml	2.5 ml	-
ddH ₂ O	4.2 ml	2.5 ml	2.5 ml
10% APS	50 μ L	50 μ L	20.8 μ L
TEMED	5 μ L	5 μ L	4.17 μ L

2.2.4.3. Western Blot Protocol

The heat-inactivated samples were loaded to wells at 15-20 μ g protein per well and were run in western blot running buffer at a constant voltage of 100 V for 90 minutes. Then, the gels were placed in wet transfer cassettes with nitrocellulose membrane. The transfer was conducted at 250 mA as constant for 150 minutes. Subsequently, the membranes were stained with Ponceau S solution to ensure the efficiency of the transfer. After the membranes were briefly rinsed with dH₂O, they were cut into sections to be incubated with different primary antibodies. The sections were blocked with 5% milk blocking solution -recipe indicated in the materials section- for 1 hour at room temperature. The membranes, then, were incubated with primary antibodies overnight at 4°C.

Following the primary antibody incubation, the membranes were washed three times with 1X TBS-T -recipe in the materials part- for five minutes at RT. Then, the membranes were incubated with secondary antibodies for 1 hour at RT. The washing step was repeated. After the membranes were placed in dH₂O, they were incubated with Pierce™ ECL Western Blotting Substrate according to the manufacturer's protocol. The chemiluminescent images were generated with Amersham Imager 600. The band quantification was conducted with ImageJ tool. The normalization of western blot bands had two steps. Firstly, each group was normalized to GAPDH control and secondly to the DMSO control.

2.2.5. RNA Isolation, cDNA Synthesis and qPCR

For RNA isolation, the cells were seeded at a density given in **Table 2.6** to 10-cm plates in maintenance medium. The following day the cells were washed with 1X PBS and the media were replenished to 4% tFBS + 1 µg/ml doxycycline in RPMI for LNCaP, C4-2 and PC3 mock/PTEN-WT lines and to 5% FBS + 2 mM L-Glu in RPMI for PNT1A cells. The cells were incubated for 72 hours. Then, the cells were taken on ice and washed once with 1X sterile ice-cold PBS and PBS was aspirated very carefully not to leave any remnants. Then, 350 µL lysis buffer of Aurum™ Total RNA Mini Kit, prepared by the manufacturer's protocol, was directly added to the cells. The cells were collected in the lysis buffer with sterile scrapers and vigorously homogenized with 18-gauge needle and syringe. The manufacturer's protocol was followed. The eluted RNA was quantified with NanoDrop™ One Microvolume UV-Vis Spectrophotometer (Thermo Scientific, Catalog no: 13-400-518). The purified RNAs were stored at -80°C.

Table 2. 6. Cell seeding densities for RNA isolation to 10-cm plates.

Cell Line	Cell counts to be seeded to 10-cm dish
LNCaP-rtTA-pRXTN-Mock	1.000.000
LNCaP-rtTA-pRXTN- PTEN-WT	1.200.000
C4-2-rtTA-pRXTN-Mock	900.000
C4-2-rtTA-pRXTN- PTEN-WT	1.500.000
PC3-rtTA-pRXTN-Mock	300.000
PC3-rtTA-pRXTN- PTEN-WT	500.000
PNT1A pLCV2-puro-sgSCRAMBLED	1.250.000

PNT1A pLCV2-puro-sgPTEN4	1.500.000
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The purified and quantified RNA was used for cDNA synthesis based on the instructions of iScript™ cDNA Synthesis Kit. The produced cDNA samples were stored at -20°C until use. Subsequently to cDNA conversion, the samples were proceeded with RT-qPCR reaction. The components and used concentration for qPCR reaction were indicated in **Table 2.7**. The master mix (primers and Sybr Green dye) was prepared for each target gene and distributed at 7 µL per well. The diluted cDNA samples by 1:5 ratio after synthesis, then, were distributed directly to wells. The plate was tightly sealed and spinned down. The qPCR was conducted in Applied Biosystems 7500 machine with the protocol indicated in **Table 2.8**.

Table 2. 7. The reaction components and concentrations for qPCR

Component	Amount/ Concentration
SsoAdvanced Universal Inhibitor-Tolerant SYBR Green Supermix (2X)	5 µL
Forward Primer	500 nM
Reverse Primer	500 nM
cDNA	30 ng
Nuclease-free water	Complete up to 10 µL
Total	10 µL

Table 2. 8. The protocol of two-step amplification for qPCR

	Temperature	Duration	Cycle
Preincubation	98°C	150 s	1
Two-step amplification	98°C	10 s	X 40 cycle
	60°C	30 s	
Melting	65°C	10 s	1
	95°C	300 s	

The relative mRNA levels were calculated by $2^{-\Delta\Delta CT}$ method to estimate fold changes upon PTEN re-expression relative to housekeeping genes (GAPDH or RPL19).

2.2.6. Cell Viability Assays

To assess cell viability and growth, crystal violet (CV) and sulforhodamine B (SRB) assays were used. Crystal violet assay was employed only for the characterization of PNT1A cell lines. As for the IC₅₀ determination experiments and dual combinations, SRB assay was utilized.

2.2.6.1. Crystal Violet

The PNT1A cells were seeded at the density of 10.000 cells per well of a 12-well plate. The cell medium was changed into 5% FBS+ 2mM L-Glu in RPMI on the following day. At the indicated time points, the cell medium was aspirated and cells were fixed with 4% PFA at RT for 15 minutes. The PFA was discarded and the cells were stained with 0.4% CV dye for 1 hour in dark. The cells were washed with dH₂O and air dried before scanning. After scanning, the cells were destained (the recipe indicated in the materials part) with the destaining solution on the shaker for 30 minutes with mild agitation in dark. After destaining 1:10 diluted destained solution from cells were transferred to 96-well and the OD was measured at 595 nm by spectrophotometer. For PNT1A cells, the growth of each day point was normalized to day 0.

2.2.6.2. Sulforhodamine B (SRB) Assay

For IC₅₀ determination and combination experiments, the cells were seeded at the given density to 96-well plate in 100 μ L of 8%tFBS RPMI media as indicated in **Table 2.9**.

Table 2. 9. Cell counts for SRB Experiments

Cell Line	Cell counts to be seeded to 96-well plate/well
LNCaP Parental	6000
LNCaP-rtTA-pRXN-Mock	5000
LNCaP-rtTA-pRXN- PTEN-WT	7500
C4-2 Parental	6000
C4-2-rtTA-pRXN-Mock	5000
C4-2-rtTA-pRXN- PTEN-WT	10.000

PC3 Parental	1300
PC3-rtTA-pRXTN-Mock	1200
PC3-rtTA-pRXTN- PTEN-WT	1500
PNT1A pLCV2-puro-sgSCRAMBLED	4000
PNT1A pLCV2-puro-sgPTEN4	3500

The following day, the doxycycline (for the Mock/WT cells) along with the inhibitors were prepared at 2X concentration delivered in 100 μ L RPMI to the cells. By doing so, the final concentration of FBS and also the inhibitors were reduced to 4%. The cells were incubated at 37°C and 5% CO₂ for four days. The cells, then, were fixed with 10%TCA overnight at 4°C or at room temperature for 1 hour. The cells were washed gently with dH₂O. The wells were stained with 50 μ L of 0.4% SRB dye in dark for 30 minutes (PC3) or for 60 minutes (other cell lines). Subsequently, the cells were washed with 1% acetic acid until no remnants of SRB dye left. The plates were airdried and scanned. Then, the wells were destained with 200 μ l destaining solution (the recipe indicated in the materials part) at a mild agitation for 30 minutes in dark. The OD measurement was conducted with spectrophotometer at 564 nm.

2.2.7. Metabolomics Sample Prep

On the initial day, the cells were seeded in maintenance medium. The next day, the medium was changed into 2%tFBS + 1 μ g/ml doxycycline in RPMI for Mock and PTEN-WT cells and into 5%FBS + 2mM L-Glu in RPMI for PNT1A cells. The cells were incubated for 72 hours at 37°C and CO₂. At the end of the incubation, the cells were taken on ice and washed twice with 1X Sterile ice-cold PBS. Then, the cells were collected in ice cold PBS. The cells were counted twice using the hemocytometer and the average count was taken into account. 5 X 10⁶ cells were pelleted and snap-frozen to be stored at -80°C for further processing.

2.2.8. Statistical Analysis

The statistical analysis was conducted with the help of GraphPad Software (Prism 10.4.1). The student's t-test was employed to compare the differences between two groups. On the other hand, the multiple t-test was applied when the dual comparisons of multiple pairs were required at the same time. One-way ANOVA was used for comparisons involving more than two groups

whereas two-way ANOVA was employed when two or more independent variables were evaluated for their effects on the outcome.

The co-efficient of drug interactions were calculated by the following formula:

$$CDI = AB / (A \times B)$$

Where A and B indicate the OD measurement value of drug A/ B-treated group while AB indicates the OD value of the combination. If $CDI < 1$, it indicates synergism while $CDI = 1$ means additive effect and $CDI > 1$ shows a potential antagonistic effect of the two drugs.



3. RESULTS

3.1. Generation of PTEN-knockout and control PNT1A cell lines using a CRISPR-Cas9 strategy

PNT1A is an immortalized and non-transformed prostate epithelial cell line. For this reason, it is prevalently used as a control group in comparison with prostate cancer cell lines. (Avancès et al., 2001) In our experimental setting, we used PNT1A cell line to model early PTEN loss during prostate carcinogenesis.

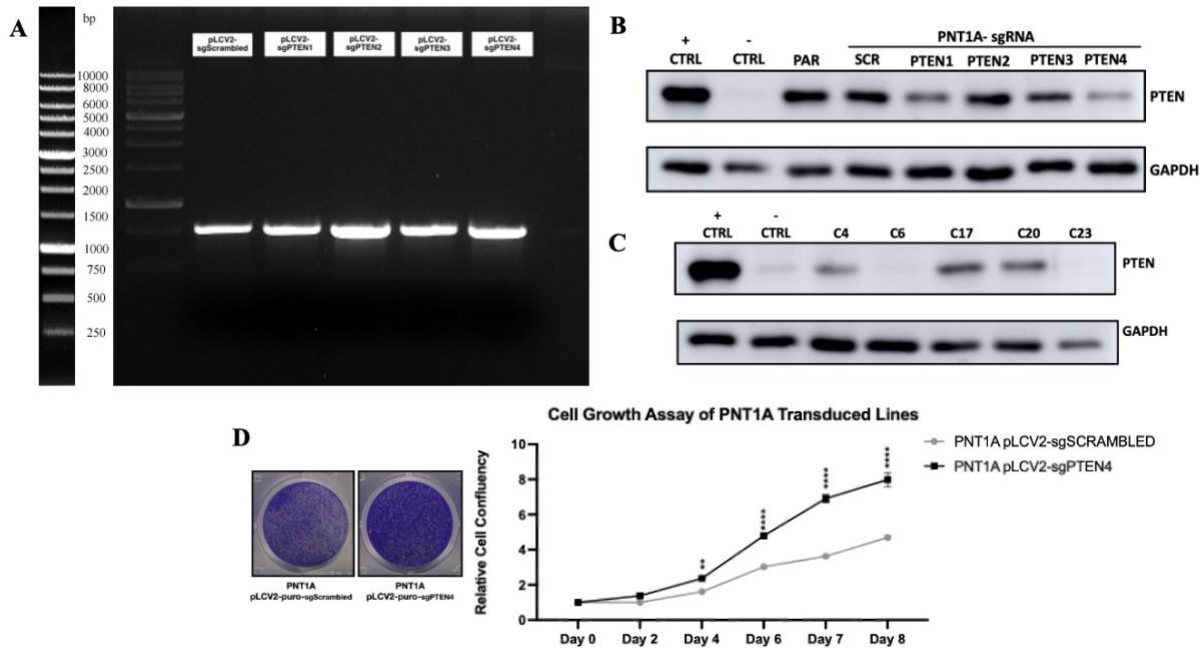


Figure 3. 1. Cloning of control along with PTEN-targeting guide RNAs into the pLCV2-puro vector and the generation of PTEN-null and control PNT1A cell lines by CRISPR-Cas9 strategy. **A)** Agarose gel electrophoresis image of the verification of cloning by colony PCR using the forward strand of gRNA design as the forward primer and the reverse primer aligned on the vector backbone to generate the amplicon at 770 bp. **B)** Western blot images belonging to the PTEN expression levels of PNT1A cell lines. As the positive control, PC3 PTEN-WT^{DOX} was used after 72 hours induction with 1 μ g/ml doxycycline and as the negative control, mock vector containing counterpart was used. PAR stood for Parental PNT1A cell line which indicated the cell line was non-transduced. SCR was an abbreviation for scrambled indicating the control gRNA. GAPDH was used as the loading control. **C)** Western blot image showing PTEN expression levels

of sgPTEN4 colonies were derived from the monoclonal cell expansion using limiting factor dilution protocol to achieve deep PTEN deletion for the next experiments. GAPDH was used as the loading control. The positive and negative controls belong to the lysates were used in panel B. The colonies numbered as C6 and C23 were expanded further and stored. **D)** The growth dynamics of PNT1A pLCV2-sgScrambled and PNT1A pLCV2-sgPTEN4 were determined by using crystal violet viability assay. Representative images of wells from two experiments group belonging to day 8, which was dyed with 0.4% crystal violet stain. Cell growth plot of PNT1A pLCV2-puro-sgSCRAMBLED and -sgPTEN4 at different time points, which was normalized to the initial day. The error bars show the SEM of three biological replicates. The statistical analysis was determined by 2-way ANOVA with Šídák's multiple comparisons test. (n=6, * p<0.05, **p<0.01, *** p<0.001, **** p<0.0001).

To do so, we cloned our PTEN-targeting single guide RNAs into all-in-one CRISPR plasmid named as lentiCRISPR v2 (pLCV2) to knock out PTEN in PNT1A cells. After cloning experiments, we verified the insertion of guide RNA into pLCV2 backbone using colony PCR approach. **(Figure 3.1 – panel A)** Upon the confirmation, we lentivirally transduced PNT1A cells with the produced constructs and selected with 0.4 µg/ml puromycin. We assessed the efficacy of our PTEN-targeting designs with western blot analysis. **(Figure 3.1 – panel B)** According to our results, we decided to continue our experiments with the design named as sgPTEN4 given that it was the most efficient construct amongst all. Then, we generated single cell clones from PNT1A pLCV2-puro-sgPTEN4 cells to obtain a cell population with deep PTEN deletion. The subclones C6 and C23 were expanded and stored for further experiments. **(Figure 3.1 – panel C)**

PTEN loss is characterized as an early and frequent alteration in prostate cancer progression and gives rise to benign growth of prostate epithelia known as prostate hyperplasia. (Trotman et al, 2003) To model PTEN loss in the growth dynamics of non-transformed prostate epithelial cells, we grew PTEN-KO and sgScrambled cell lines in 4% FBS and fixed cell lines at indicated time points in **Figure 3.1 – panel D**. Our results showed that PTEN loss significantly potentiated the growth of PNT1A cell lines.

3.2. PTEN loss leads to an increased dependency on particular PI3K Class IA catalytic subunits

In the literature, PTEN loss has been associated with p110 β dependency. (Wee et al, 2008) We aimed to further investigate dependency shifts among PI3K Class IA catalytic subunits namely p110 α , p110 β and p110 δ .

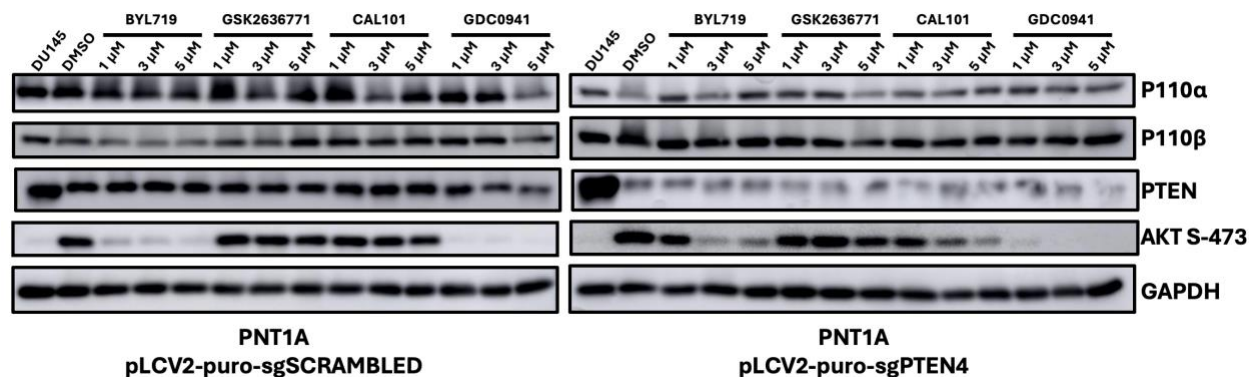


Figure 3. 2. PI3K class IA isoform dependency partially shifts in PNT1A cells upon PTEN loss. Immunoblot of PNT1A pLCV2-puro-sgSCRAMBLED and -sgPTEN4 showing AKT p-S473 as well as p110 α/β levels following treatment with different isoform-specific and pan-PI3K Class IA inhibitors for 5 hours in 5% FBS media. Representative images from three independent biological replicates were shown. DU145 cell lysate was loaded as a PTEN-WT positive control.

In order to detect the contribution of different isoforms to PI3K activity, we assessed AKT phosphorylation on S473 as a key marker of PI3K activity. We treated PNT1A PTEN-KO and -WT cells with isoform specific and pan-PI3K inhibitors. Our results in **Figure 3.2** suggested that 1 μ M p110 α -specific inhibitor BYL719 (Alpelisib) was sufficient to diminish S473 phosphorylation in PNT1A control cell line; however, it was not sufficient to do so in PTEN-KO PNT1A cell line. Furthermore, p110 δ -specific inhibitor CAL101 (Idelalisib) gradually reduced AKT phosphorylation in PTEN-KO PNT1A cell line with the increasing concentrations, when compared to the control counterpart which did not display any significant changes in phosphorylation levels upon CAL101 treatment. As for p110 β specific inhibitor GSK2636771, 5 μ M of GSK2636771 led to a mild decrease in AKT p-S473 in PNT1A PTEN-KO cell line and did not have any effect in PNT1A control cells. When pan-PI3K inhibitor GDC0941 (Pictilisib) was used, the phosphorylations diminished in both cell lines as expected.

Table 3. 1. The IC50 values of PNT1A cell lines for the indicated isoform-specific PI3K inhibitors determined by SRB viability assay. The concentrations were calculated by using three independent biological replicates. (IC50: half maximal inhibitory concentration)

IC50 Values	BYL719	GSK2636771	CAL101
sgSCRAMBLED	10.4 μ M	89.5 μ M	91.6 μ M
sgPTEN4	14.1 μ M	77.5 μ M	85.6 μ M

As the second mean of examining p110 isoform dependency, we conducted cell viability assays using formerly explained inhibitors on PNT1A PTEN-deficient and -proficient cell lines. As it is indicated in **Table 3.1**, we observed an increase in IC50 value for BYL719 and a slight decrease in both CAL101 and GSK2636771 IC50 values upon PTEN deletion in PNT1A cell line. This finding was in line with our western findings, which collectively suggested that PTEN deficiency results in a partial shift towards both p110 β and p110 δ in non-transformed prostate epithelia.

3.3. Doxycycline induction of LNCaP, C4-2 and PC3 Tet-ON expression systems

Our lab utilizes from a temporally controllable expression system to re-express PTEN in endogenously PTEN-null prostate cancer cell lines namely LNCaP, C4-2 and PC3. Our motive behind using Tet-On expression system is to achieve PTEN expression shortly prior to our experiments, which is approximately 72 hours, so that our cells cannot develop adaptations against PTEN presence, but instead, display acute effects of PTEN. Even though our lab well-characterized these cell lines in our previous publication Kaysudu et al., 2023; the experiment was repeated with three independent biological replicates. (**Figure 3.3**)

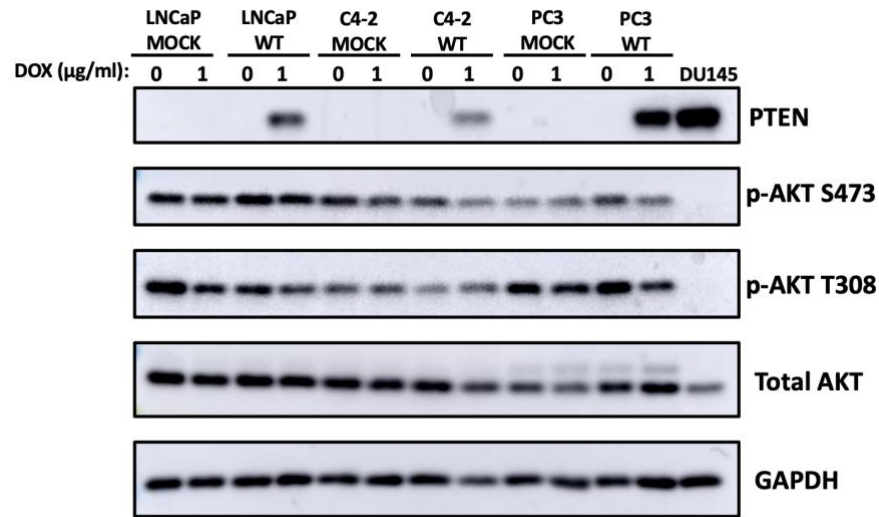


Figure 3. 3. Verification of doxycycline dosages for Mock and PTEN-WT Tet-On expression system containing prostate cancer cell lines. Western blot images of PTEN, AKT p-S473, AKT p-T308, total AKT and GAPDH as the loading control. DOX induction status is indicated on the above of images. The used cell lines were LNCaP, C4-2 and PC3. DU145 cell line was used as PTEN-intact control.

3.4. Our rationale behind this project is built on the targetable SPL metabolism and PI3K-dependent vulnerabilities upon PTEN-loss

In our previous metabolomics-based project, we were able to show that PTEN loss caused alterations mostly in sphingolipid metabolism amongst variety of different metabolite classes in both LNCaP and C4-2 cell lines according to our metabolite set enrichment analysis. (**Figure 3.4. – panel B and C**) Since we observed SPL metabolism alteration both in ADT-naïve LNCaP cell line and its ADT-resistant subclone C4-2 cell line (Liu et al., 2004), we reasoned that the association between PTEN status and SPL alteration might not be specific to ADT-resistance, but rather reflect a general mechanism across PTEN-deficient prostate epithelia. To test this hypothesis, we planned to conduct targeted metabolomics on PNT1A sgPTEN4, LNCaP, C4-2 and PC3 cell lines along with their PTEN-intact controls. The experimental setting included 72 hours of 1 μg/ml doxycycline induction in 2% tFBS for LNCaP, C4-2 and PC3 Tet-On systems while the culture conditions was to 5% FBS for 72 hours for PNT1A cells prior to metabolomics sample harvesting. (**Figure 3.4. – panel A**)

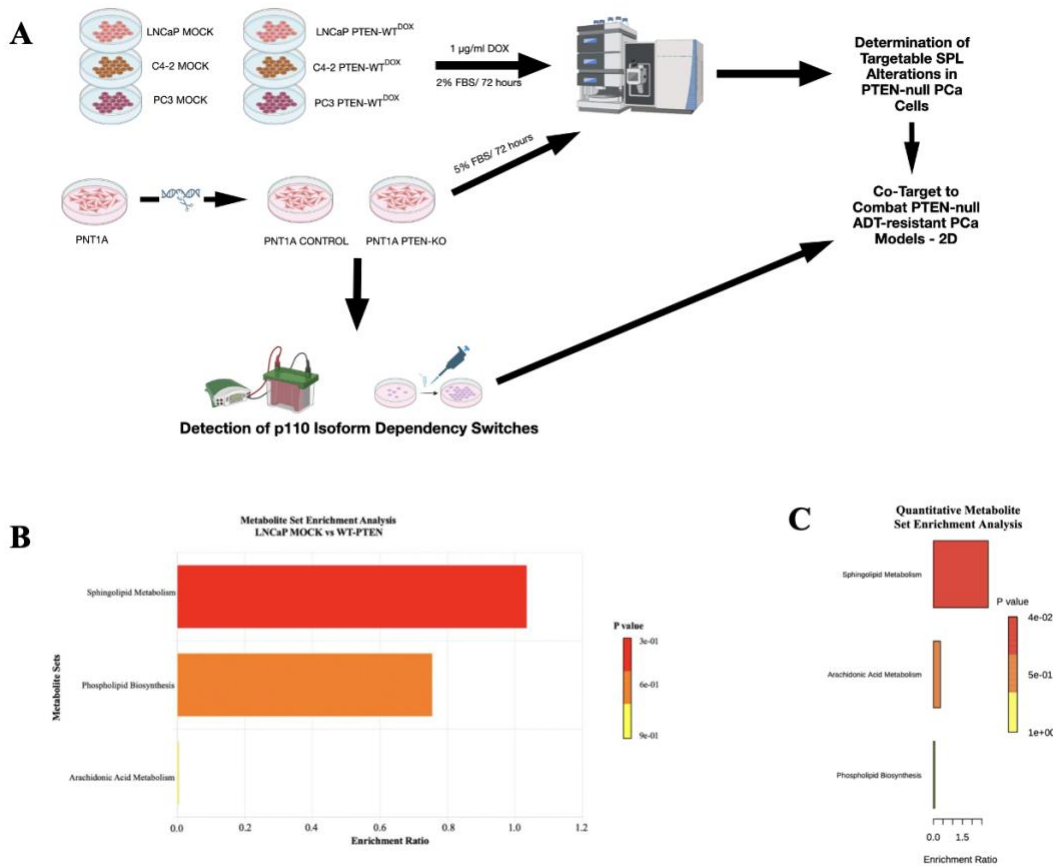


Figure 3. 4. The rationale behind joining the isoform dependency shifts and SPL metabolism changes. **A)** The schematic workflow depicting our experimental methodologies to develop our novel combinatory strategies. Created with BioRender **B)** The mostly altered metabolic classes in LNCaP cells upon PTEN expression taken from Irmak Kaysudu MSc Thesis. **C)** The mostly altered metabolic classes in C4-2 cells upon PTEN expression taken from Buğra Güngül MSc Thesis.

Our plan after determining differentially regulated SPL metabolites upon PTEN loss was to co-target these metabolites alongside p110 β or p110 δ given that the dependency on those were also regulated by PTEN status. (**Figure 3.4. – panel A**)

3.5. Targeted metabolomics uncovers PTEN as a regulator of sphingolipid metabolic reprogramming

Our procedure for sample collection was fairly straightforward as shown in **Figure 3.5.1. - panel A**. We collected cells in ice cold PBS and counted using hemocytometer to achieve 5×10^6 cells precisely. Then, we snap-froze our sample and shipped them to Haznedaroglu Lab. The

remainder of cells was pelleted and resuspended in WB lysis buffer to validate the equal efficacy of doxycycline induction across replicates.

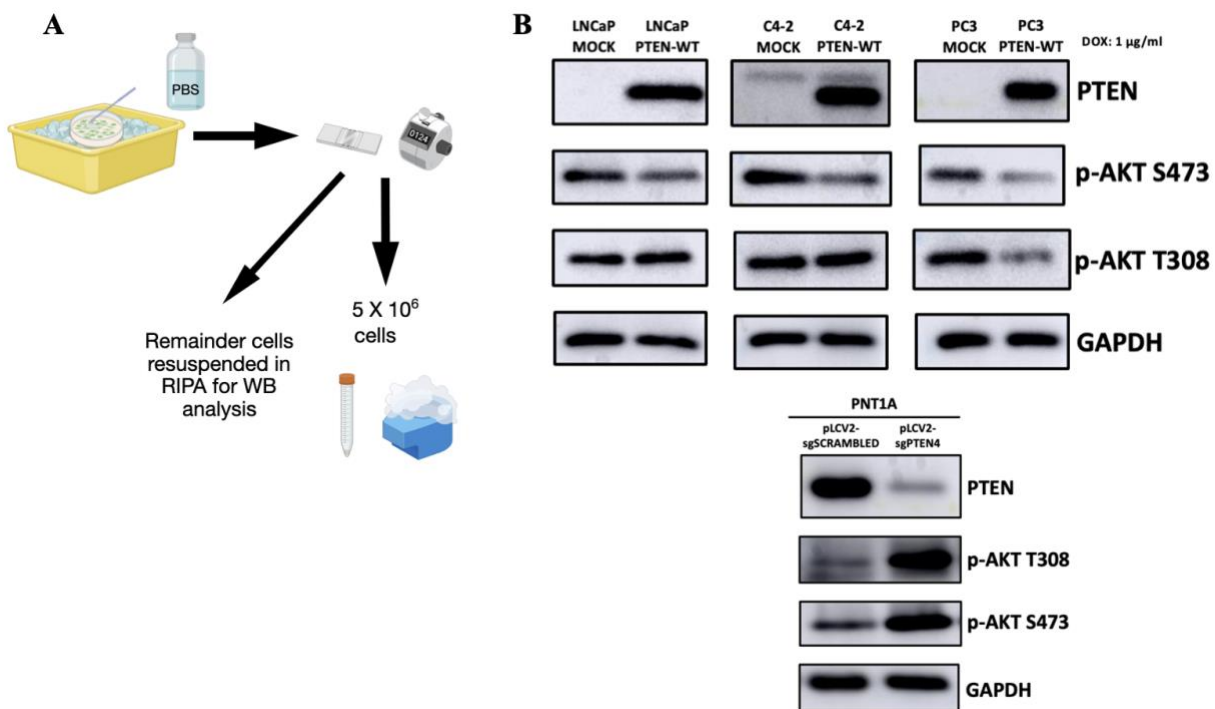


Figure 3.5.1. The harvesting and the verification of targeted metabolomics samples. **A)** The animated representation of metabolomics sample harvesting protocol. After being snap-frozen, the samples were shipped to Haznedaroglu Lab for further processing. Created with BioRender **B)** The western blot results showing the PTEN status and pAKT levels of representative replicates. GAPDH is used as the loading control.

We confirmed PTEN re-expression and its phenotypic effect on pAKT levels as well as the impact of PTEN deletion in PNT1A cell line by using western blot analysis for each biological replicate. **(Figure 3.5.1. - panel B)** We observed a consistent reduction in AKT p-S473 levels in LNCaP, C4-2 and PC3 cell lysates with doxycycline induction. Nevertheless, a decrease in AKT p-T308 levels could be achieved only in PC3 cell line with PTEN re-expression. PNT1A cell line, on the other hand, showed a strong phenotypic reaction to PTEN-knockout with a sharp increase both in AKT p-S473 and p-T308 levels. **(Figure 3.5.1. - panel B)**

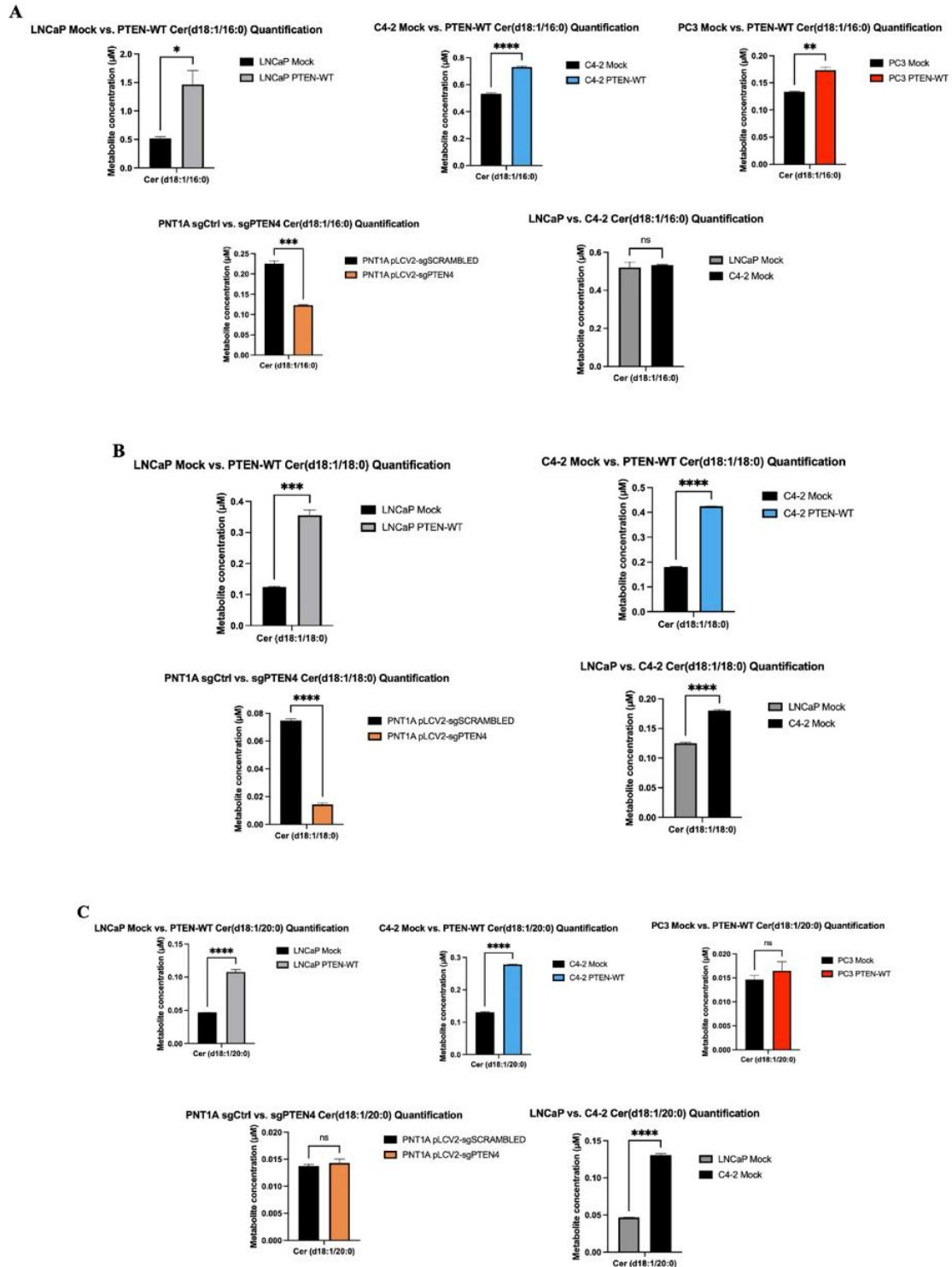


Figure 3. 5. 2. C16, C18 and C20 ceramide concentrations increase with functional PTEN presence in prostate cancer cell lines and non-transformed prostate epithelial cell line PNT1A. A, B, C) The bar plots depicting C16, C18 and C20 ceramide concentrations in order, in

LNCaP, C4-2, PC3 and PNT1A cells in the presence and absence of functional PTEN. The error bars showed SEM of at least three independent biological replicates determined through the targeted metabolomics approach. The statistical significance was determined via Student's T-test. (3<n<5, * p<0.05, **p<0.01, *** p<0.001, **** p<0.0001)

As our targeted metabolomics results demonstrates, C16 and C18 ceramide species showed a strong correlation with PTEN presence since all detected metabolite levels of LNCaP, C4-2, PC3 and PNT1A cell lines depicted a statistically significant increase in the intracellular concentration of these metabolites in PTEN-proficient condition. **(Figure 3.5.2 – panel A and B)** C20 ceramide levels, nevertheless, did not seem to be highly dependent on PTEN status given that C20 ceramide levels did not significantly change in PNT1A and PC3 cell lines with PTEN status although C20 levels showed a similar pattern with other Cer species in LNCaP and C4-2 cell lines. **(Figure 3.5.2 – panel C)**

As for the comparison between ADT-naïve LNCaP and its ADT-resistant counterpart C4-2, we observed an enrichment of ceramide metabolites in C4-2 cell lines. Our interpretation is that this showed the upregulation of SPL metabolism in ADT-resistant C4-2 cell line given that ceramides are core species of sphingolipid metabolism, which can be easily converted to any SPL metabolite. (Ogretmen, 2017) **(Figure 3.5.2 – panel A, B and C)**

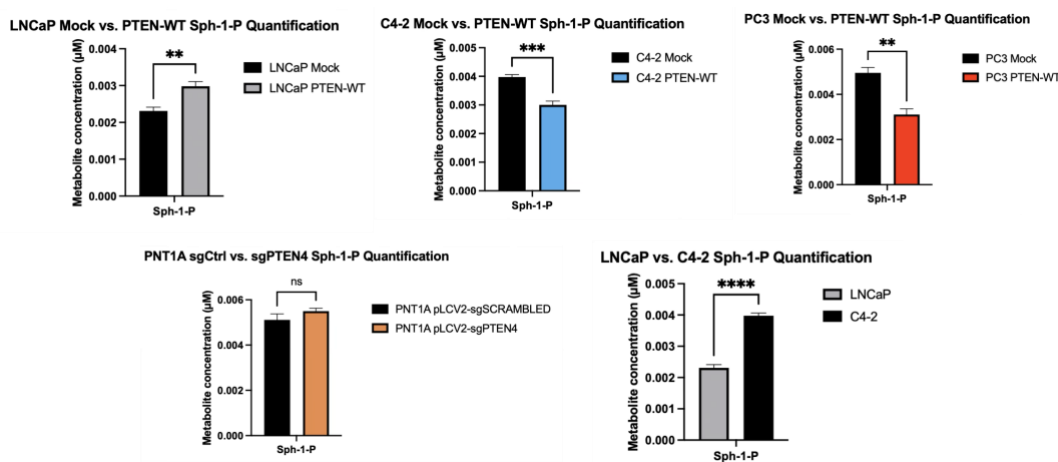


Figure 3. 5. 3. Sphingosine-1-Phosphate (S1P) concentrations in LNCaP, C4-2, PC3 and PNT1A cells in the presence and absence of functional PTEN. The error bars showed SEM of at least three independent biological replicates determined through the targeted metabolomics

approach. The statistical significance was determined via Student's T-test. ($3 < n < 5$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

After ceramide species, we analyzed the intracellular level of a well-known oncogenic metabolite sphingosine-1-phosphate (S1P). S1P levels remained unchanged between control and PTEN-KO conditions in PNT1A cell line. LNCaP cells, however, unexpectedly showed an increase in S1P levels with PTEN re-expression. On the contrary, both ADT-resistant cell lines used in our screening, which were C4-2 and PC3, experienced a marked decrease in S1P levels upon PTEN re-expression. This contrasting regulation led us to hypothesize that the influence of PTEN on S1P metabolism may be modulated by the ADT-resistance status of the cells. In line with our hypothesis, baseline S1P levels were elevated in C4-2 cells relative to those in LNCaP cells. **(Figure 3.5.3)**

3.6. Comparison of the relative mRNA levels of the genes regulating S1P metabolism and signaling between PTEN-null and intact conditions

After our metabolomics screen suggested a metabolic shift towards S1P metabolites in advanced prostate cancer cell lines, we assessed the mRNA levels of S1P related genes under PTEN-null and -proficient conditions.

PNT1A cells did not show any statistically significant results except for a slight increase in the relative mRNA levels of SPHK2 and S1PR2 in PNT1A pLCV2-sgPTEN4 cell line when compared to control cell line. **(Figure 3.6.1 – panel A)**

The genes responsible for S1P production as well as signaling seem to be regulated by PTEN status in LNCaP cell lines with strong downregulation of SPHK1 and S1PR receptors upon PTEN re-expression. **(Figure 3.6.1 – panel B)**

As both for C4-2 and PC3 cell lines, the expression of SPHK1 expression was reduced markedly in PTEN-WT condition, which showed that S1P production might be regulated by PTEN. S1PR gene expression, additionally, seems to be generally decreased with PTEN presence as well. The expression of SGPL1 which is responsible for irreversible S1P turnover, was also higher in C4-2 and PC3 mock cell lines suggesting that both S1P production and neutralization might be more dynamic in ADT-resistant PCa cells. **(Figure 3.6.1 – panel C and D)**

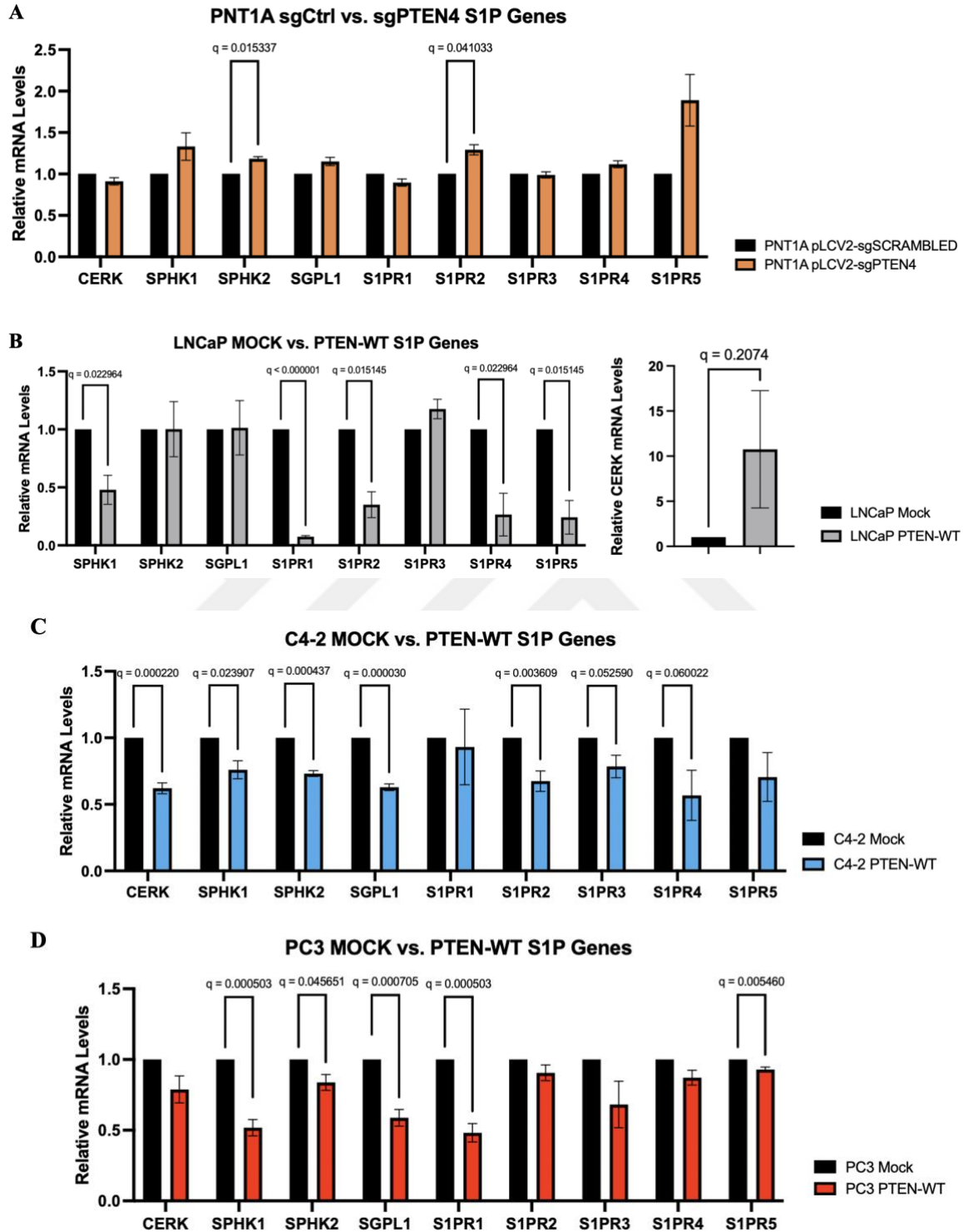


Figure 3. 6. 1. Relative mRNA expression levels of the genes involved in S1P metabolism and signaling in PTEN-null and PTEN-intact cell lines. B, C, D) LNCaP, C4-2 and PC3 cells were induced with 1 μ g/ml doxycycline in 4% tFBS medium for 72 hours while A) PNT1A cells were

incubated in 5% FBS medium for 72 hours prior to RNA isolation. Following the RNA isolation, cDNA synthesis and RT-qPCR were conducted in order. GAPDH or RPL19 was used as the housekeeping control. Fold changes were calculated by the $2^{-\Delta\Delta CT}$ formula. The statistical significance was determined by multiple unpaired t-test with a false discovery rate approach. Adjusted p-values were indicated above the genes yielding statistically significant results. The error bars depicted the SEM of at least three biological replicates ($3 \leq n \leq 4$)

3.7. PTEN-null cell lines showed increased sensitivity to S1PR signaling in comparison to PTEN-WT cells

To identify if elevated S1P levels constitutes a targetable therapeutic window, we assessed the reliance of PCa cells on S1PR in the presence versus absence of PTEN by using non-isoform selective S1PR inhibitor FTY720. The viability of cells was assayed by SRB method. The cells were incubated for four days with the indicated doses of FTY720 in 4%FBS.

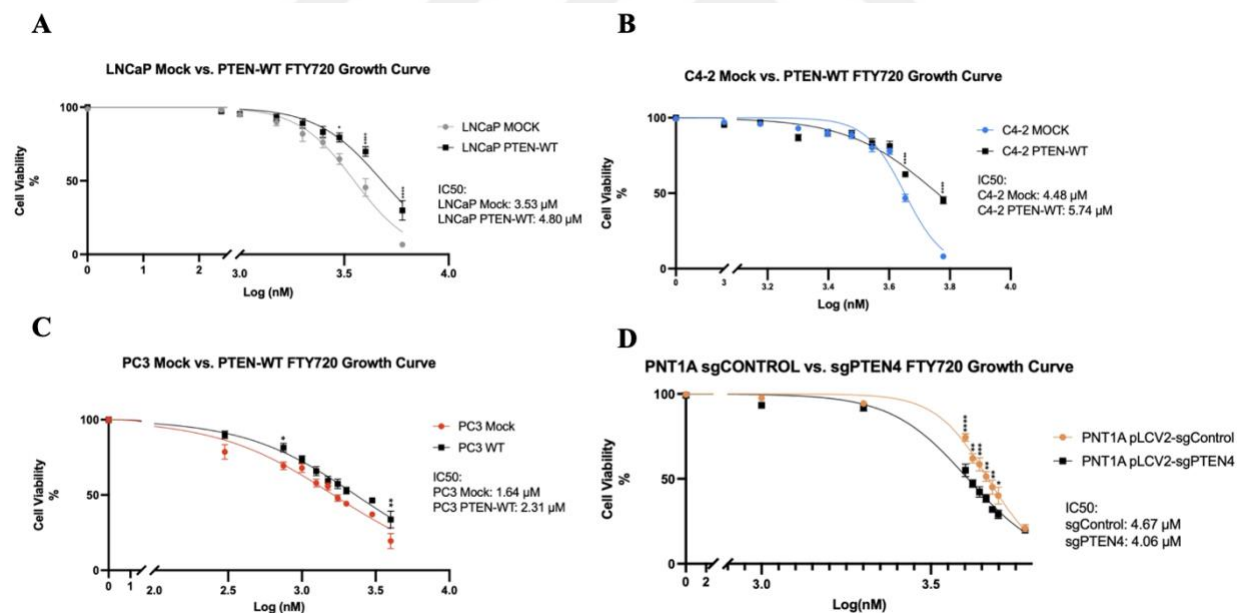


Figure 3. 7. 1. Growth inhibition curve for LNCaP, C4-2, PC3 and PNT1A cell lines in the presence vs absence of functional PTEN across increasing doses of non-isoform selective S1PR inhibitor FTY720. A, B, C, D) The viability of LNCaP, C4-2, PC3 and PNT1A cell lines was assessed by SRB viability assay. The error bars depicted SEM of three different biological replicates. The statistical significance was determined by two-way ANOVA with Šídák's multiple

comparisons test. (IC50: half maximal inhibitory concentration, n=3, * p<0.05, **p<0.01, *** p<0.001, **** p<0.0001)

Both PCa cell lines and non-transformed PNT1A cell line seemed to be sensitized to S1PR inhibition under PTEN-deficient condition as opposed to PTEN-intact counterparts. (**Figure 3.7.1**) This implies that the downregulation of PTEN activity makes cells more dependent on S1PR signaling for growth and survival.

Given that the S1P receptors are members of GPCR superfamily (O'Sullivan and Dev, 2013) which is known to activate mainly p110 β , we investigated if PI3K signaling is one of its effectors. Given that PC3 cell line showed the strongest dependency on S1PR signaling with an IC50 of 1.64 μ M (**Figure 3.7.1 – panel C**), we selected PC3 cells to address this question.

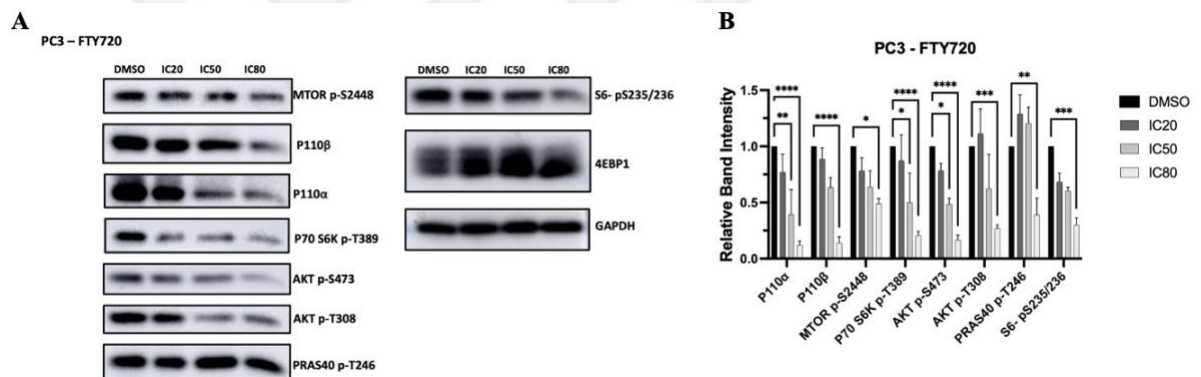


Figure 3. 7. 2. Inhibition of S1PR signaling leads to downregulation of PI3K pathway and downstream phosphorylations. **A)** Western blot results showing PI3K downstream phosphorylations in PC3 cell line following treatment by FTY720 at indicated doses. **B)** The bar plots showing the band quantification of three independent biological replicates, normalized to the internal control GAPDH and then to DMSO negative control. The quantification was conducted with the help of ImageJ. The error bars depict SEM of biological replicates. The statistical significance was determined by two-way ANOVA with Dunnett's multiple comparisons test. (n=3, * p<0.05, **p<0.01, *** p<0.001, **** p<0.0001)

FTY720-mediated S1P signaling inhibition in PC3 cells drastically affected the phosphorylation levels of PI3K signaling components. Interestingly, both p110 α and p110 β levels appeared to drop with the increasing FTY720 doses. This might be a possible explanation why all downstream phosphorylations were decreased. In more detail, both PI3K- mediated S-473 and T-

308 phosphorylations of AKT were dropped gradually with S1PR inhibition. Even though PRAS40 p-T246 phosphorylation - which is catalyzed by AKT- seemed to be reduced to a smaller extent, significant changes in the phosphorylation status of mTORC1 substrates such as 4EBP1, S6K were observed. (**Figure 3.7.2**) These results overall demonstrate that PI3K pathway is one of the effectors functioning downstream of S1P receptors.

Since S1PR1 mRNA expression was downregulated in PTEN re-expressing LNCaP and PC3 cells (**Figure 3.6.1**), we repeated the viability assay by using S1PR1 specific inhibitor called ACT128800 (Ponesimod) (Ianniello and Pozzilli, 2021) to assess the role of S1PR1 isoform in PTEN-null PCa cells.

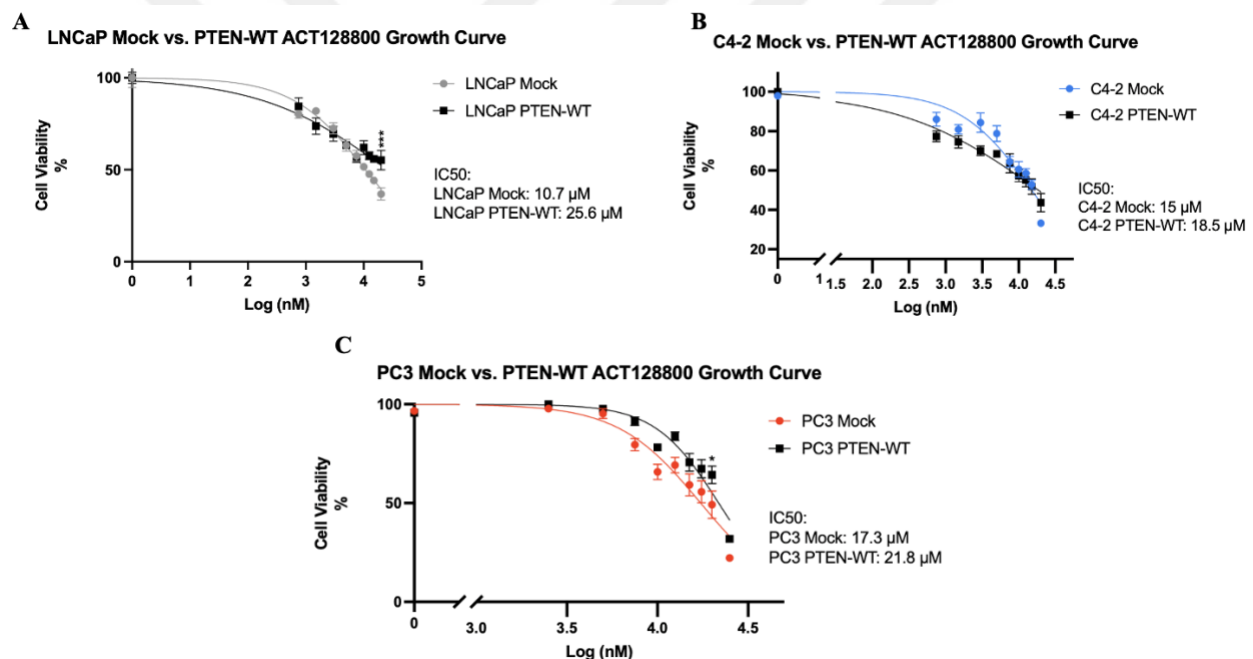


Figure 3. 7. 3. Growth inhibition curve for LNCaP, C4-2 and PC3 cell lines in the presence vs absence of functional PTEN across increasing doses of isoform selective S1PR1 inhibitor ACT128800. A, B, C) The viability of LNCaP, C4-2, PC3 cells was assessed by SRB viability assay. The error bars depicted SEM of three different biological replicates for C4-2 and PC3 cell lines while it depicted the SEM of two independent biological replicates for LNCaP cell line. The statistical significance was determined by two-way ANOVA with Šídák's multiple comparisons test. (IC50: half maximal inhibitory concentration, n=3, * p<0.05, **p<0.01, *** p<0.001, **** p<0.0001)

The isoform-specific inhibition of S1PR1 reduced the viability of PTEN-null PCa cell lines more efficiently than that of PTEN re-expressing PCa counterparts. (**Figure 3.7.3**) This result was in line with our previous hypothesis that PTEN presence confers protection against S1PR inhibition. Nevertheless, the IC₅₀ values for ACT128800 were markedly higher than IC₅₀ values for FTY720 in LNCaP, C4-2 and PC3 cell lines, which showed that S1PR1 might not be the primary isoform required for cell growth in PTEN-deficient cells.

3.8. Inhibition of S1P production through SPHK1 and SPHK2 reduces the cell viability of PCa cells

After showing PTEN status regulated effectiveness of S1PR inhibition in PTEN-null prostate cancer cells, we aimed to strengthen our hypothesis that bioactive S1P signaling was a targetable node in PTEN-null prostate cancer cells. To do so, we targeted S1P production through either inhibiting SPHK1 or SPHK2 alternatively to our previous strategy to inhibit S1PR. Given that our qPCR results strongly led us towards SPHK1 isoform of sphingosine kinases (**Figure 3.6.1**), we compared SPHK1 protein levels of PTEN re-expressing and control cell lines. (**Figure 3.8.1**)

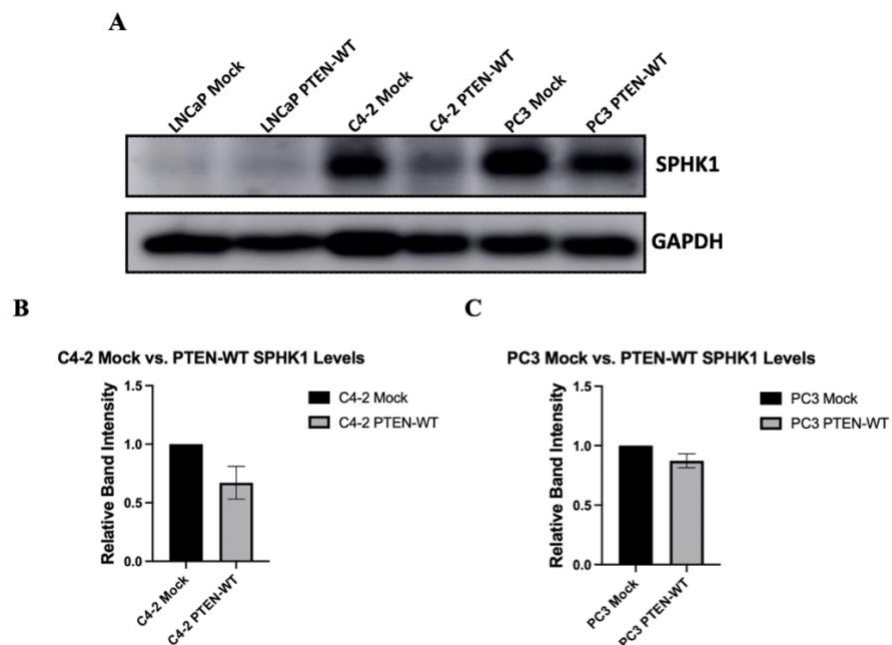


Figure 3. 8. 1. Western blot analysis of SPHK1 protein levels in PTEN-null and PTEN-WT LNCaP, C4-2 and PC3 cells. A) Western blot images belonging to the representative replicate of

the experiment. GAPDH was loaded as a housekeeping control. The cells were incubated in 1 $\mu\text{g/ml}$ doxycycline containing 4%tFBS RPMI for 72 hours before cell harvesting. **B, C)** The band quantification results of C4-2 and PC3 cells respectively, which were conducted with two independent biological replicates. The band intensity measurements were performed in ImageJ tool. The band intensities were initially normalized to GAPDH. Then, each cell line was normalized to their Mock control.

We observed significant downregulation of SPHK1 protein levels upon PTEN expression in C4-2 and PC3 cells. Nevertheless, we did not detect a visible band in LNCaP cell lines which suggests that LNCaP cells had intrinsically low levels of SPHK1 proteins when compared to C4-2 and PC3 cells. We also quantified band intensities due to unequally loaded GAPDH, both our biological replicates yielded very close results which confirms the PTEN-mediated downregulation of SPHK1 in ADT-resistant PTEN-null PCa cells. (**Figure 3.8.1**)

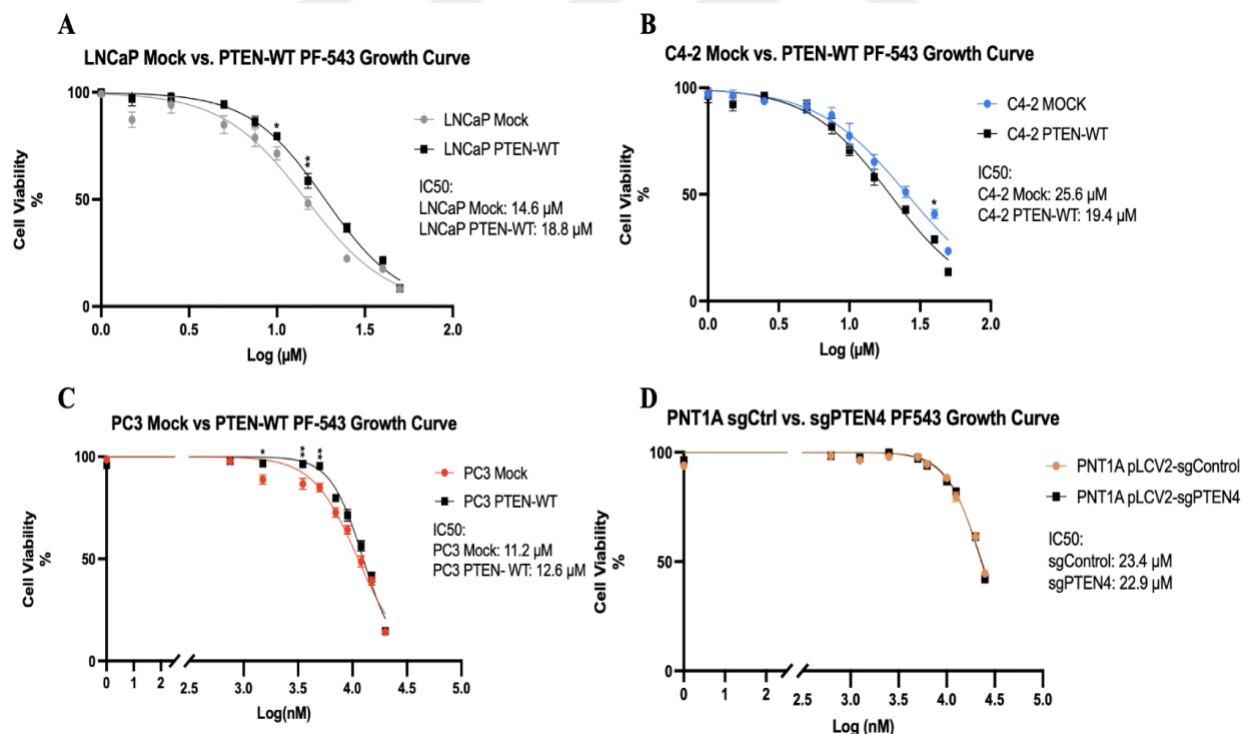


Figure 3. 8. 2. Growth inhibition curve for LNCaP, C4-2, PC3 and PNT1A cell lines in the presence vs absence of functional PTEN across increasing doses of isoform specific SPHK1 inhibitor PF543. A, B, C, D) The viability was measured with SRB assay. The error bars depicted SEM of three different biological replicates. The statistical significance was determined by two-

way ANOVA with Šídák's multiple comparisons test. (IC₅₀: half maximal inhibitory concentration, n=3, * p<0.05, **p<0.01, *** p<0.001, **** p<0.0001)

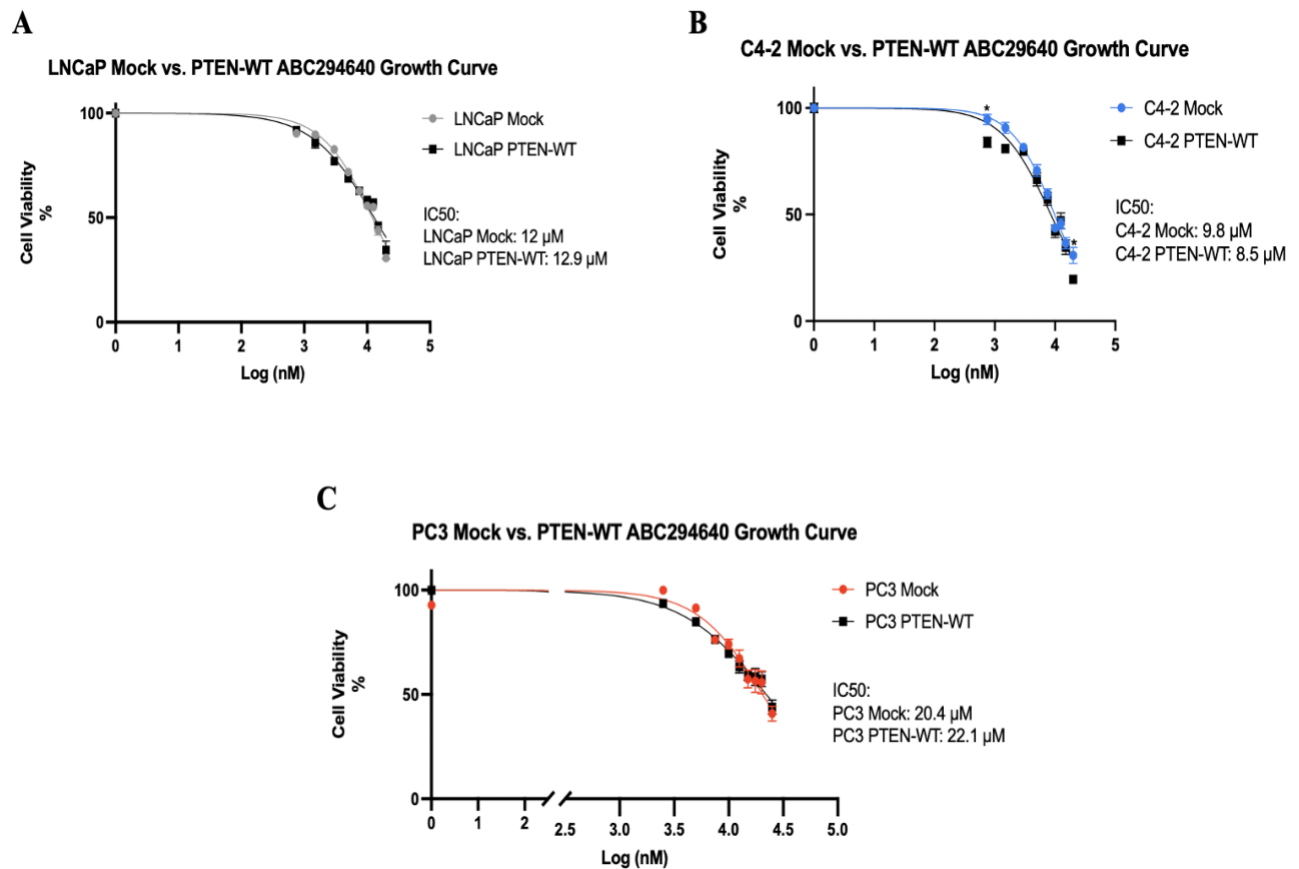


Figure 3. 8. 3. Growth inhibition curve for LNCaP, C4-2 and PC3 cell lines in the presence vs absence of functional PTEN across increasing doses of isoform specific SPHK2 inhibitor ABC294640. A, B, C) The viability was assessed using SRB assay. The error bars depicted SEM of three different biological replicates. The statistical significance was determined by two-way ANOVA with Šídák's multiple comparisons test. (IC₅₀: half maximal inhibitory concentration, n=3, * p<0.05, **p<0.01, * p<0.001, **** p<0.0001)**

Our strategy against SPHK isoforms demonstrated that PTEN expression in different prostate cancer lines might lead to different outcomes. In LNCaP lines, PTEN re-expression protected cells against SPHK1 inhibition while it had minimal impact on the sensitivity to SPHK2 inhibition. Additionally, PF543 exhibited a higher IC₅₀ value than ABC294640 in LNCaP cell

lines, which implies that LNCaP cells might depend more on SPHK2 isoform. (**Figure 3.8.2 – panel A, Figure 3.8.3 – Panel A**)

C4-2 cells displayed an unexpected response to both SPHK1 and SPHK2 inhibition. PTEN increased their sensitivity to SPHK inhibition. Furthermore, C4-2 cells appeared to rely more on SPHK2 isoform according to the IC50 values of two drugs. (**Figure 3.8.2 – panel B, Figure 3.8.3 – Panel B**)

PC3 cell line also displayed dependency on SPHK1 isoform based on IC50 values. PTEN expression, nevertheless, provided very minimal protection against the inhibition of both isoforms. (**Figure 3.8.2 – panel C, Figure 3.8.3 – Panel C**)

PNT1A cells were tested only for SPHK1 inhibition using PF543 and PTEN-knockout slightly sensitized the cells to SPHK1 inhibition. (**Figure 3.8.2 – panel D**)

3.9. Dual combinations of p110 β and p110 δ isoforms with S1P production/signaling synergistically hinder the growth of PTEN-null prostate cancer cells

The most critical aspect of our study was to unite the metabolic and PI3K-driven vulnerabilities emerged from PTEN deficiency in prostate cancer cells to minimize the potential toxicity to PTEN-WT cells. In this regard, we co-targeted p110 β or p110 δ alongside the bioactive S1P metabolite by combining either p110 β i or p110 δ i with FTY720 or PF543. We interpreted our results based on the empirical CDI model formula which returns a coefficient of drug interaction (CDI) value to describe the interaction dynamics of two drugs. (Kaysudu et al, 2023)

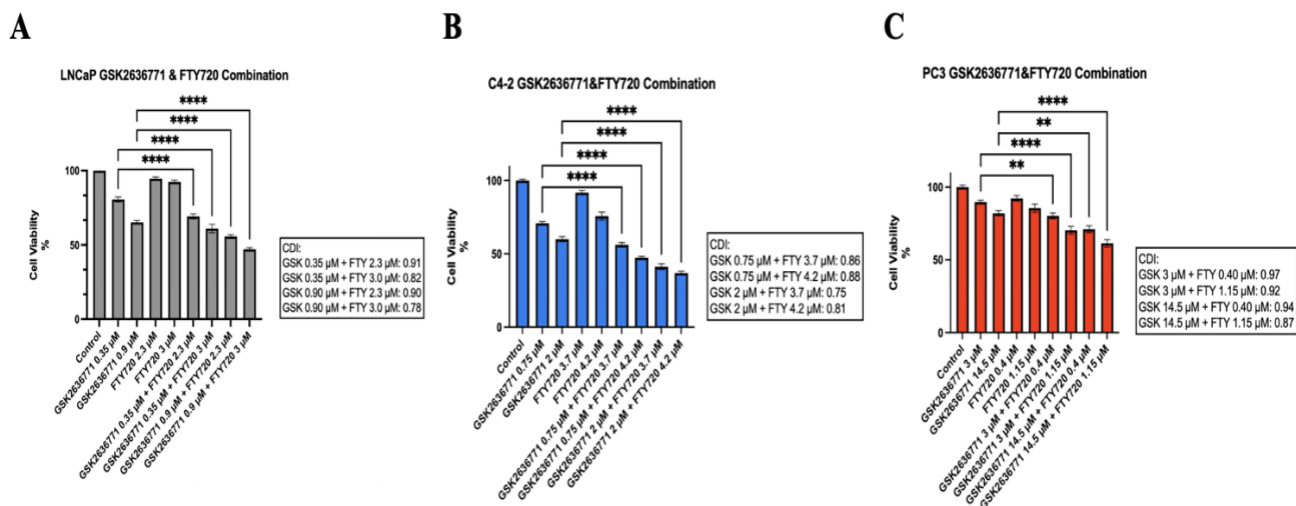


Figure 3.9.1. Co-inhibition of p110 β via GSK2636771 and S1PR inhibit the growth of PTEN-null PCa cells. The cell viability was measured using SRB assay and the viability values were normalized to DMSO control. The interaction between drugs were examined by the simplified empirical CDI model. **A, B, C)** Bar plots showing the cell viability in the combination of GSK2636771 and S1PR inhibitor FTY720 for LNCaP, C4-2 and PC3 cell lines. The statistical significance was determined by ordinary one-way ANOVA with Šídák's multiple comparisons test. The bar plot showing three independent biological replicates (CDI: coefficient of drug interaction, n=3, * p<0.05, **p<0.01, *** p<0.001, **** p<0.0001)

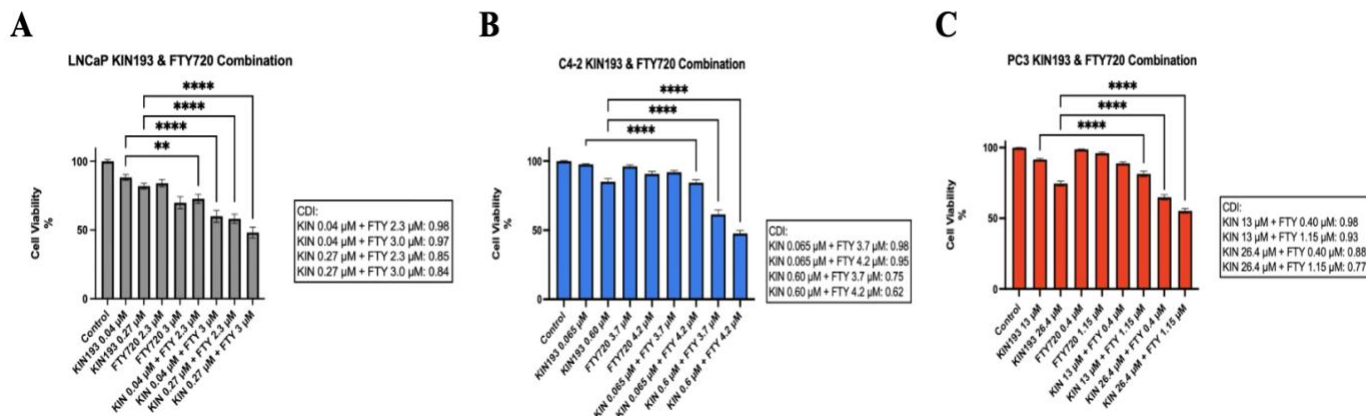


Figure 3.9.2. Co-inhibition of p110 β via KIN193 and S1PR inhibit the growth of PTEN-null PCa cells. The cell viability was measured using SRB assay and the viability values were normalized to DMSO control. The interaction between drugs were examined by the simplified empirical CDI model. **A, B, C)** Bar plots showing the cell viability in the combination of KIN193 and S1PR inhibitor FTY720 for LNCaP, C4-2 and PC3 cell lines. The statistical significance was assessed by ordinary one-way ANOVA with Šídák's multiple comparisons test. The bar plot showing three independent biological replicates (CDI: coefficient of drug interaction, n=3, * p<0.05, **p<0.01, *** p<0.001, **** p<0.0001)

We utilized two structurally irrelevant p110 β inhibitors KIN193 and GSK294640 to test if co-targeting S1P signaling alongside p110 β isoform potentiated the effect of single treatments further. Two doses of agents were combined and CDI value was calculated for each. For ADT-sensitive LNCaP line, the co-inhibition of p110 β and S1PR with FTY720 returned 0.78 as the lowest CDI value among the dose combinations, which show moderate synergism. (Kaysudu et al, 2023) (**Figure 3.9.1 and Figure 3.9.2 -panel A**) Our co-targeting strategy in C4-2 cells, on the other hand, was more effective with 0.62 CDI value indicating synergy. (**Figure 3.9.1 and Figure 3.9.2 -panel B**) As for PC3 cell line, the interaction of used drugs was moderately synergistic with the lowest CDI value as 0.77. (**Figure 3.9.1 and Figure 3.9.2 -panel C**)

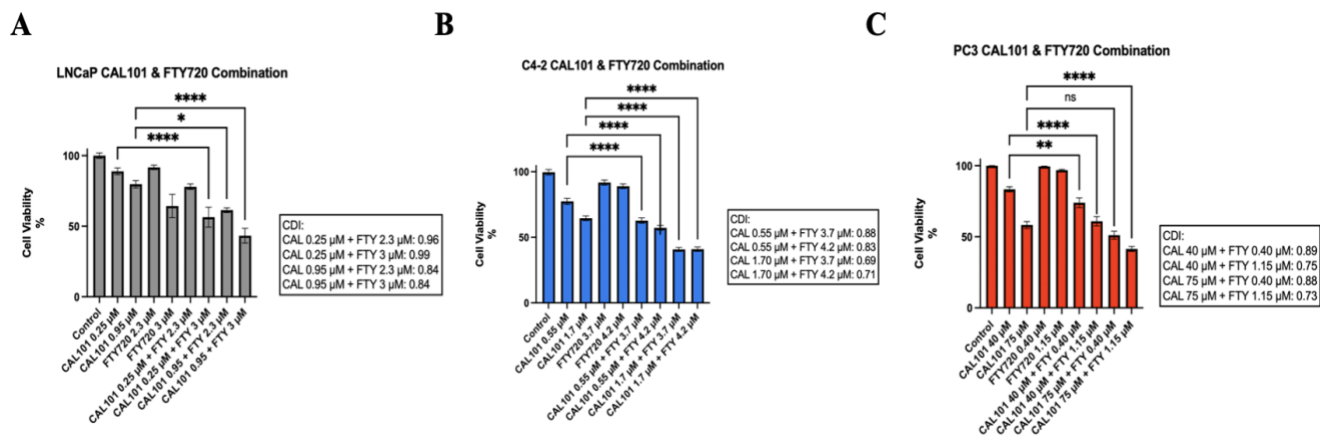


Figure 3.9.3. Co-inhibition of p110 δ and S1PR suppresses the growth of PCa cells. The cell viability values were normalized to DMSO control while CDI values were calculated by the simplified empirical CDI model. **A, B, C)** Bar plots showing the cell viability of LNCaP, C4-2 and PC3 cell lines after the combination of CAL101 and S1PR inhibitor FTY720. Cell viability was assayed by using SRB viability test. The statistical significance was calculated by ordinary one-way ANOVA with Šídák's multiple comparisons test. The bar plot showed three independent biological replicates (CDI: coefficient of drug interaction, n=3, * p<0.05, **p<0.01, *** p<0.001, **** p<0.0001)

Since we observed a partial dependency shift towards p110 δ upon PTEN loss (**Figure 3.2 and Table 1**), we recapitulated our co-targeting strategy by replacing p110 β inhibitor KIN193 and GSK2636771 with p110 δ inhibitor CAL101. Therefore, we co-targeted p110 δ along with S1PR. Our results of this co-targeting strategy were perfectly in line with the results obtained from p110 β and S1PR co-inhibition. In other words, the combination yielded moderately synergistic growth inhibition in LNCaP and PC3 cell lines while the effect of the combination was greater in C4-2 cells with a synergistic CDI value. (**Figure 3.9.3**)

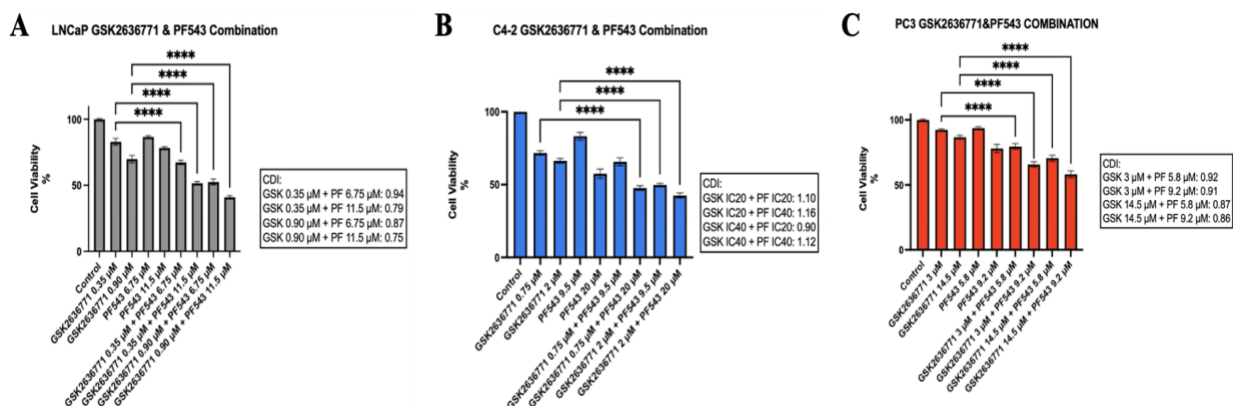


Figure 3.9.4. Co-inhibition of p110 β with GSK2636771 and SPHK1 had a synergistic inhibitory effect on the growth of LNCaP and PC3 cells. The cell viability values were normalized to the internal DMSO control. CDI values were calculated by the simplified empirical CDI model. **A, B, C)** Bar plots showing the cell viability belonging to the combination of GSK2636771 and SPHK1 inhibitor PF543 of LNCaP, C4-2 and PC3 cell lines. SRB viability assay was used to measure cell growth. The statistical significance was determined by ordinary one-way ANOVA with Šídák's multiple comparisons test. The bar plot showed three independent biological replicates (CDI: co-efficient of drug interaction, n=3, * p<0.05, **p<0.01, *** p<0.001, **** p<0.0001)

By completing the experiments depicted in **Figure 3.9.1**, **Figure 3.9.2** and **Figure 3.9.3**, we showed the efficacy of co-targeting bioactive S1P signaling with PI3K Class IA isoforms. We aimed to expand the scale of our co-targeting strategy by targeting S1P production. In this regard, we decided to target SPHK1, but not SPHK2, given that SPHK1 expression was significantly downregulated in all three PCa cell lines with PTEN re-expression. (**Figure 3.6.1**) Similarly to our previous approach, we utilized two different p110 β inhibitors namely KIN193 and GSK2636771 to validate our results. We used the isoform-specific SPHK1 inhibitor PF543 to suppress S1P production through SPHK1. The co-inhibition of p110 β and SPHK1 almost synergistically inhibited LNCaP cell growth with the lowest CDI value as 0.75. (**Figure 3.9.4** and **Figure 3.9.5 – panel A**) This combination was even more potent to suppress PC3 cell growth with a synergistic CDI value 0.65. (**Figure 3.9.4 panel C** and **Figure 3.9.5 panel B**) Since C4-2 cells were previously identified to depend more on SPHK2 isoform for growth (**Figure 3.8.2** and **Figure**

3.8.3), this combinatory treatment returned only additive effect in C4-2 cells with CDI value around 1.0. (Figure 3.9.4 – panel B)

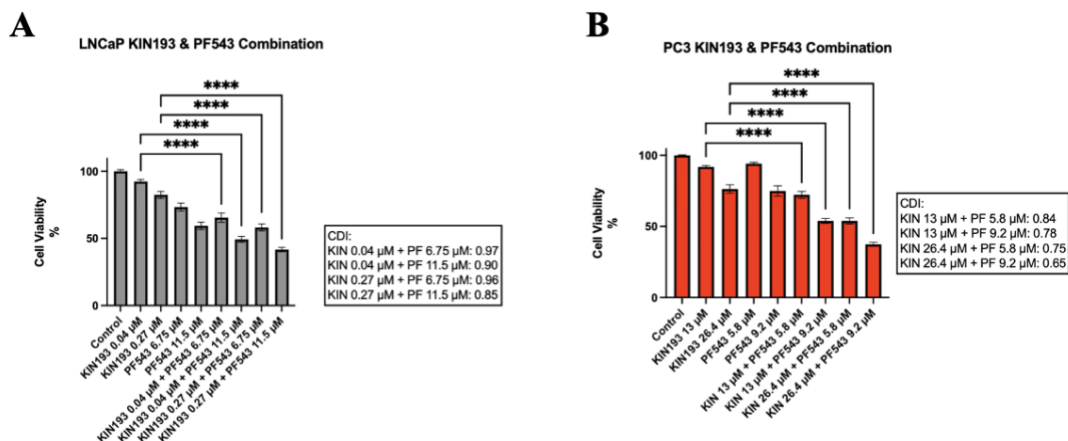


Figure 3. 9. 5. Co-inhibition of p110β using KIN193 and SPHK1 had a synergistic inhibitory effect on the growth of LNCaP and PC3 cells. The cell viability values were normalized to the internal DMSO control. CDI values were calculated by the simplified empirical CDI model. **A, B)** Bar plots showing the cell viability belonging to the combination of KIN193 and SPHK1 inhibitor PF543 of LNCaP and PC3 cell lines. SRB viability assay was used to quantify cell growth. The statistical significance was assessed by ordinary one-way ANOVA with Šidák's multiple comparisons test. The bar plot showed three independent biological replicates (CDI: co-efficient of drug interaction, n=3, * p<0.05, **p<0.01, *** p<0.001, **** p<0.0001)

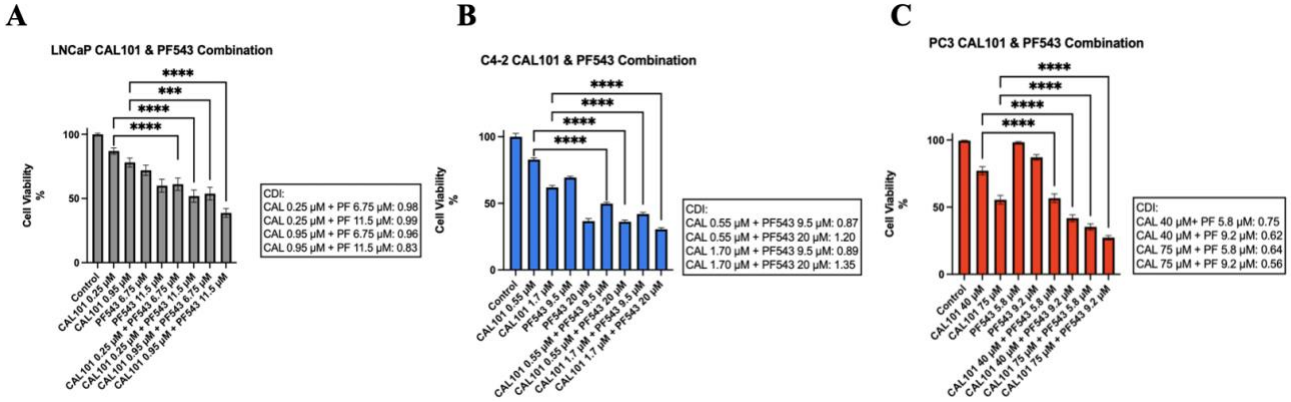


Figure 3.9.6. Co-inhibition of p110 δ and SPHK1 synergistically inhibited the growth of LNCaP, C4-2 and PC3 cells. The cell viability values were normalized to DMSO control and CDI values were calculated by empirical CDI formula. **A, B, C)** Bar plots showing the cell viability in the combination of CAL101 and SPHK1 isoform specific inhibitor PF543 for LNCaP, C4-2 and PC3 cell lines in order. Cell viability was measured using SRB assay. The statistical significance was assessed by ordinary one-way ANOVA with Šidák's multiple comparisons test. The bar plot showed three independent biological replicates. (CDI: co-efficient of drug interaction, n=3, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001)

The results of our experimental strategy to co-inhibit SPHK1 and PI3K with p110 δ inhibitor CAL101 were parallel to our results with p110 β inhibition. The potency of co-targeting p110 δ and SPHK1 was moderately synergistic in LNCaP cells. The growth of C4-2 cells was inhibited additively, which supports the previous finding that C4-2 cells rely on SPHK2 isoform selectively. PC3 cells, on the other hand, were sensitive to p110 δ and SPHK1 co-inhibition and returned a significant synergy score as 0.56. (**Figure 3.9.6**)

4. DISCUSSION

In 2022, 1.5 million men were diagnosed with prostate cancer and prostate cancer-related deaths were reported at approximately 0.4 million. (The Global Cancer Observatory, 2024) Given that current treatment options fail to reduce these numbers, new therapeutic target identification plays a key role. To achieve this, the dysregulated cellular mechanisms including frequent genomic alterations, phosphorylations and metabolites have been extensively investigated. PTEN, TP53 deletions, AR gene locus amplifications, ETS rearrangements and TMPRSS2-ERG gene fusions are the most frequent genomic alterations. (Barbieri et al., 2013) Among this, PTEN loss is associated with hamartoma syndrome and occurs early in prostate cancer progression. (Lee et al., 2018) In this regard, we previously identified that PTEN loss leads to a metabolic reprogramming in prostate cancer (Kaysudu et al, 2023) and SPL metabolism is one of the significantly altered metabolic pathways (**Figure 3.4 - panel B and C**).

In this project, we aimed to work on the impact of PTEN loss in prostate cancer cells. The cell lines we chose for our study was LNCaP, C4-2 and PC3. To elucidate more on the co-occurring PCa-related genomic alterations, p53 status of these cells should be addressed given that p53 and PTEN co-deletion is highly reported in PCa (**Figure 1.1**). LNCaP and C4-2 cells are p53^{WT/WT} indicating the expression of functional p53 protein. The knockout of p53 gene in LNCaP cells, furthermore, was reported to enable LNCaP cells in androgen-deprived environments. (Vinall et al., 2006) Therefore, p53 gene function is crucial for LNCaP cell line's ADT-naïve characteristics. As for PC3 cells, they lack functional p53 protein and they are characterized as p53-null. (Chappell et al., 2012) From this aspect, we utilized both p53-intact and p53-null cells to investigate the impact of PTEN loss on the metabolic shift between bioactive sphingolipid metabolites. In this manner, we achieved to show that ceramide downregulation and S1P enrichment in ADT cells constituted a node regulated by PTEN status irrelevant of p53 status.

To elucidate the consequences of PTEN loss in prostate cancer progression, we focused on PI3K class IA signal and SPL metabolite level alterations in PTEN-knockout and control PNT1A cells along with control and PTEN re-expressing prostate cancer cells. To this end, we started with the generation of PNT1A PTEN-KO and control cell lines. Although we also generated subclones with deep PTEN deletion (**Figure 3.1 – Panel C**), we proceeded with our polyclonal PNT1A pLCV2-puro-sgPTEN4 for metabolomics screening and subsequent experiments since tumor cells

in vivo harbor genetic heterogeneity and are not genetically identical. (Burrell et al., 2013) We tested if PTEN deletion alone was sufficient to alter the growth dynamics of non-transformed PNT1A cells. Our time lapse growth experiment showed that PTEN deletion considerably increases growth dynamics of PNT1A cells (**Figure 3.1 – panel D**), which is in line with the fact that PTEN loss leads to hamartoma formation in many tissues.

After confirming and phenotypically characterizing PTEN deletion in PNT1A cells, we investigated its impact on PI3K Class IA isoform dependency. To do so, we conducted both cell viability and western blot analysis by using isoform specific as well as pan-PI3K inhibitors. Our results (**Figure 3.2, Table 3.1**) collectively highlighted that PTEN loss in prostate epithelia does not directly lead to a change in predominant isoform, which remains as p110 α . Nevertheless, PTEN deleted prostate epithelial cells acquire an increased reliance on p110 β and p110 δ isoforms for survival and growth.

The mechanisms behind the acquisition of partial p110 β / p110 δ dependency acquisition upon PTEN deletion can be commented further by highlighting crucial findings from literature. The mechanism linking PTEN deletion to p110 β dependency was attributed to the non-raft localization of PTEN. In healthy prostate, PTEN dephosphorylates PIP₃ species in non-raft regions while p110 β is activated by GPCRs in lipid rafts, a process mediated by Rac1. The loss of PTEN activity, thus, allows the further propagation of p110 β signaling to non-raft regions. In parallel, the viability of PTEN-null PC3 cells was effectively decreased upon the disruption of lipid raft formation. (Cizmecioglu et al. 2016)

Since p110 α is localized to non-raft regions naturally, its function is not upregulated upon PTEN loss given that p110 α cannot be activated by GPCRs in lipid rafts. Nevertheless, p110 α can also induce raft-mediated PI3K activation through GPCR-EFGR transactivation when extrinsically targeted to lipid rafts. (Cizmecioglu et al. 2016)

p110 δ dependency was associated with leukocytes and B cell malignancies. Here, we showed p110 δ as a novel player which emerges upon PTEN deletion. The activation of p110 δ in solid tumors is poorly defined. The possibility of lipid raft-mediated p110 δ dependency represents an intriguing hypothesis that warrants further investigation.

As our last step before metabolomics screening, we confirmed the efficacy of the DOX induction doses in previously described PTEN-null PCa cells transduced with PTEN-WT^{DOX} vectors. (Kaysudu et al., 2023) We decided the doxycycline dose based on its effectiveness in generating a response in p-AKT levels. 1 µg/ml doxycycline seemed to reduce both S473 and T308 phosphorylations in all three cell lines LNCaP, C4-2 and PC3. **(Figure 3.3)** PTEN expression levels, nevertheless, were not comparable between the cell lines. The reason is that C4-2 cells cannot tolerate high levels of PTEN expression and we observed an accumulation of death cells when the increased doxycycline doses were tried to achieve a greater PTEN expression.

We verified the efficacy of our doxycycline induction and culture conditions by checking PTEN protein levels and AKT phosphorylations. Our results confirmed that our cell culture conditions for metabolomics were suitable to display the impact of PTEN presence. All four cell lines LNCaP, C4-2, PC3 and PNT1A showed the impact of PTEN in AKT phosphorylations. **(Figure 3.5.1 – panel B)**

Our samples were shipped to Haznedaroglu lab for further preparation and targeted mass spectrometry. Since we had the preliminary data suggesting that sphingolipid metabolites were the most altered ones upon PTEN re-expression in LNCaP and C4-2 cell lines, **(Figure 3.4 – panel B and C)** we focused only on SPL metabolites including ceramides, ceramide-1-phosphate, sphingosine-1-phosphate and sphingomyelins – the data belonging to sphingomyelin and ceramide-1-phosphate quantification were not exhibited and discussed in my thesis.

Our screening quantified the intracellular concentrations of three different ceramides species classified as Cer(d18:1/18:0), Cer(d18:1/16:0) and Cer(d18:1/20:0). The nomenclature of ceramides defines the composition of the two acyl chains. The first one is “d18:1” which is common in all ceramides since it belongs to the sphingosine backbone containing 18 carbon length tail with a double bond. The second one varies in length and defines the specific ceramide species. (Liebisch et al., 2013)

Our results revealed that PTEN expression led to the enrichment of Cer(d18:1/16:0) as well as Cer(d18:1/18:0) species in LNCaP, C4-2, PC3 (Cer(d18:1/18:0) was undetectable in PC3 lines) and PNT1A cell lines, which indicates that PTEN regulates Cer(d18:1/16:0) and Cer(d18:1/18:0) levels independently of the cellular transformation status. **(Figure 3.5.2 – panel A and B)** As for the Cer(d18:1/20:0) metabolite, significant elevation upon PTEN expression could be observed

only in LNCaP and C4-2 lines while PTEN expression did not change the level of this ceramide in other cell lines. (**Figure 3.5.2 – panel C**)

We also compared the level of these metabolites between LNCaP and C4-2 cells to analyze if ADT-resistance has an impact on SPL metabolism. Our results showed that the levels of Cer(d18:1/18:0) and Cer(d18:1/20:0) species elevated in C4-2 cells when compared to LNCaP cells while the level of Cer(d18:1/16:0) remained unchanged. (**Figure 3.5.2**) Our interpretation is that SPL metabolism is upregulated upon the acquisition of ADT-resistance given that ceramides are the core metabolites of SPL metabolism that can be used as a starting molecule of multiple metabolites. (**Figure 1.4**)

The reason why the different ceramide species did not follow the same pattern could be explained by the fact that their synthesis is under the control of different ceramide synthases (CERSs). CERS1 and CERS4 are responsible for C18-20 ceramide synthesis; CERS5 and CERS6 produce C14-16 ceramides while CERS2 selectively generates C22-24 ceramides. (Kraveka et al., 2007 and Raichur et al., 2014) According to our results, CERS1,4,5 and 6 should be regulated by PTEN activity. (**Figure 3.5.2**)

Apart from the central molecule ceramide, our screening measured the levels of well-characterized sphingolipid species including sphingosine-1-phosphate. Our results revealed that the oncogenic S1P metabolite levels dropped significantly following PTEN re-expression in AR-independent cell lines PC3 and C4-2. In line, C4-2 cells had higher abundance of S1P when compared to their AR-dependent counterpart LNCaP. This inclined us to speculate that S1P metabolites could facilitate ADT-resistant stage emergence in PTEN-null cells. The S1P levels did not seem to be affected by PTEN status in PNT1A cells while it followed an unexpected pattern in LNCaP cell lines with an increase of S1P metabolites upon PTEN expression. (**Figure 3.5.3**)

We further investigated the impact of PTEN on S1P metabolism through qPCR analysis to detect if PTEN controls the mRNA transcription of S1P metabolism regulating enzymes. We used the cell lines used in our metabolomics screening by keeping the experimental conditions still. Our results suggested that LNCaP, C4-2 and PC3 cells upregulated SPHK1 and also S1PR gene expressions in PTEN-null cells. (**Figure 3.6.1**) In PC3 cells, an increase in SPHK2 expression was also observed in PTEN-null cells. By following these findings which are in line with our metabolomics screening, we proceeded with drugs that target S1PR signaling and SPHKs.

To start, we used an FDA-approved and non-isoform selective S1PR inhibitor called FTY720. (Sharma et al, 2011) All the cell lines we tested in cell viability assays, which were LNCaP, C4-2, PC3 and PNT1A, showed decreased sensitivity to S1PR inhibition under PTEN-intact condition. These results imply that PTEN-null cells appear to rely more on S1PR signaling for growth and survival. Among our cells, the most sensitive cells to FTY720 were PC3 line with a IC50 value of 1.64 μ M followed by LNCaP, C4-2 and PNT1A in order. (**Figure 3.7.1**)

We, then, investigated if FTY720 acted through PI3K pathway. To this end, we treated PC3 cells with increasing doses of FTY720 and conducted western blot analysis to detect changes in PI3K-driven phosphorylation events. The reason why we proceeded with PC3 cells was that they had shown greater reliance on S1PR signaling compared to the other prostate cancer cell lines in our previous experiments. Our results highlighted PI3K pathway as one of the S1PR signaling effectors. Furthermore, p110 α and p110 β protein levels were decreased upon FTY720 treatment, which may explain the underlying mechanism of FTY720 action on PI3K pathway. (**Figure 3.7.2**)

Completing our analysis with non-isoform selective S1PR modulator FTY720, we revised our experimental workflow with an FDA-approved isoform-specific S1PR modulator called ACT128800 acting on S1PR1 (Piali et al., 2011) since S1PR1 isoform was regulated by PTEN in multiple cell lines in our qPCR analysis. (**Figure 3.6.1**) Our experiments with ACT128800 yielded parallel results with FTY720 experiments showing that PTEN presence has a protective effect against S1PR1 inhibition in prostate cancer cells. (**Figure 3.7.3**)

We focused not only on S1P signaling by targeting S1PRs but also on the production of S1P through targeting SPHKs. Our western analysis showed that SPHK1 protein levels were reduced in C4-2 and PC3 cell lines upon PTEN re-expression while we could not detect an expression in LNCaP lines compared to C4-2 and PC3 cells. (**Figure 3.8.1**) This finding supports our qPCR results (**Figure 3.6.1**) and identifies SPHK1 levels as an effector regulated by PTEN status.

We, then, conducted cell viability experiments with isoform specific drugs against SPHK1 and SPHK2 named as PF543 and ABC294640, respectively. The PTEN status of LNCaP and PC3 cells seem to mildly affect the response to SPHK1/2 inhibition in an expected manner by exhibiting PTEN presence as a protective mechanism. On contrary, C4-2 cells showed an increased reliance on SPHK1/2 for survival in PTEN-WT condition, which was an interesting finding. PNT1A cells,

nevertheless, were not markedly affected by PTEN status in their SPHK1 inhibition response. **(Figure 3.8.2 and Figure 3.8.3)**

Since we identified a partial reliance on p110 β , p110 δ isoforms and S1P/S1PR signaling as targetable vulnerabilities of PTEN-null metastatic prostate cancers, we aimed to generate a novel strategy to co-target these to efficiently eliminate PTEN-null cells while sparing the PTEN-intact ones. Our experimental setting, therefore, utilized the use of p110 β , p110 δ isoforms and S1P/S1PR inhibitors in dual combinations. We assessed the cell viability with SRB assay and started our experiment with increased number of cells compared to IC50 experiments to see how efficient our combination to destroy well-populated PTEN-null prostate cancer cells. Our approach of evaluating synergy was to conduct CDI calculation based on the differences between single-dose and combination response. Although multiple mathematical models exist to assess drug interactions -including Bliss independence, Loewe additivity, Highest single agent (HSA), zero interaction potency-, we proceeded with CDI calculation because it offers a simple approach with minimal assumptions regarding the mechanisms of the drugs used. (Duarte and Vale, 2022)

To test the efficacy of p110 β and S1PR co-inhibition, we utilized two structurally independent p110 β inhibitors namely KIN193 and GSK2636771 and also FTY720 as S1PR inhibitor. GSK2636771 & FTY720 and KIN193 & FTY720 combinations yielded results parallel to one another, as expected. To get into more detail, LNCaP cells showed a moderate synergistic response with the lowest CDI value as 0.78. C4-2 cells, on the other hand, displayed a significant synergy with the CDI value of 0.62 while PC3 cells also responded moderately synergistic with the 0.77 CDI value as the lowest. **(Figure 3.9.1 and Figure 3.9.2)**

We repeated the combinatory approach by targeting p110 δ isoform using CAL101 and FTY720. The results followed the same pattern as p110 β inhibiting combinations. In other words, LNCaP and PC3 cells returned a moderately synergistic response with 0.84 and 0.73 as the lowest CDI values respectively while C4-2 showed a synergistic response with 0.69 CDI value. **(Figure 3.9.3)**

Since we highlighted that both mRNA and protein levels of SPHK1 are regulated by PTEN status **(Figure 3.6 and Figure 3.8.1)**, we utilized SPHK1 inhibitor PF543 for our combinatory strategy by combining it with GSK2636771, KIN193 and CAL101. C4-2 cells did not return any synergistic interaction with these combinations. The CDI values pointed only additive responses

with CDI values around 1. (**Figure 3.9.4 and Figure 3.9.6**) We speculate that the reason might be the selective reliance on SPHK2 isoform in C4-2 cells given that IC50 values for ABC294640 was significantly lower than that of PF543 in C4-2 cells. (**Figure 3.8.2 and Figure 3.8.3**) On the other hand, LNCaP cells had a moderately synergistic response with the lowest CDI value of 0.75 in p110 β and SPHK1 co-inhibition while the lowest CDI value was determined as 0.83 in p110 δ and SPHK1 co-inhibition. PC3 cells responded synergistically to both p110 β i & SPHK1i and p110 δ i & SPHK1 inhibitions with 0.65 and 0.56 as the lowest CDI values respectively. (**Figure 3.9.4, Figure 3.9.5 and Figure 3.9.6**)

The co-targeting either p110 β or p110 δ along with S1P production/S1PR signaling constitutes a targetable synergistic opportunity for the treatment of PTEN-null metastatic prostate cancers. Our approach, however, becomes limited for other PTEN-depleted cancers. It has been shown that PTEN deletion does not uniformly lead to p110 β dependency across all cancer types. For example, PTEN deletion leads to p110 β in prostate cancer while it does not alter p110 α dependency in pheochromocytoma and also in glioblastomas. (Berenjeno et al., 2012) In addition, the impact of PTEN deletion on isoform dependency is also regulated by the co-occurring genomic alterations. Schmit et al. 2014 showed that ovarian cancer cells remain dependent on p110 β when Kras gene is wild-type. Nevertheless, cells become dependent on p110 α upon the expression of KRasG12D. In a nutshell, our proposed combinatory strategy remains limited to PTEN-null prostate cancers.

5. CONCLUDING REMARKS

PTEN loss is an early and frequent event in prostate cancer progression with increased occurrence in metastatic incidents. (Barbieri et al., 2013) For this reason, we focused on the effect of PTEN status on prostate epithelia in terms of metabolism and PI3K signaling to design a novel therapeutic strategy. As discussed, we identified that PTEN loss partially alters PI3K class IA reliance from p110 α to both p110 β and p110 δ through western blot and cell viability analysis. Additionally, we previously conducted metabolite set enrichment analysis and identified that sphingolipid metabolism is the most altered metabolic pathway upon PTEN loss. In order to detect the reprogramming of sphingolipid metabolism, we employed targeted metabolomics approach and concluded that metabolic shift occurs from ceramide species to sphingosine-1-phosphate metabolism in PTEN-null and ADT-resistant C4-2 and PC3 cells. In parallel to this finding, we identified PI3K pathway as an effector of S1PR signaling in PC3 cells. SPHK1 protein and mRNA levels, moreover, were upregulated upon PTEN loss in C4-2 and PC3 cells. In other words, PTEN elevates S1P levels by upregulating SPHK1 enzyme and S1PR activates PI3K in return.

To efficiently eliminate PTEN-null metastatic PCa cells, we co-targeted p110 β /p110 δ with S1PR/SPHK1 and obtained synergistic results indicating that these drugs potentiate the toxicity of one another.

As depicted in **Figure 5.1**, our rationale behind the combinatory treatment is to co-inhibit oncogenic S1PR-driven signaling and aberrantly active p110 β /p110 δ to synergistically reduce the viability of PTEN-deficient PCa cells. Although PI3K pathway is a downstream effector of S1PR signaling, we could obtain synergistic results probably due to a potential crosstalk between other effector pathways.

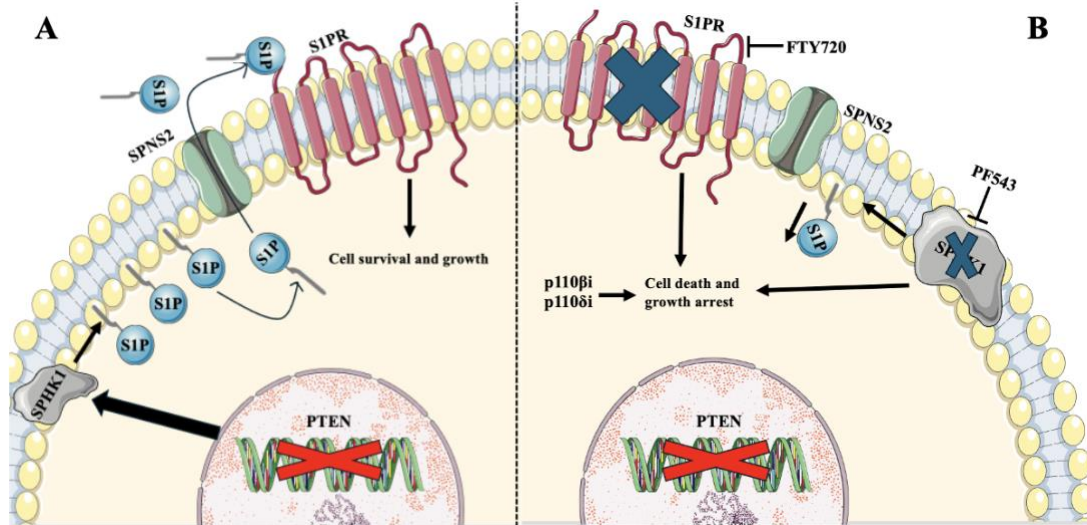


Figure 5. 1. Schematic representation of PTEN-regulated S1P metabolism and co-targeting of PTEN loss-driven alterations in the S1P and PI3K pathways in PTEN-null and hormone refractory prostate cancer. A) The aberrantly upregulated S1P production and signaling in PTEN-null and ADT-resistant prostate cancer to promote cell survival and growth. B) The action mechanism of our novel combinatory approach which relies on dual inhibition of p110β/p110δ alongside S1P production/signaling to unite two PTEN-regulated targetable nodes.

6. FUTURE PERSPECTIVES

In the scope of this project, we managed to show effective growth inhibition of PTEN-null prostate cancer with co-targeting p110 β /p110 δ and S1PR. The next step to further validate our approach is to recapitulate our combinatory treatment in a more physiologically relevant settings such as spheroid models or anchorage-independent soft agar growth experiments. Since combinatory treatments can lead to systemic toxicity or immune dysregulation, our approach should be also tested in *in vivo* models such as nude mice xenografts and orthotopic prostate cancer models.

Additionally, the mechanism behind the synergism should be investigated further if it arises from shared downstream effectors or parallel compensatory pathways since this would guide patient stratification.

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