

**DETECTION OF PHOSPHORYLATION SIGNATURES
SPECIFIC TO CANCER-RELATED PI3-KINASE ISOFORMS
P110 α AND P110 β**

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By
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DETECTION OF PHOSPHORYLATION SIGNATURES
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P110 α AND P110 β

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January 2023

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

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ABSTRACT
DETECTION OF PHOSPHORYLATION SIGNATURES
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M.S. in Molecular Biology and Genetics

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The PI3K signaling pathway is required for many physiological activities, but it is commonly disrupted during cancer formation. The PI3K p110 α and β isoforms, encoded by the PIK3CA and PIK3CB genes, are lipid kinases that phosphorylate PIP2 to PIP3 to activate the PI3K pathway. However, the distinct molecular targets of these isoforms have yet to be discovered, making targeted treatment problematic. According to cancer genomics research, the PIK3CA gene is commonly altered in cancers, but the PIK3CB gene is frequently amplified. The clinical usage of Pan-PI3K inhibitors has resulted in significant side effects, prompting the development of isoform-specific inhibitors. However, it has been shown that these drugs trigger alternate signaling systems downstream, leading in resistance to single-agent treatment. Our research intends to uncover distinctive protein-protein interactions of PI3K isoforms, as well as the consequent different phospho-proteomic signatures, which might be crucial determinants of specific cellular activities. This will be accomplished by using

isogenic MEF cells that are only dependent on the p110 α or p110 β isoforms, isoform-specific pharmacological inhibitors BYL-719 and KIN 193, and a high-resolution mass spectrometry-based method to determine the phosphorylation levels of these protein samples. The predictive biomarkers discovered in this study can be utilized to identify patients who will benefit from PI3K-targeted drugs and to better understand the resistance mechanisms that may arise in response to pathway inhibition.

Keywords: PI3K signalling, PIK3CA isoform, PIK3CB isoform, phosphoproteomics, BYL719, KIN193, cancer, resistance, mass spectrometry, IMAC, phosphorylation

ÖZET

KANSERLE İLİŞKİLİ PI3-KINAZ P110 α VE P110 β IZOFORMALARINA ÖZEL FOSFORILASYON İMZALARININ BELİRLENMESİ

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PI3K sinyal yolu, birçok fizyolojik aktivite için gereklidir, ancak genellikle kanser oluşumu sırasında bozulur. PIK3CA ve PIK3CB genleri tarafından kodlanan PI3K p110 alfa ve beta izoformları, PI3K yolağını aktive etmek için PIP2'yi PIP3'e fosforile eden lipid kinazlardır. Bununla birlikte, bu izoformların farklı moleküler hedefleri henüz keşfedilmemiştir, bu da hedefe yönelik tedaviyi sorunlu hale getirir. Kanser genomik araştırmasına göre, PIK3CA geni kanserlerde yaygın olarak değiştirilir, ancak PIK3CB geni sıklıkla amplifiye edilir. Pan-PI3K inhibitörlerinin klinik kullanımı yan etkilere yol açtığından, izoforma özgü inhibitörlerin geliştirilmesine yol açmıştır. Bununla birlikte, bu ilaçların aşağı yönde alternatif sinyal sistemlerini tetikleyerek tek ajan tedavisine direnç yol açtığı gösterilmiştir. Araştırmamız, PI3K izoformlarının ayırt edici protein-protein etkileşimlerinin yanı sıra, bunun sonucunda ortaya çıkan, belirli hücresel aktivitelerin çok önemli belirteci olabilecek farklı fosfo-proteomik imzaları ortaya çıkarmayı amaçlamaktadır. Bu, yalnızca p110 alfa veya p110 β izoformlarına bağımlı olan izojenik MEF hücreleri, izoforma özgü

farmakolojik inhibitörler BYL-719 ve KIN 193, ve fosforilasyon seviyelerini belirlemek için yüksek çözünürlüklü kütle spektrometresi tabanlı bir yöntem kullanılarak gerçekleştirilecektir. Bu çalışmada keşfedilen öngörücü biyobelirteçler, PI3K hedefli ilaçlardan fayda görecektir hastaları belirlemek ve yolak inhibisyonuna yanıt olarak ortaya çıkabilecek direnç mekanizmalarını daha iyi anlamak için kullanılabilir.

Anahtar Sözcükler: PI3K sinyal yolağı, PIK3CA, PIK3CB, fosfoproteomiks, byl719, kin193, kanser, direnç, kütle spektrometresi, imak, fosforilasyon



For the hope of those battling with cancer

In memory of those we lost

For the joy of those who beat it

And

For their families and friends.

To Nana, Nanu

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ABBREVIATIONS:

PTEN Phosphatase and tensin homolog

PI3K Phosphatidylinositol-3 kinase

PIP3 Phosphatidylinositol-3,4,5-triphosphate

PIP2 Phosphatidylinositol-4,5-bisphosphate

AKT Serine/threonine kinase

PDK1 3-phosphoinositide-dependent protein kinase 1

mTOR Mammalian target of rapamycin

PH Pleckstrin homology

Btk Bruton's tyrosine kinase

PDGF Platelet-derived growth factor

LPA Lysophosphatidic acid

DKO Double Knock out

WCL Whole Cell lysate

IP. Immunoprecipitation

MEF Mouse Embryonic Fibroblas

AURKA Aurora Kinase A

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CHAPTER 1: INTRODUCTION

1.1 Phosphoinositide 3-kinases (PI3Ks)

Phosphoinositide 3-kinases (PI3Ks) are a family of enzymes that play a role in a variety of cellular processes, including cell growth, proliferation, differentiation, motility, survival, and intracellular trafficking. These enzymes are activated by various stimuli, including growth factors, hormones, and stress signals, and they act by phosphorylating the 3-position hydroxyl group of the inositol ring of phosphoinositides.

PI3Ks are enzymes that phosphorylate the 3' position of the inositol ring of phosphatidylinositol (PI) and its derivatives, a process known as phosphorylation. This reaction produces a number of new lipid products, including PI(3)P, PI(3,4)P₂, and PI(3,4,5)P₃, which are called second messengers. These second messengers have the ability to influence several downstream pathways by recruiting the effector proteins to the cellular membranes .

PI3Ks are important therapeutic targets for a variety of diseases, including cancer, diabetes, and autoimmune disorders. Inhibitors of PI3Ks are being developed as potential treatments for these diseases.

1.1.1 Classification of PI3Kinases:

There are three classes of PI3Ks: Class I, Class II, and Class III which are classified depending on their substrate preference and structural homology [2]

Class I PI3Ks has four members, namely p110 α , p110 β , p110 δ , and p110 γ , which are further sub-divided in accordance with which regulatory subunit they associate with. p110 α , p110 β , and p110 δ , that form the Class I A isoforms associate with the p85

subunit, whereas p110 γ binds to either p101 or p87 subunit. These differences in regulatory subunit binding may contribute to the distinct functions of these subunits [2]

Class I PI3Ks are the most well-studied and are further divided into three subclasses: Class IA, Class IB, and Class IC. Class IA PI3Ks, also known as p110 α , p110 β , and p110 δ , are activated by receptor tyrosine kinases and are involved in cell growth and proliferation. They are activated by growth factors, hormones, and other stimuli, and they act by phosphorylating the 3-position hydroxyl group of the inositol ring of phosphoinositides.

Class IB PI3Ks, also known as p110 γ , are activated by G protein-coupled receptors and are involved in intracellular trafficking and other processes. They are activated by hormones and neurotransmitters, and they act by phosphorylating the 3-position hydroxyl group of the inositol ring of phosphoinositides.

Class IC PI3Ks, also known as p110 η , are activated by receptor tyrosine kinases and are involved in immune cell activation and migration. They are activated by immune stimuli, such as antibodies and cytokines, and they act by phosphorylating the 3-position hydroxyl group of the inositol ring of phosphoinositides.

Class I PI3Ks are important therapeutic targets for a variety of diseases, including cancer, diabetes, and autoimmune disorders. Inhibitors of Class I PI3Ks are being developed as potential treatments for these diseases.

1.1.2 Structure of Class I PI3K

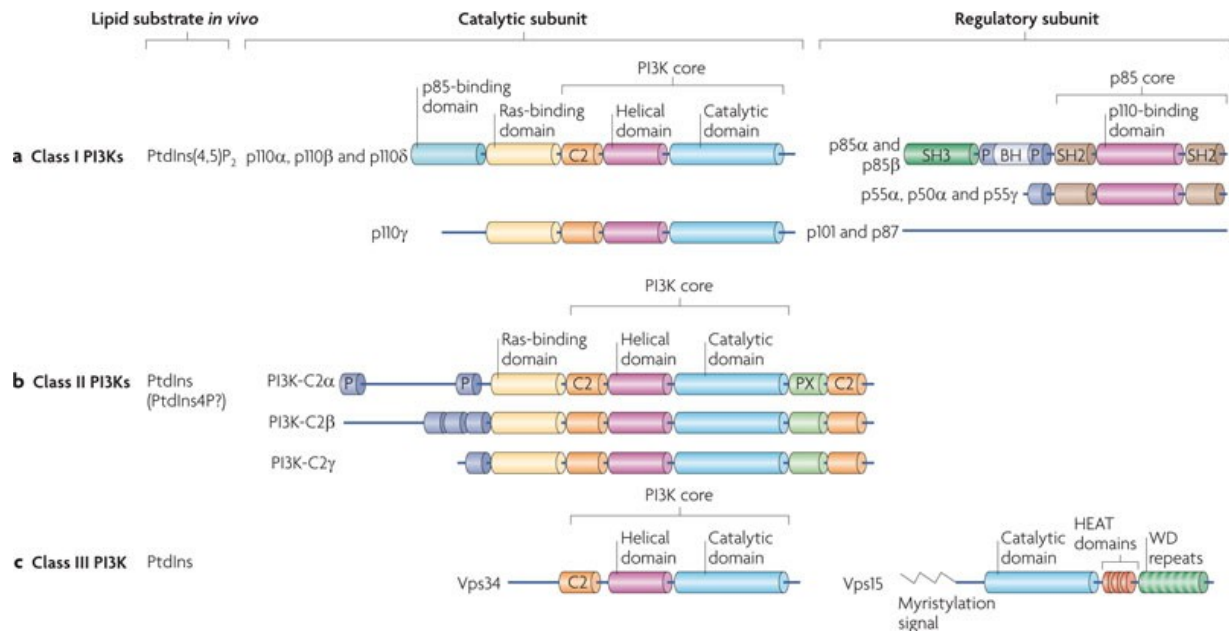
Catalytic subunits of PI3Ks are kept inactive and closed in resting cells by the Class I regulatory subunits of PI3Ks [3]. As the catalytic subunits are unstable on their own, the regulatory subunits recruit the catalytic subunits to active receptors upon stimulation and stabilise them[4,5]. Class IA catalytic subunits of PI3Ks have an extra

p85-binding domain, while all Class I catalytic subunits share a conserved PI3K core and a Ras-binding domain. This enables them to bind to p85 regulatory subunits preferentially[5]. The proline-rich region and the p110-binding domain with SH2 domains that bind to phosphorylated tyrosine residues are two of the domains that make up the p85 regulatory subunits[1]. Additional proline-rich regions, Bcr homology, and SH3 domains found on the p85 and p85 subunits may also aid in signalling. Although the role and structure of the p87 and p101 regulatory subunits in p110 signalling are unknown, they can assist in bringing p110 to Ras and G protein-coupled receptors [1].

Proteins with lipid-binding domains, like pleckstrin homology (PH) domains, can be recruited to the plasma membrane by Class I PI3Ks, which catalyse the production of PIP3 from PI(4,5)P2 [7]. All cell types contain class I PI3Ks, though the distribution of the various isoforms varies: p110 γ and p110 δ are enriched in leukocytes, whereas p110 α and p110 β are expressed ubiquitously in other cell types [6]. Mice lacking these isoforms exhibit embryonic lethality, while those lacking p110 γ and p110 δ are viable but have immune defects. This isoform lethality may be caused by the ubiquitous tissue distribution of p110 α and p110 β [8]. It is imperative to remember that PI3K signalling can vary in various cell- and signal-specific contexts, both upstream and downstream.

PI3Ks also exhibit protein kinase activity in addition to their lipid kinase activity [9]. The PI3Ks themselves are the best understood substrates for this action; p110 phosphorylates p85 at Ser608 whereas the other isoforms autophosphorylate the p110 subunit [10]. It has been suggested that (auto)phosphorylation may function as a potential negative regulatory mechanism for the class IA isoforms since it decreases

their lipid kinase activity. Although little research has been done to determine the probable functions of such phosphorylations, exogenous substrates have also been identified.



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Figure 1.1: Classification of PI3Kinases A) The four class I PI3Kinases are heterodimers made of a catalytic subunit and a regulatory subunit, that have a high degree of structural homology. B) Class II PI3Kinases are monomers that have a PI3K core composed of Ras bindind domain, helical and catalytic domain C) Class III PI3K heterodimer which is membrane bound [1]

1.2 Upstream Signalling of the PI3K pathway

Class I PI3Ks were first classified based on variations in how activation by RTKs and GPCRs was recognised, as well as regulatory subunits (Fig. 1.2.). The actual situation is, however, much more complicated than a straightforward division into classes IA and IB. Ras can also activate p110α and p110δ, however the p85/p110 complex gets recruited to pTyr residues in RTKs or adaptor proteins is the primary mechanism [11,

12]. In contrast to p110 γ , which is activated downstream of Ras and G protein coupled receptor (GPCR) G subunits, p110 β is activated by all three of these mechanisms as well as perhaps by other families of small GTPases, such as the Rab5 family.

Canonical Class I activation is triggered in a downstream manner, after the activation of growth factor receptors [2]. When platelet-derived growth factor (PDGF) attaches to its receptor, it triggers the receptor's activation and autophosphorylation of intracellular regions. Class IA PI3Ks are recruited to the plasma membrane, where they catalyze the synthesis of PIP3 from PI(4,5)P2, upon recognition and binding of these pTyr residues by their respective SH2 domains. Furthermore, adaptor proteins including Grb2 and the insulin receptor substrate (IRS) -1 can mediate the indirect recruitment of class I heterodimers to RTKs. To be completely active, p110 α may only need the N-terminal SH2 domain to bind to pTyr [13], whereas p110 β and p110 δ seem to need both SH2 domains of p85 to be linked to pTyr. The fact that p110 α is the sole PI3K identified mutated in cancer [14] may be due to its less stringent requirement for activation and widespread tissue expression.

Class IB PI3K p110 γ is activated after GPCR activation [15]. Although the precise method by which the G $\beta\gamma$ subunit switches from inactive to active state by dissociating from G α and recruiting p110 γ is not yet known. Evidence suggests that GPCRs are upstream of p110 β [17], which is also triggered by direct contact with the G $\beta\gamma$ subunit.

Ras family members have long been hypothesized to activate all four class I isoforms due to the presence of a Ras binding domain (RBD) in each of these proteins. New data, however, reveals that p110 β is an outlier and is activated only after other small GTPases [18]. Ras is a large family with many members; Ras/PI3K interactions likely vary depending on the kind of cell and the agonist present. Since p85 may inhibit Ras

stimulation, it follows that class IA isoform activation occurs downstream of Ras but via RTKs [19]. These contrasting dependencies on upstream agonists are also seen between p101 and p87 for p110 γ .

Although p110 α and p110 β share a great deal of sequence similarity, they also differ in the RBD, which may account for their dissimilar sensitivities to small GTPases [18]. Rab and the Rho family of GTPases may also have a role in activating p110 β [18], complementing the inputs from RTKs and GPCRs. It has been hypothesized that p110 β plays a function in endosome formation since Rab5 is localized to these organelles and because p110 β is bound to clathrin-coated vesicles [22, 21]. It was previously believed that Ras activated p110 β , but new data suggests that this interaction does not occur and that the Rho superfamily is actually upstream of p110 [13]. It's possible that the intricacy and situations surrounding the relationship between PI3K and different families of small GTPases need a reevaluation of the existing understanding.

Concerns persist about the relevance of the several regulatory isoforms and the degree of overlap between the various inputs, highlighting the intricacy of upstream PI3K activation. It has been established that there is some functional redundancy across isoforms of PI3Ks, and that the input to and output from PI3Ks will change depending on the specific geographical and temporal context. Signalling studies that often only

track the response of one agonist oversimplify the true physiological spectrum of stimuli since cells and tissues are not confronted with a single input.

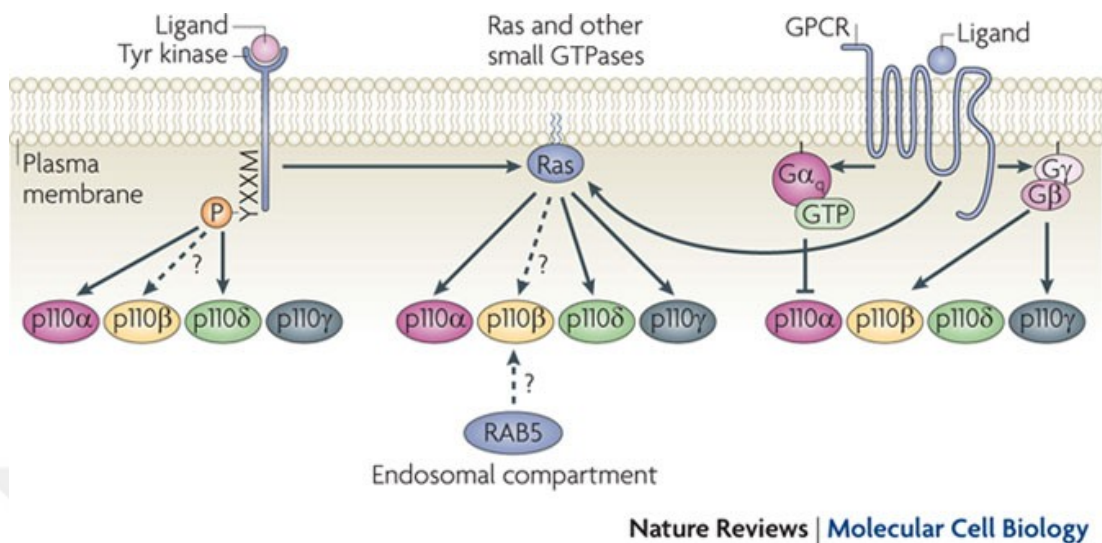


Figure 1.2 Upstream signalling of class I PI3Kinases RTKs regulate class IA isoforms while p110γ is a downstream component GPCRs, and Ras influencing all isoforms. p110β is also controlled by GPCRs, with indication of the involvement of additional small GTPases [1].

1.3 Downstream Signalling of class I PI3Kinases

PI3Ks have the capacity to phosphorylate both lipid molecules and proteins due to their dual selectivity [1, 19].

1.3.1 Lipid kinase activity of PI3Ks

The physiological effects of PI3Ks are mostly caused by their lipid kinase activity, which is the most well-studied function of these kinases.

Effector proteins with lipid-binding domains (such as PH or PX domains) recognize phosphoinositide species with various degrees of selectivity, resulting in membrane recruitment [23]. The function, activity, or binding partners of the protein may then be impacted by this shift in localization. The class I isoforms all catalyze the acute

synthesis of PIP3 from PI(4,5)P2, a lipid species that is present in all cells, despite the wide range of upstream signaling [24]. PIP3 is a transient protein that is recognized by proteins with PH domains. The serine/threonine kinase Akt/protein kinase B (PKB), which is the most well-studied PI3K effector, is addressed in more depth below for its function. [25].

Effector proteins can prolong the signaling cascade by being recruited to the plasma membrane. For instance, the kinase PDK1 [26], which is susceptible to PIP3 levels, phosphorylates and activates Akt. Following then, Akt continues to phosphorylate a variety of proteins, changing many processes including, survival, metabolism and proliferation [27].

1.3.2 Akt

One of the most thoroughly studied PI3K effectors is Akt. Akt is a member of the AGC family of protein kinases, which also includes PKA, PDK, and serum- and glucocorticoid-induced protein kinase (SGK), and was first discovered by its similarity with previously known protein kinases [27]. The kinase has three isoforms, Akt1, 2, and 3, each of which is encoded by a distinct gene and has a varied distribution in tissues. There are three conserved domains that are shared by all the isoforms a membrane binding N-terminal lipid binding PH domain, a catalytic kinase domain, and a C-terminal hydrophobic motif that is present in other AGC family members in the regulation of metabolism [30]. Brain and metabolic abnormalities are caused by Akt binds to both PI(3,4)P2 and the PIP3 through its PH domain, which causes relocalization to the plasma membrane from the cytoplasm [27]. The Akt gets localized to the membrane which brings it into a closer proximity with the activating kinases upstream to it. This causes a conformational change in it which cause an increase in

its kinase activity slightly but two more phosphorylation events that follow increases its activity by 1000 times. PDK1 that gets recruited to the plasma membrane by its PDH domain first phosphorylates Thr308 [26]. As a result, Akt's kinase activity is increased by around 100 times, and a further 10 times by phosphorylating the Ser473 residue [32], a process mTORC2 catalyzes [33].

These two residues, which are found in different domains of the kinase, must be phosphorylated in order for the kinase to be fully activated. The activation or T-loop region, where the Thr308 residue of Akt1 is situated, is a domain shared by all kinases. The Ser473 residue is part of a C-terminal hydrophobic motif, and other members of the AGC family are frequently activated by phosphorylation of residues in this area [34]. Protein phosphatase 2A (PP2A) and PH domain and Leucine rich repeat Protein Phosphatase (PHLPP) both target Ser473 and remove the phosphorylations from the proteins, respectively [35, 36]. These phosphorylations, however, are not necessarily a precise indicator of the activity of Akt or of upstream or downstream pathways.

Akt is a crucial component of many signaling pathways. It links growth factor signaling and PI3K activity to many cell processes, many of which have been linked to the development of cancer. These pathways are not controlled by just one Akt substrate, and some substrates may have an effect on more than one output. Targets of AKt can change depending on the signal and the cell, which lets the response be fine-tuned. This lets Akt control many different types of cellular signaling pathways, such as survival, metabolism, and growth [27].

Mechanistic target of rapamycin complex 1 activation is one of the most well-known outcomes of Akt activation (mTORC1). Akt participates in the regulation of mTORC1 when it phosphorylates and inhibits, TSC2 [42, 43, 44] and (PRAS40) which are

negative regulators of mtorc1. [41, 45]. mTORC1 is a crucial regulator of cell growth, proliferation, and metabolism.

Although Akt is predominantly associated with the PI3K signaling pathway, it is also somewhat involved in other signaling axes. Since Akt interacts with a variety of signaling pathways, including mitogen-activated protein kinase (MAPK) pathways, it is possible to fine-tune the signaling process more than would initially be the case [54, 55, 56].

1.3.3 mTOR

A serine/threonine protein kinase called mTOR controls key signaling pathways through its enzymatic activity. In order to regulate the nutrients in the environment and cellular development, it is able to respond to environmental stimuli by responding to growth factors and stress with metabolism and cell destiny. mTOR is frequently viewed as a sensor that enables the cell to alternate between metabolic expansion when nutrients and cellular building blocks are abundantly accessible and energy conservation and recycling during times of deprivation. Due to its crucial role as a regulator, it has been linked to metabolic disorders including diabetes [57] and cancer [58], where it serves as the cell's energy source.

mTOR is a member of the PI3K-signalling axis and that it exists as two complexes (mTORC1 and mTORC2) made up of different regulatory proteins allows the two complexes to perform distinct activities independently of one another [59]. Through feedback loops, the mTOR complexes are activated, which affects PI3K signaling [63]. In order to suppress IRS1, p70 ribosomal S6 kinase (S6K) is activated by mTORC1 (Manning 2004, [64]). On the other hand, Akt is further stimulated by a positive feedback loop in which mTORC2 participates [33].

The mTOR complexes are important controllers of cell growth and metabolism. The more well-studied of the two complexes, mTORC1, positively controls anabolic processes like protein and lipid synthesis and negatively controls catabolic activities like autophagy[57]. [66]. mTORC2 has the ability to regulate a variety of functions, including metabolism, survival, growth, and cytoskeletal polarity.

1.3.4 Protein Kinase Activity

PI3Ks have the ability to phosphorylate proteins in addition to their conventional lipid kinase activity [9]. All three PI3K classes have reported this activity, although there are still a lot of unanswered concerns. Although class I PI3Ks' protein kinase activity has been known about for decades, it has not been well studied, despite the fact that its lipid kinase activity has been fairly well characterized. Although numerous substrates have been found for other isoforms and autophosphorylation is a potential mechanism for self-regulation, it is not known if the protein kinase activity holds any relevance in vivo.

1.4 PTEN phosphatase activity

These proteins transduce the PI3K signal, whereas others terminate it. PTEN, a lipid phosphatase, removes the 3' phosphate group of PIP3 to form PI(4,5)P2, ending the PI3K-signal [67, 68]. It is considered a crucial regulator in the progression of cancer; PTEN comes second to p53 in frequency where it is lost in cancer, by being missing on chromosomal level or produced in truncated forms [69]. PTEN can function as both a lipid and a protein phosphatase, contrary to how PI3Ks may function as both lipid and protein kinases [70, 69]. The crystal structure of PTEN [72] revealed that its active site is big enough to tolerate phosphorylated serine, threonine, and tyrosine.

1.5 Isoform-specific functions of class IA PI3K:

There are similarities between the roles played by p110 α and p110 β in metabolic, proliferative, and other downstream processes. Although the difference in effects made by the upstream activators of these two isoforms had been, some functional differences have also been shown using animal models and pharmacological techniques.

In mice, homozygous p110 deletion results in embryonic lethality [7]. Embryonic fatality is seen when a kinase dead version of p110 α is knocked in [73]. Although they are less sensitive to insulin, when mice are knocked in for the allele in a heterozygous fashion, they are alive and reproductive [73]. The use of pharmacological agents demonstrated that p110 α is crucially located downstream of the insulin receptor, whereas p110 β has little role [74]. p110 α is also vitally important in the regulation of angiogenesis. When class IA isoforms are compared, only p110 α , working through the small GTPase RhoA, was found to be essential for endothelial cell migration and vascular remodelling [75].

Mice with a homozygous deletion of p110 β are likewise embryonally fatal [76], whereas heterozygous litters are viable and fertile. It's interesting to note that whereas animals that express larger quantities of the same gene survive into adulthood, those who express lower levels of kinase-dead p110 are similarly embryonic fatal [77]. This shows that p110 β 's kinase activity is optional and could serve as scaffolding. The use of inhibitors revealed an early, isoform-specific involvement for p110 β in platelet function and thrombosis. Inhibition of p110 β decreased integrin bond binding and stability [78]. When p110 β was conditionally knocked off in platelets, it was discovered that this isoform was in charge of maintaining appropriate signaling downstream of both RTKs and GPCRs and that platelet aggregation was flawed [79]. These findings show the potential of p110 β as a thrombosis therapy target. Due to its

ability to bind the endocytotic regulator Rab5, p110 β has also been linked to endocytosis [10, 21]. Given that p110 β ablation results in abnormalities in receptor internalization that may be corrected by expressing kinase-dead p110 β , this may be a kinase-independent function [78, 80].

Both p110 α and p110 β appear to contribute differently to cancer, as will be explored below. In a nutshell, p110 α is mainly the PI3K that is shown to be mutated in cancer [81], and it has been demonstrated that p110 β is necessary for oncogenesis in certain PTEN-null prostate tumors [82].

The two isoforms abundant in haematopoietic cells are capable of producing various biological outcomes, just as p110 α and p110 β have a tissue distribution but have different roles that they may regulate. According to research on illness, p110 γ has been linked to immunology, the tumor microenvironment, and cardiac function [83]. As previously mentioned, there are variations in the substrates used by the isoforms' protein kinase activity [84].

1.6 PI3K isoforms' roles in diseases

Considering that the PI3K-Akt-mTOR axis forms the center of essential pathways such as cell growth, survival, and metabolism, it stands to reason that their signaling is frequently deregulated in a variety of illnesses. It makes sense that because PI3Ks are extensively expressed in immune cells and are activated downstream of growth hormones like insulin, they are involved in pathologies like diabetes and inflammatory disorders. The PI3K/Akt/mTOR pathway is responsible for a large number of the aberrations that cause a cell to proliferate rapidly and transform, hence mutations in this system are frequently seen in cancer [85]. Tumor development and survival are influenced by mutations that either cause gain of function mutation or cause loss of function mutations. The PI3K pathway is affected by both forms of mutation in many

malignancies, and mutations can take place at several nodes [85]. These alterations can be in RTK and ras that are upstream of PI3K either in Akt that is downstream, and in the regulatory subunit p85. These activating mutations take place near the SH2 domains and reduce inhibition of the class I PI3K catalytic isoforms [101]. Two of the most common mutations are in the p110 α [82] and PTEN genes in the PI3K pathway, which also happen to be two of the most frequently mutated genes in cancer. With mutations concentrating in two "hotspots" in the helical and kinase domains, p110 α is the widely found PI3K isoform that has been discovered to be mutated in cancer [82]. The H1047R mutation is in the kinase domain that mimics the activation by Ras[87, 88], but the E542K or E545K mutations in the helical domain alleviate p85's inhibition of p110 by simulating the association of pTyr residues.

P110 β isoform is mutated in approximately 5% of all cancer types when analysed in Pan-cancer analysis of whole genomes (ICGC/TCGA, Nature 2020) database, and most of the alteration is the amplification of the gene. Some PIK3CB mutations have also been discovered by the examination of commonly altered residues in tumor samples from distinct cell lineages and genome-wide screening. Cell proliferation, PI3K signaling, and cancer have been reported to be driven by the kinase domain mutation D1067V and the helical domain mutation E633K. (Whale et al., 2017). Another kinase domain mutation, E1051K, was discovered in metastatic castrate-resistant prostate cancer. The study of this mutant's functional properties reveals that it is a gain-of-function mutation that causes p110 β dependency and drives activation of the PI3K pathway, cell proliferation, and cancer (Whale et al., 2017).

The PI3K pathway can also be activated by losing the phosphatase PTEN, which has been linked to a dependence on p110 β in the setting of prostate cancer [82]. Tumor development was inhibited in PTEN-null mice by loss of p110 β but not p110 α [80].

PTEN-null cell line proliferation and colony formation, as well as tumor development, were adversely affected in other prostate cancer models by inhibition or siRNA-mediated knockdown of p110 β [89] [82].

Other PI3Ks than p110 α are involved in signaling paths that are crucial for the development and conservation of tumors, thus it maybe unforeseen that p110 α is the only PI3K that is vastly mutated in cancer. This might be explained by a variety of aspects of p110 α 's structure and signaling, as well as the fact that it can aid with many hallmarks of cancer. Although not as a result of mutation, p110 α can provide tumors [75] a chance at survival since it plays a unique function in angiogenesis, which other isoforms do not share. Tumors cannot grow past a size of 1 mm³ without a blood supply. Second, while in principle p110 γ might only contribute to haematological malignancies, p110 α is ubiquitously expressed [90], which means it can contribute to a variety of tumor types. This does not, however, account for the absence of typical oncogenic mutations in p110 β , which is likewise widely distributed throughout tissue. Different inhibitory forces that the regulatory subunit applies to the p110 protein have been indicated by structural analysis of the class I PI3K isoforms. While other class I isoforms have an additional inhibitory interaction at the cSH2, p110 α has a stronger affinity for the nSH2 and iSH2 domains of p85, rendering it more susceptible to oncogenic alteration than the other class I PI3Ks [13]. This is also true of upstream signaling: p110 α is largely activated by RTKs, as opposed to p110 β and p110 γ , which [91]. Interestingly, despite the fact that H1047 is the same residue that is altered in many malignancies, it was discovered that in these disorders the mutation causes benign mosaic hyperplasia rather than tumor growth. This adds an intriguing layer to the discussion of p110 α 's potential role in the development and maintenance of tumors.

Current findings has characterized a similar mutation in the p110 δ kinase domain. A single amino acid change (E1021K) boosted the isoform's activation and membrane association [92], which caused a variety of clinical characteristics such as respiratory illness and splenomegaly.

1.7 Intervention by pharmacological drugs

There has been an effort to target different nodes with pharmacological agents since the PI3K-Akt-mTOR signaling pathway is important in a number of diseases and therapeutic sets. Since down-regulation of an enzyme is typically easier to perform than up-regulation, efforts to limit kinase activity rather than boost phosphatase activity have seen the most advancement.

The human genome contains 518 kinases, and most, if not all, signal transduction cascades include them [93]. Because of this, kinases have been popular targets for therapeutic intervention for a long time, and both targeted and broad inhibitors are created to be investigated in labs and during clinical trials. The ATP-binding site of the kinase is often the target of these inhibitors, which are typically tiny compounds [94]. ATP competitive inhibitors of types I and II, as well as allosteric inhibitors, can be used to target the active site.

Since the catalytic region of kinases is highly conserved among family members, it can be challenging to achieve specificity for different kinases and isoforms; therefore, next-generation inhibitors may try to take advantage of differences in the rest of the molecule to achieve this desired specificity. The rapid growth of tumours and the selective pressure of drugs on kinases both contribute to the rapid emergence of drug-resistant cells in malignant tumours.

Even though there has been a lot of work put into trying to inhibit the PI3K pathway at different stages and in different malignancies, there hasn't been much success [86].

This is true when both groups with and without PI3K pathway mutations have received treatment. It would appear that inhibiting PI3K or its partners is insufficient to have significant and long-lasting positive impacts in the clinic, despite its crucial role in numerous pathways that cancer cells depend on.

It is becoming more and more obvious that targeting many enzymes or signaling pathways is important for the best treatment outcome, both in the laboratory and in the clinic. This is also true in terms of inhibitor resistance because it is more difficult for a tumour to develop resistance to more than one therapy. However, when feedback loops are taken into account, the usage of numerous inhibitors might make it more difficult to read out their effects. A more focused approach is currently being adopted in terms of the design of new inhibitors thanks to the accumulation of information of individual and combined signaling pathways and structural insights derived from crystallography. Similar to how our understanding of the target and associated kinases influences drug design, genetic variables and biomarker readout are starting to influence which patients receive these medications.

In order to make broad observations on PI3K activity, the pan-PI3K-isoform inhibitors wortmannin and LY294002 were originally introduced in the 90s. Both drugs were first believed to be more specific than they really are, and since they have off-target effects like inhibiting mTOR, findings gained from their early use may need to be reassessed. But for the early descriptions of PI3K function, they were really helpful. Inhibitors of class I PI3Ks, pan-Akt, and mTORC1/2 have recently become accessible; they are shown in Table 1. These inhibitors are both selective and pan-isoform. While CAL101 is in the last stages of clinical testing for use in haematological malignancies, IC87114, which targets p110 δ , was the first isoform-specific drug disclosed. These translational outcomes have been particularly encouraging for p110 δ inhibitors.

Despite the fact that p110 δ is not mutated in cancer, it has been discovered that targeting p110 can have observable benefits in a therapeutic context.

P110 α is an appealing target for inhibition since it is the only PI3K isoform identified to be widely altered in cancer, and specific inhibitors have just recently become accessible. Clinical studies for several of these substances, notably BYL-719, are now under process. The E545K and H1047R mutations can also be inhibited by the drug A66, which has around 40-fold higher selectivity for p110 α than for the other class I isoforms [95].

Despite these encouraging results, a growing body of evidence suggests PI3Ks may have a more nuanced role in cancer than was previously appreciated. While there is no association between PIK3CA and PTEN mutation and the effectiveness of p110 α -specific/pan-class I PI3K inhibitors to reverse tumour development, investigations utilising selective or pan-isoform PI3K inhibitors have been less successful than expected at causing cancer cell death. Despite the common occurrence of PI3K pathway mutations in malignancies, this is the case. Many anti-class I PI3K medicines fall short in clinical settings because they block negative feedback loops, which ultimately leads to increased rather than decreased signalling downstream of PI3K [63]. Akt has also been subject to the development of inhibitors [96], and phase II clinical studies for the pan-isoform allosteric inhibitor MK-2206, which is being used both alone and in conjunction with other medications to treat a variety of carcinomas, are currently underway. Although it is understood that the various Akt isoforms perform distinct functions, isoform-specific drugs are not yet accessible. The discovery of the macrolide rapamycin marked the beginning of mTOR treatments, which advanced when it was shown that rapamycin acutely targets mTORC1 through FKBP12, however long-term exposure can also influence mTORC2. Since the

discovery of rapamycin, other rapalogs have been produced and introduced into clinical trials with variable degrees of success, such as Everolimus. Rapamycin has immune-suppressive properties. Inhibitors that are specific for one or both mTOR complexes are available, and next-generation drugs (some of which also target PI3K) are in early stage studies for use in solid tumors to treat a variety of carcinomas, are currently underway. Although it is understood that the various Akt isoforms perform distinct functions, isoform-specific drugs are not yet accessible.

Inhibitor compound	Targets	Clinical use
LY294002	Pan-PI3K, mTOR	shown dermal toxicity in addition to its <i>in vivo</i> anti-tumor effects.
Wortmannin	Pan-PI3K, mTOR	<i>in vivo</i> anti-tumour effects, Anti-inflammatory, but also liver toxicity
GDC-0941	Pan-class I PI3K	Trials in solid tumours (including ovarian, melanoma, breast, pancreatic)
BYL-719	p110 α	Trials in solid tumours (including cervical, endometrial, breast, ovarian, neck and head)
KIN193	p110 β	Not in clinical trials
TGX221	p110 β	Thrombosis
MK-2206	Akt	Trials for solid tumours (including neuroendocrine)
Rapamycin	mTORC1 (mTORC2 with long-term treatment)	Approved for kidney cancer, in trials for solid tumors and ageing.
Everolimus (RAD0019)	mTORC1	Renal and other tumors

Table1: Pharmacological inhibitors used to target different components of the PI3K pathway and their use in the clinics.

1.8 Post translational Modifications and Phosphoproteomics

Signalling pathways coordinate and synchronize cellular activities and the extracellular environment [97]. Protein expression does not always indicate signalling capability, and other variables impact activity. PTMs regulate and integrate signalling pathways by affecting effector protein localization and stability. They also work faster than gene expression changesPTMs modify proteins by attaching a chemical tag to the

protein's side chain. Some posttranslational modifications (PTMs) are well recognised, such as how ubiquitination on specific lysine residues directs the protein for varied functions (including destruction) [98, 34], or how phosphorylation of kinases in specific patterns typically boosts activity. Phosphorylation, ubiquitination, acetylation, methylation, and palmitoylation are among hundreds of PTMs. The range of changes, the ability for dynamic insertion and removal, and the numerous functional impacts allow quick and precise fine-tuning of signalling pathways, both intra- and intercellularly [97]. Phosphorylation is one of the most researched PTMs, in part because it is easy to detect with existing methods, which continue to improve due to their widespread usage.

PTMs that regulate important networks can cause disease since their proper operation is necessary for healthy cell signalling. Mutations are notably common in phosphorylation kinases and phosphatases. Delineating signalling networks, assessing PTM functioning, and assigning substrates to effector enzymes is increasingly crucial in understanding and treating illnesses like cancer.

In more than half of proteins, a phosphate group is covalently attached to a serine, threonine, or tyrosine residue, resulting in a reversible post-translational modification [99]. Although less explored, additional amino acids can be phosphorylated. Over 500 kinases and 150 phosphatases in the human genome carry out phosphorylation [93]. Phosphorylation controls protein target quantity, activity, and cellular location by changing protein structure with the addition of the negatively charged bulky phosphate group [100]. To this end, it permits both positive and negative feedback and input from a plethora of signalling networks, therefore regulating the vast majority of cellular pathways at each level [63]. However, the existence or activity of the cellular protein product does not always coincide with the presence or activity of the genomic or

transcriptomic data, which might provide valuable information about a cellular or biological system. Because of this, proteomics is being used more and more to detect and evaluate the outputs of pathways and signalling. Many phosphoproteome sites alter protein activity or localization, whereas some do not. Monitoring protein phosphorylation can reveal a system's signalling output better than genomic or transcriptomic data.



1.9 AIM of the research

Amongst the Class-I PI3-kinases, p110 α and p110 β isoforms encoded by the PIK3CA and PIK3CB genes are ubiquitously expressed lipid kinases, comprising of catalytic subunits p110 α and p110 β , which phosphorylate PIP2 to PIP3 to activate the PI3K pathway [103, 102]. Even though both isoforms are expressed in all tissues and catalyze the same reaction, their unique molecular targets have not yet been elucidated, which majorly becomes the root problem in treatment when tumors develop alternative signaling mechanisms downstream in the pathway to compensate for the silenced component, leading to the problem of resistance to single-agent therapy [105]. Circumventing this problem requires an understanding of the unique downstream targets of the PI3K isoforms, p110 α and p110 β , so that they may be targeted uniquely in individual cases.

For this purpose, our study aims to identify specific protein-protein interactions of the PI3K isoforms and the resulting distinct phospho-proteomic signatures that could be important determinants of specific cellular activities. To address these goals, our study design utilizes isogenic MEF cells dependent on either p11 α or p110 β isoform only, the isoform-specific pharmacological inhibitors BYL-719 and KIN 193, and a high-resolution mass spectrometry-based IMAC method to determine the phosphorylation levels of these protein samples. The identified candidate phosphoproteins are then biochemically validated in parental WT MEFs and cancer models. The predictive biomarkers identified can be used in the longer term to identify patients who will benefit from PI3K-focused therapies and to understand the modalities of resistance that may develop against pathway inhibition.

Clinical data has shown that several Pan-PI3K pharmacological inhibitors used in clinics have resulted in severe adverse effects such as hyperglycemia and liver toxicity [104]. However, preclinical studies with some of these drugs have revealed that alternative signaling mechanisms downstream in the pathway may get activated to compensate for the silenced component, leading to the problem of resistance to single-agent therapy [105]. Circumventing this problem requires an understanding of the unique downstream targets of the PI3K isoforms, p110 α and p110 β , so that they may be targeted uniquely in individual cases.

Even though both p110 α and p110 β share a great deal of homology, their distinct specific activities in cellular processes have not yet been fully identified and are yet to be delineated. As a result, when deciding on targeted therapy and which isoform to silence in which patient group, determining the activity of individual PI3K isoforms in a biological sample is critical.

For this purpose, our study aims to identify specific protein-protein interactions of the PI3K isoforms and the resulting distinct phospho-proteomic signatures that could be important determinants of specific cellular activities. To address these goals, our study design utilizes isogenic MEF cells dependent on either p11 α or p110 β isoform only, the isoform-specific pharmacological inhibitors BYL-719 and KIN 193, and a high-resolution mass spectrometry-based method to determine the phosphorylation levels of these protein samples. The identified candidate phosphoproteins are then biochemically validated in parental WT MEFs and cancer models. The predictive biomarkers identified can be used in the longer term to identify patients who will

benefit from PI3K-focused therapies and to understand the modalities of resistance that may develop against pathway inhibition.



CHAPTER 2. MATERIALS AND METHODS

2.1 Materials

Table 2.1.1 Buffers and Solutions

Solutions	Ingredients
8M Urea lysis Buffer	8M Urea 1X Protease inhibitor 1X Phosphatase inhibitor mix 1 μ M DTT 1 mM Na ₃ VO ₄
1x NP-40 Lysis buffer	50mM Tris-HCl pH 7.5 150mM NaCl 0.5% w/v NP-40 5mM EDTA 1mM DTT 0.1mM Na ₃ VO ₄ 1x. Protease Inhibitor 1x Phosphatase Inhibitor Mix
1 x RIPA cell lysis buffer	1X RIPA 1X Protease inhibitor 1X Phosphatase inhibitor mix 1 μ M DTT 1 mM Na ₃ VO ₄
10x Western Blotting Running buffer	30 g Trizma-Base 144 g Glycine 10 g SDS Complete volume up to 1 liter with ddH ₂ O
1X Western Blotting Transfer Buffer	100 mL 10X Transfer buffer 200 mL Methanol 700 mL ddH ₂ O
10X Western Blotting Running Buffer	30 g Trizma-Base 144 g Glycine 10 g SDS Complete volume up to 1 liter with ddH ₂ O

Resolving Gel Buffer (pH=8.8)	187 g Trizma-Base pH adjusted to 8.8 with HCl Complete volume up to 1 liter with ddH ₂ O
Stacking Gel Buffer (pH=6.8)	60.5 g Trizma-Base pH adjusted to 6.8 with HCl Complete volume up to 1 liter with ddH ₂ O
10X TBS	80 g NaCl 2 g KCl 30 g Trizma-Base pH adjusted 7.4 with HCl Complete volume up to 1 liter with ddH ₂ O
1X TBS-T	1 mL Tween-20 1 liter 1X TBS
10x PBS	80 g NaCl 7.64 g Na ₂ HPO ₄ 2 g KCl 2 g KH ₂ PO ₄ 1 L ddH ₂ O
Crystal Violet Staining Solution	0.4% crystal violet powder 20% Ethanol complete to desired volume with ddH ₂ O
Destaining Solution	10% Acetic Acid 0.5% SDS Complete to desired volume with ddH ₂ O
Fixation Solution	100 ml acetic acid 100 ml ethanol 800 ml dH ₂ O

Table 2.1.2 Cell Culture Reagents

Catalog Number	Product Name	Brand
41966-029	DMEM	Gibco, US
RPMI	P04-17500	Pan-Biotech, Germany
S1810-500	Fetal Bovine Serum (FBS)	Biowest, US
15140-122	Penicillin- Streptomycin	Gibco, US
25300-054	Trypsin-EDTA (0.05%)	Gibco, US
A111138-03	Puromycin	Gibco, US
AB-101L	Nourseothricin (NAT)	Jena Biocience, Germany
10131-035	Geneticin (G418 Sulfate)	Gibco, US
D9891-1G	Doxycycline Hyclate	Sigma-Aldrich, US
	PDGF	
	LPA	
1H72652	PBS 10x pH 7.4	Bioshop, Canada
5100-0001	Mr. Frosty Freezing Container	Thermo Scientific, US

Table 2.1.3 List of inhibitors

Catalogue Number	Inhibitor	Brand
S2814	BYL719	Shellect Chem
S1462	KIN193	Shellect Chem
S1120	RAD001 (Everolimus)	Shellect Chem
S1078	MK2206	Shellect Chem

Table 2.1.4 Western Blotting Reagents

Catalogue Number	Product Name	Brand
161-0747	4x Laemmli Buffer	Bio-Rad, US
10688.01	Acrylamide/Bisacrylamide (30w/v)	Serva, Germany
K-12045-D20	WesternBright ECL Kit	Advansta, US
11930.03	Albumin Bovine Fraction V pH 7.0	Serva, Germany
773301	Prime- Step Protein Ladder	Biolegend, US
Glycine	1154KG001	BioFroxx
39055.01	Phosphatase Inhibitor Mix	Serva, Germany
P0758S	Sodium Orthovanadate	New England Biolabs, US
11836153001	cOmplete, Mini Protease Inhibitor Cocktail	Merck, Germany
2502	ReBlot Plus Mild Stripping Solution, 10x	Merck, Germany
2504	Reblot Strong Stripping solution, 10x	Merck, Germany
GE10600003	Nitrocellulose Blotting Membrane	Merck, Germany
23225	Pierce™ BCA Protein Assay Kit	ThermoScientific, US
	Coomassie Blue	
	PVDF Blotting membrane	
1.107.320.100	TEMED	Millipore
24137-2.5L-R	Isopropapanol	Sigma-Aldrich
24229-2.5L-R	Methanol	Sigma-Aldrich
10426890	Whatman Gel Blot Paper	GE Healthcare Life Sciences

8.22050.100	SDS	Millipore
2- Betamercaptoethanol	M3148- 250ML	Sigma-Aldrich
Ponceau Solution	P7170-1L	Sigma-Aldrich
Tween 20	39796.51	Serva

Table 2.1.5 Routinely used chemical list

Name	Catalogue Number	Brand
Acetic acid	27222-2.5L- R	Sigma- Aldrich
Crystal Violet	131762.1608	AppliChem
Ethanol	32221-2.5L	Sigma Aldrich
Potassium phosphate monobasic	04243-1KG	Sigma Aldrich
Potassium chloride	1.04936.1000	Millipore
Sodium chloride	31434-1KG- R	Sigma Aldrich
Trizma Base	T1503-1KG	Sigma-Aldrich

Table 2.1.6 Antibodies

Catalogue Number	Name	Brand
2997S	Phospho-PRAS40 (Thr246) (C77D7)	Cell Signaling Technology
9272S	Akt	Cell Signaling Technology
9614S	Phospho-Akt Substrate (RXXS*/I*) (110B7E)	Cell Signaling Technology
13038S	Phospho-Akt (Thr308) (D25E6) XP	Cell Signaling Technology
2317S	S6 Ribosomal Protein (54D2)	Cell Signaling Technology
4249S	PI3 Kinase p110 α (C73F8)	Cell Signaling Technology
3011S	PI3 Kinase p110 β (C33D4)	Cell Signaling Technology
2974T	Phospho-mTOR (Ser2481)	Cell Signaling Technology
2215S	Phospho-S6 Ribosomal Protein (Ser240/244)	Cell Signaling Technology
2367S	HA-Tag (6E2)	Cell Signaling Technology
9271S	Phospho-Akt (Ser473)	Cell Signaling Technology
2118S	GAPDH (14C10)	Cell Signaling Technology
sc-376641	PI 3-kinase p110 β	SantaCruz Biotechnology
sc-55589	PI 3-kinase p110 δ	SantaCruz Biotechnology
5536	Phospho-mTOR (Ser2448)	Cell Signaling Technology
2972	mTOR	Cell Signaling Technology
2691	PRAS40	Cell Signaling Technology
AF3740	p-NPM1 S125	Affinity
3837S	α tubulin	Cell Signaling Technology
Sc-8432	β actin	santacruz
9451s	Phosphor-4ebp1 S65	Cell Signaling Technology
619602	Phosphor 4ebp1 T70	Biolegend
9452S	Eif4Ebp1	Cell Signaling Technology
sc-55579	Cortactin	Santa Cruz
7074S	Anti-rabbit IgG, HRP-linked	Cell Signaling Technology
7076S	Anti-mouse IgG, HRP-linked	Cell Signaling Technology

Table 2.1.7 Equipments

Mini-Protean Tetra Cell	Bio-Rad, US
Amersham Imager 600	GE Healthcare, US
Centrifuges	Hettich, Germany Nuve, Turkey
Cell Culture Hood	Nua, re, US
CO2 incubator	Thermo Scientific, US
Synergy HT Microplate Reader	Biotek, US
Hemocytometer	

2.2 Methods

2.2.1 Cell culture

All cell culture work was done in a laminar flow hood under standard sterile conditions. Cell lines were grown at 37°C in 5% CO₂ using the media listed in Table 2.8

Cell line	Cell type	Species	Growth media
MEFs	Immortalized Mouse Embryonic Fibroblast	Mouse	DMEM supplemented with 8% FBS, 1% Pen/Strep
T47-D	Breast	Humans	RPMI supplemented with 8% FBS, 1% P/S, 0.04% insulin
MCF-7	Breast	Humans	RPMI supplemented with 8% FBS, 1% P/S, 0.04% insulin

Table 2.1.8 Cell Lines used, cell types, species of cell lines and their growth media

2.2.1A Passaging and Growth Maintenance

Cells were maintained in 7-8ml of growth media. Cells were passaged when they reached 80% confluency. For passaging, cells were washed with 6-7 mL of sterile 1X PBS and incubated with 0.05% Trypsin/EDTA for 5 minutes, at 37°C and 5% CO₂ in the incubator. Cells were checked under the microscope for detachment, and trypsin was inhibited by adding excess FBS containing growth media. The cells suspension was then collected in a falcon and centrifuged at 1500rpm for 5 minutes. The media was aspirated and the cell pellet was diluted using growth media in the desired ratio

for splitting. The cells were then seeded on 10cm plates completing the total volume to 8-10ml.

Cell line	Selection marker	Concentration
p110 α , β Cre+ α -wt (MEFs)	Puromycin	0.5ug/ml
p110 α , β Cre+ β -wt (MEFs)	Puromycin	0.5ug/ml
p110 α , β Cre+ p110 H1047R (MEFs)	Nourseothricin (NAT) Geneticin (G418) Doxycycline	50ug/ml 50ug/ml 0.25ug/ml
p110 α , β Cre+ p110 H1047R (MEFs)	Nourseothricin (NAT) Geneticin (G418) Doxycycline	50ug/ml 50ug/ml 0.25ug/ml

Table 2.1.9 Antibiotic selection markers for the modified cell lines

2.2.1B Freezing and Thawing Cells from Cryovials

Cells were frozen in their log growth phase. Cells are trypsinized in the same way as described in chapter 2.2.3. After the cells are centrifuged and media discarded, cells are resuspended in freezing media that contains 50% media, 40% FBS and 10% DMSO. 3ml of Freezing media is added drop by drop on top of the cells so cells do not undergo an osmotic shock. Cells are resuspended in the freezing media and split into 3 cryovials, each containing 1ml of cell suspension. The cryovials are labelled and placed in Mr. Frosty overnight, after which the cells are transferred in liquid nitrogen or -80C

Previously frozen cells were thawed in water bath at 37C till some frost was left. Cells are collected gently from the cryovial using a 1000ul micropipette and transferred into

thawing media made of 10% FBS containing media. Pen/Strep is not added till the cells recover so as to not cause stress on the cells. Cells are centrifuged at 1500rpm for 5 minutes to get rid of DMSO containing freezing media. Media is aspirated out and cells are resuspended in fresh thawing media. The cells are then seeded on 6cm plates in 5ml of thawing media.

2.2.2 Cell Harvesting and Cell Lysis

2.2.2A Cells Harvestation

Cells are harvested by scraping them. Cells are washed with 5-6ml of cold 1x PBS. PBS is discarded followed by adding 4ml of 1x cold PBS on top of the cells. The cells are then scraped using a sterile scraper. The cells are collected using a serological pipette in a 15ml falcon. The cell suspension is then spun in a cold centrifuge at 1500rpm for 10 mins. PBS is discarded and the cell pellet is snap frozen in liquid nitrogen and stored at -80C for further use in lysis.

2.2.2B Urea Lysis for LC-Mass Spectrometry

This lysis method was used for DKO-HA p110 α and DKO- HA-p110 β cells to lyse the cells and prepare samples for mass spectrometry analysis. 8M urea lysis buffer as described in Table 2.1 is used for lysis. For 1mg of protein, cells from 8x 10cm dishes are pooled together. The frozen cell pellets are kept in ice and lysis buffer is added in the ratio of 1:1 to the pellet. The pellet is flicked every 10 mins for 30 mins. Pellet can also be pipetted up and down. If the pellets are in falcons transfer them in eppendorfs. The cells are sonicated using a probe sonicator. Sonicate each sample for 5 seconds in **ice slush**. Sonicate thrice by giving 2-3 mins break in between each sonication Settings: Pulse on off: 15s, Amp: 40%. Cells are then spun in the cold centrifuge at

13000rpm for 15mins. Supernatant is transferred to another Eppendorf and the pellet is discarded and the lysate is snap frozen liquid nitrogen and stored in -80C.

Protocol inspired from DOI: and doi.org/10.1038/nprot.2013.010

2.2.2C RIPA Lysis for Western Blot Analysis

This lysis method was used to prepare lysates for western blot analysis. 1x RIPA lysis buffer is used as described in Table 2.1. The frozen harvested cell pellet is kept on ice till thawed and RIPA lysis buffer is added in the ratio of 1:3. The pellet is flicked or pipetted every 10 mins for 30 mins. If the pellets are in falcons transfer them to eppendorfs. Supernatant is transferred to another Eppendorf and the pellet is discarded and the lysate is snap frozen liquid nitrogen in -80C.

2.2.2D NP-40 Lysis buffer for Immunoprecipitation

NP-40 lysis buffer as described in Table 2.1 is used for performing cell lysis for immunoprecipitation as it is a mild buffer so it does not disrupt the interactions of proteins. Cells with NP-40 lysis buffer are lysed in the same way as 2.3.3. However, at every 10 minutes the cells are not pipetted, only flicked very gently to not disrupt protein interactions.

2.2.3 Starvation and Stimulation

Cells were starved, stimulated and inhibited with stimulants and isoform specific inhibitors to observe the differential isoformic functions of PIK3CA and PIK3CB.

On the day of the experiment, the confluency should be between 70%- 80%.

Starvation

1. Old media is aspirated and the cells are washed thoroughly with PBS and then PBS is then aspirated too.
2. 5ml of empty DMEM (*devoid of FBS and P/S*) is added to a 10 cm plate and the cells are starved for 4 hours.

Inhibition

1. After the 3.5-hour mark, the cells are treated with inhibitors.
2. The inhibitory mixture is pre-prepared so it can be directly added to the cells after 3.5 hours.
3. For each plate 5ml of inhibitory media is required. Dilute the inhibitor appropriately in empty dmem according to the number of plates and the treatment concentration.
4. At 3.5 hours, the empty media is aspirated from the plate and 5ml of inhibitory media is added to the plate.
5. Cells are inhibited for 30 mins so 3.5 hours + 30 mins = 4hours, time for stimulation.

Stimulation

1. After 4 hours, the cells are stimulated with the stimulant for 5-7 mins.
2. Stimulant is added to the media in the plate and the plate is swirled in the 8 shape multiple times to make sure the stimulant reaches all cells.
3. After 5-7 mins, media is aspirated and the cells are harvested in cold PBS.

Inhibitor	Concentration
BYL-719	0.5uM – Mass Spectrometry 0.5uM, 2uM- Western Blot
KIN -193	0.5uM – Mass Spectrometry 0.5uM, 2uM- Western Blot

Table 2.10: PIK3CA and PIK3CB isoform specific inhibitors used for starvation stimulation experiments

Stimulant	Concentration
PDGF	20ng/mL
LPA	20uM

Table 2.11 Stimulants used for starvation stimulation experiments

2.2.4 BCA Assay

1. This protocol is adopted and a modified version of Microplate procedure of PierceTM BCA Protein Assay kit (#23225).
2. Calculate the volume of working reagent (WR) mixture needed by using the formula below.
3. $\text{Total } V_{WR} = (n_{\text{Standards}} + n_{\text{Unknowns}}) * (n_{\text{Replicates}} = 3) * (V_{WR} \text{ per Sample} = 200)$

$V_{WR} = (\text{Solution A} + \text{Solution B})$; Solution A and Solution B are added in the ratio of 50:1

4. Prepare the Total Volume of WR and Wrap it with aluminium foil because it is light sensitive.
5. Use the following table to prepare the required standards according to the
6. experiment with the

Standards/ μg	Volume of the Standard BSA (2 $\mu\text{g}/\mu\text{l}$) / μl	ddH ₂ O / μl
0	0	10
1	0.5	9.5
2	1	9
3	1.5	8.5
5	2.5	7.5
7	3.5	6.5
10	5	5
15	7.5	2.5
20	10	0

Table 2.12 BCA standards

7. Add 1 μl of unknown sample to the wells in triplicates.
8. Top of the unknown samples by 9 μl of ddH₂O to a total volume of 10 μl
9. Add 200 μl of WR over each well and cover the plates with aluminium foil.
10. Place the plate on an orbital shaker for 30 seconds and place it in an incubator for 30 minutes at 37°C.
11. Use a microplate reader to record the OD readings at 562nm.
12. Equation of the standard curve is obtained by plotting the OD values against the concentration. The equation $y=mx+c$, is used to obtain the concentrations of the unknown samples.

2.2.12 SDS- PAGE

For SDS- PAGE 4% stacking gel, and 10% or 13% Resolving gels were prepared depending on the size of proteins needed to be detected. Gels were prepared according to the volumes in the following table

	10% Resolving	13% Resolving	4% Stacking
ddH ₂ O	4.1ml	3.1	6.1
Acrylamide/bisacrylamide (30w/v)	3.3	4.3	1.3
Resolving Gel Buffer (pH=8.8)	2.5	2.5	-
Stacking Gel Buffer (6.8)	-	-	2.5

Table 2.13 Recipe for Resolving and stacking gels

Depending on the experiment, 15ug-30ug of proteins were loaded and run on the SDS-PAGE. For sample preparation of proteins, the proteins are normalized after quantification and denatured with 4x Laemmli sample buffer containing 90% β -mercaptoethanol by boiling them on a heatblock at 95C for 7 minutes. The samples are then centrifuged for 2 minutes at 3000rpm and the desired grams of proteins are loaded on the gel with the protein ladder in the first and the last lane. The samples are run at 80V in running buffer till they stack and then increased to 100V for 120 minutes, till they run the complete gel to the end.

2.2.6 Western Blot and ImmunoBlot

1. After the proteins are run on the gel, they are then transferred on to the nitrocellulose membrane or PVDF using the wet-transfer setup. In the case of PVDF it is first activated with methanol, then soaked in water, followed by equilibration in transfer buffer whereas nitrocellulose is dipped in transfer buffer for few seconds. Transfer sandwich is prepared from the black side of the sandwich (negative pole). Assembly is performed by first placing a sponge, then 2 wattman paper soaked in transfer buffer, the gel, membrane, 2 wattman papers and sponge. After the membrane and whatmman papers are placed on the gel, a roller is used to roll out any bubbles between the gel and the

membrane to ensure efficient transfer. The sandwich is placed in the transfer cassette in the tank. Cool pack is placed and the tank is filled completely with cold 1x- methanol containing transfer buffer. The gel is run at 250mA, at constant current for 150 minutes in a box filled with ice.

2. Transfer efficiency is controlled by dipping the membrane in Ponceau stain and dipping the gel in Coomassie blue stain.
3. The membrane is then washed with 1x TBS-T to wash off the ponceau and then cut at the desirable molecular weights.
4. Membranes are blocked with 5% BSA containing TBS-T solution for phosphor-specific blots, otherwise with 5% skimmed milk containing TBS-T solution.
5. Membranes are incubated overnight with the desired primary antibodies at +4 degrees on a shaker. After the incubations, the antibodies are collected and the membranes are washed 3x for 5 minutes with 1x TBS-T.
6. Membranes are incubated for 1hr, at room temperature on a shaker with the corresponding HRP-conjugated mouse or rabbit antibody. After the incubation the membranes are washed 3x with 1x TBS-T.
7. Membranes are then incubated with ECL- solution in accordance with the manufacturer's instructions and the membranes are developed and visualized by Amersham Imager 600.
8. Analysis of the immunoblots were performed by ImageJ 1.53k.
9. Phosphorylation signals were normalized by first normalizing the total proteins to the loading control and then normalizing the phosphor-proteins to the normalized total proteins.

2.2.7 Immunoprecipitation and Co-Immunoprecipitation

1. After the cells are lysed using NP-40 lysis buffer, perform BCA/Bradford and obtain the concentrations. The samples are then diluted to 1ug/ul using lysis buffer.
2. IP can be performed using 300ug-1mg or greater mass of protein depending on the experimental setup.

This protocol is for 500ug of protein

A. PRE-CLEARING

1. Lysate needs to be clarified using protein A/G sepharose or agarose beads.

2. The beads need to be equilibrated first:

- a. Add 20ul unpacked beads to 500ul of NP-40 lysis buffer and place it on ice for 5 mins.

Cut the tip in a slanted fashion otherwise the tip might harm the beads

- b. The beads are spun for 5mins at 3000 rpm at 4 C.
- c. Lysis buffer is discarded. Resuspend the beads in 50uL of lysis buffer.

DO NOT pipette. Shake or flick a little

3. 500ul (500ug) of protein lysate is added on top of the beads and incubated on the rotating wheel for 30 minutes at 4C.
4. The bead-lysate mixture is centrifuged at 3000rpm for 5mins at 4C. The clarified cell lysate is collected and the pelleted beads are discarded.

B. INCUBATION WITH ANTIBODY:

- 1) Calculate the antibody volume that would be used for the pull down. Refer to the data sheet of the antibody being used.
- 2) For negative control Random rabbit/mouse IGgs are used.
- 3) The calculated antibody is added to the clarified lysate and incubated in an icebox at 4C overnight.

Bury the Eppendorf in ice and keep the ice box in +4 room or fridge

- a. Lysis buffer is discarded. Resuspend the beads in 50uL of lysis buffer.

DO NOT pipette. Shake or flick a little

C. Ab-LYSATE CONJUGATION WITH BEADS:

- 1) The A/G coupled agarose beads are equilibrated. 50ul of packed beads for 500ug of protein. *Refer to the bead equilibration in Part A*
- 2) The Ab-protein conjugated lysate is added on top of the equilibrated beads and incubated on a rotating wheel at 4C for 30 mins.
- 3) The mixture is spun at 3000rpm for 5 mins at 4C and the supernatant is discarded.

D. WASHING OF BEADS-Ab-PROTEIN CONJUGATE

1. Wash the bead-ab-protein conjugate with lysis buffer 3 times.
2. Lysis buffer is added on top of the conjugate and spun at 3000rpm for 5 mins at 4C and the supernatant is discarded. This is done thrice.

Do not pipette. Invert the eppendorf after adding lysis buffer few times, VERY gently.

E. ELUTION

1. 30ul of 4x laem containing 10% b mercaptoethanol is added on top of the bead conjugate

2. The mixture is heated on the heat block for 10-15 mins at 95 C
3. The sample is spun at 3000rpm for 5mins at RT and the supernatant is collected carefully without disturbing the beads

Elute can be stored at -20 for few days.

Continue with Western blot and Immunoblot to detect the pull downs.

F. Co-Immunoprecipitation

1. Anything co-immunoprecipitated with the IP can be detected by doing an Immunoblot using the specific Ab.

2.2.8 Crystal Violet Growth Assay

6 well plates were used for this growth assay. DKO- HA α and DKO-HA β cells were characterized for their isoform dependency. The assay was performed in triplicates for each condition to reduce handling error.

Cell counting and seeding

1. Cells are trypsinized as described in **2.3.1**
2. Resuspend the cells in 4ml of growth media and make a cell suspension.
3. Mix 200 ul of cell suspension with 200ul of trypan blue in another Eppendorf and resuspend them till properly homogenised.
4. Pipette 10ul of the trypan blue-cell suspension into the chamber of the hemocytometer and count the cells.

Cell Counting

1. By using light microscope, count the cells in each 1mm² squares. Minimum of 4 squares needed to be counted.
2. Calculate the average number of cells and dilution factor in one square.

Average #of cells/square = viable cells /squares

Dilution factor= volume of trypan blue + volume of cells/ volume of cells

3. Calculate the number of cells in 1ml.

Concentration (Viable cells/ml) = Average # of cells /square x Dilution factor x 10⁴

4. From the concentration the volume needed to seed a certain number of cells can be calculated.

5. Add the calculated volume of cell suspension to seed the cells into 6-well plates at a density of 5×10^3 cells per well and make the final volume to 1ml by using 5%FBS media.
6. Each condition is seeded in triplicates.
7. One day post seeding the cells, after the cells have attached to the plate, drug treatment is performed.
8. Stock concentration of BYL 719 and KIN193 is 2mM; working concentration of BYL 719 and KIN193 is 2uM
9. Add 1ul of inhibitor to 1ml of media and swirl the plate in the pattern of 8.
10. The experiment usually lasts for 5 days till the DMSO control plate has reached 100% confluency.
11. On day 3, change the media and replenish the inhibitors.
12. On day 5, end the experiment and continue to the crystal violet experiment.

Fixation and Destaining

1. Gently remove the fixation solution.
2. Gently tilt the plates to wash each well with 1 ml nonsterile PBS.
3. Carefully remove the PBS.
4. Pour 1 mL of crystal violet stain into each well without disturbing the cells.
5. Incubate the plates for at least 1 hour at room temperature and in the dark.
6. Gently remove the stain.
7. Rinse each well with 1 mL of distilled water. Wash 3x to remove all the crystal violet solution.
8. Allow plates to air-dry for couple of hours. Make sure that all the water has evaporated.
9. Add 1ml of destaining solution and incubate it at RT for 1 hours .
10. Take 200ul from each well and load it into a 96 well plate. Load each well into triplicates.
11. Take spectrophotometer reading at 595nm.

Quantifying Crystal Violet Stain

1. From each well read, subtract the blank well triplicate. The values obtained represent the dye taken up by the cells hence the growth of the cells.

$$OD_{\text{Treatment}} = \text{Read obtained from treatment}$$

OD_{DMSO} = Read obtained from DMSO control

i = Percent inhibition

2. Calculate the percentage inhibition:

$$i = 100 - ((OD_{Treatment} / OD_{DMSO}) * 100)$$

2.2.9 Peptide Formation and Phosphopeptide Enrichment for Mass Spectrometry

Proteolytic degradation processes were carried out by taking 1 mg of the samples with serum deprivation and growth factor stimulation and the amount of protein was determined. The chemicals used in proteolytic degradation processes were prepared in 25 mM ABC (ammonium bicarbonate). Reduction of proteins was carried out by incubating the samples at 37 °C for 1 hour after the samples were brought to the final concentration of 5 mM DTT (1,4-Dithiotreitol). The alkylation process was carried out for 30 minutes, with the final concentration of the solutions being 10 mM IAA (iodoacetamide). carried out by incubation in the dark. Afterwards, the DTT concentration in the samples was brought to 5 mM. After the urea concentration was reduced to 4 M with 25 mM ABC, Lys-C enzyme was added to the samples (1/100, enzyme/protein, w/w) and incubated at 37 °C for 4 hours. Afterwards, the urea concentration was reduced to 1.6 M with 25 mM ABC and trypsin enzyme was added to the samples (1/100, enzyme/protein, w/w). Samples were incubated overnight at 37 °C. The next day, the samples were acidified with TFA and the proteolytic degradation process was stopped. Afterwards, desalting of the peptides was carried out using C18 solid phase cartridges. After elution, the samples were dried with a fast vacuum concentrator (SpeedVac Concentrator) and dissolved in the appropriate solvent (50%

ACN/49.9% Water/0.1% TFA, v/v/v) to enrich the phosphopeptides. Afterwards, it was divided into two equal parts and the enrichment of phosphopeptides was started.

2.2.10 Mass Spectrometric Analysis

The enrichment process of phosphopeptides was carried out by adapting the method in the literature (Thingholm et al., 2016). An immobilized metal affinity chromatography (IMAC)-based method and material was used for the enrichment of the phosphopeptides (Iron-coated PHOS-select™ metal chelate beads). Approximately 75 µL of iron-coated material was taken into 1.5 mL tubes. The materials were then washed with loading solution 2 times with 0.5 mL of loading buffer (50% ACN/49.9% Water/0.1% TFA, v/v/v). Samples dissolved in the loading buffer were mixed with the materials for 30 min at room temperature. incubated. Afterwards, each was washed 2 times with 0.5 mL of loading buffer. Elution process of the materials with 50 µL of 1% ammonia for 5 min. performed by incubation. After 40 µL of elution was mixed with 20 µL of 2% formic acid solution, analyzes were performed with Thermo Q-Exactive-Plus.

Mass spectrometric analyzes were performed with an easy-nano source integrated Thermo QExactive Plus instrument working with the Ultimate 3000 RSLC nano liquid chromatography system (Dionex/Thermo Scientific). After the enrichment process, 5 µL of the prepared samples was injected into the device. In the chromatographic separation system, Acclaim PepMap 100 trap column (100 µm × 2 cm, particle size 5 µm, Dionex/Thermo Scientific) and Acclaim PepMap RSLC C18 nano separation column (75 µm × 75 cm, particle size 3 µm, Dionex/Thermo Scientific) used. A) (0.1% Formic Acid solution containing 2% ACN) and B) (100% ACN containing 0.1% formic acid solution) solvents were used as the gradient elution as the mobile phase.

Solvent B in gradient elution 40 min. The separation of phosphopeptides was ensured by programming it to reach from 3% to 18% and then to 30% linearly in 90 minutes. Top10 method was used in the analysis. In mass spectrometric analysis, full mass spectra were taken between 300-2000 and analyzes were performed using HCD (high collision decomposition) mode under optimum mass spectrum parameters where the highest intensity was obtained. The raw data obtained were evaluated with MaxQuant software.

Mass spectrometric data was used for the detection and quantification of phosphopeptides, phosphoproteins, and MaxQuant (v1.6.17). Data from 6 replicates of each condition (3 experimental, 2 techniques) were included in the software. In protein sequencing, the taxonomy (Mus Muculus, Mouse) was selected as the database and downloaded from UNIPROT. Then it was introduced to the software. The parameters applied for the QExactive Plus system were used to illuminate the phosphopeptides. 25 was determined for the lowest score of the modified peptides. The LFQ option was selected for label-free quantification. The intensity values of the obtained data were determined for each phosphopeptide.

The data obtained with MaxQuant software were first evaluated in Perseus software. First of all, various filters were made here. Peptides with a degree of phosphorylation greater than 0.75 were evaluated first. Those with LFQ intensity value in at least 3 of the 6 replicates were included in the analysis. Log2 transform was applied to LFQ intensity values. Pearson correlation analysis and One-way Anova analysis of variance were performed on the phosphopeptides isolated as a result of this filtration.

2.2.11 Statistical Analysis

GraphPad Prism8 was used for performing statistical analysis and for the generation of graphs. Student's t-test was used to determine and show the statistical differences while comparing two groups.

Data analysis pipeline for phosphoproteomic data

Analysis of phosphoproteomics data was performed using Excel and GraphPad Prism8.

1. Phospho-peptide levels to be determined after growth factor stimulation for p110 α and p110 β dependent cells lines were divided by the level before stimulation (serum deprivation) and fold increase was determined.
2. Candidate phospho-proteins that show a statistically significant increase with a fold change >1.5 will be shortlisted.
3. Of the identified candidate-phosphoproteins, those that show a statistically significant change in the increase in phosphorylation levels at the same growth factor stimulation of the other PI3K isoform will be eliminated.
4. Among the candidate phospho-proteins that meet these two conditions, the subgroup whose phosphorylation level decreases below 1.5 times with isoform-specific inhibitor treatment will be selected and evaluated as specific targets of PI3K catalytic activity.

CHAPTER 3: RESULTS

3.1 Generation of immortalized MEF cell lines dependent on either p110 α or p110 β isoform.

MEFs were used in this study to establish the PI3K pathway in isogenic cells through a single isoform. As described in our previous study (Cizmecioglu et al. 2016), MEFs were genetically modified where the first exons of PIK3CA and PIK3CB genes encoding p110 α and p110 β respectively, were floxed. AdCre (Adenoviruses that carry a construct with Cre recombinase) treatment was used to remove the first exons of PIK3CA and PIK3CB, which eliminated endogenic expressions of the ubiquitous p110 isoforms in the cells to create double knockouts (DKO), p110 α and p110 β null cells. HA-tagged human wildtype p110 α or p110 β isoform was then ectopically overexpressed in comparable levels to establish single isogenic PI3K isoform dependent cells, p110 α,β Cre + α - wt and p110 α,β Cre + β -wt. MEFs untreated with Cre will be referred to as WT MEFs (α, β +/+ WT MEFs) in this study due to their intact p110 isoform. Single isoform dependent cells will be referred to as (DKO) HA-p110 α or HA-p110 β in this study. Fig 3.1 shows an immunoblot of DKO p110 α and DKO p110 β cells showing endogenous knockdowns and comparable exogenous expression of wt-HA-tagged p110 α or β isoform.

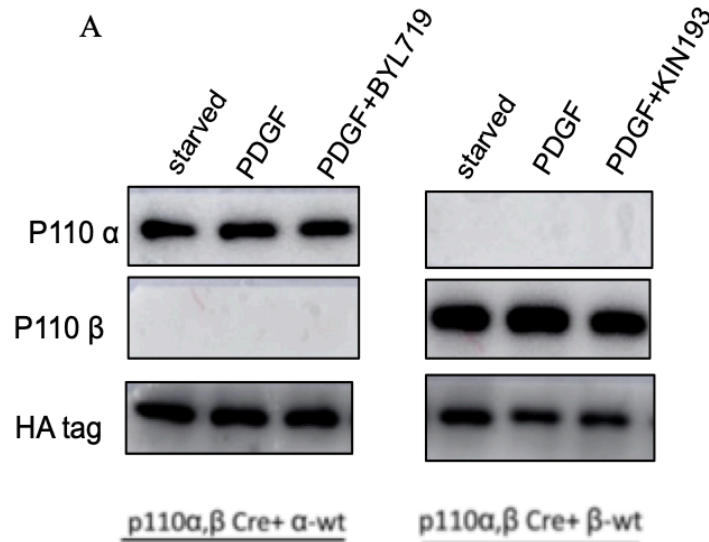


Figure 3.2 Generation of DKO-HA p110 α β and DKO- p110 β MEFs. DKO-p110 α cells show complete knockdown of p110 β isoform and DKO-p110 β cells show complete knockdown of p110 α isoform. Cells are entirely dependent on the exogenously overexpressed HA-tagged p110 isoforms. Immunoblotting for p110 α , p110 β and HA-tag. 25ug protein loaded. n=1

3.2 Confirmation of isoform-specific configuration of the PI3K pathway in DKO- HA- α , HA- β cells by growth assay.

HA- α and HA- β cells showed isoform- specific configuration of the PI3K pathway when they were treated with the isoform specific inhibitors BYL719 and KIN193. An increase in percentage inhibition is seen when the HA- α cells are treated with BYL719 (α inhibitor) as compared to when they are treated with KIN193 (β inhibitor) showing p110 α isoformic dependency of HA- α cells. The vice versa is seen for HA- β cells, they show sensitivity for the β inhibitor KIN193 and not towards the α inhibitor (Figure 3.2). Thereby it can be confirmed that the PI3K pathway is configured by the

p110 α isoform in the DKO HA α cells and by the p110 β isoform in the DKO HA β cells.

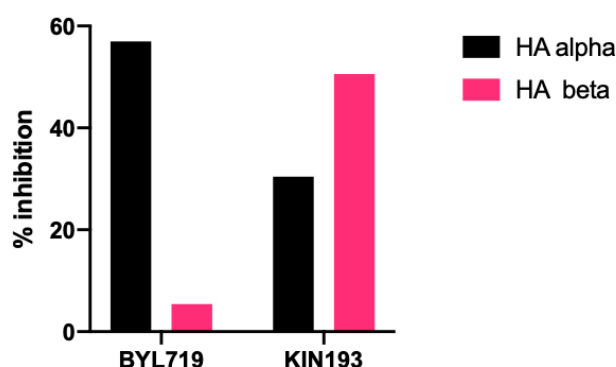


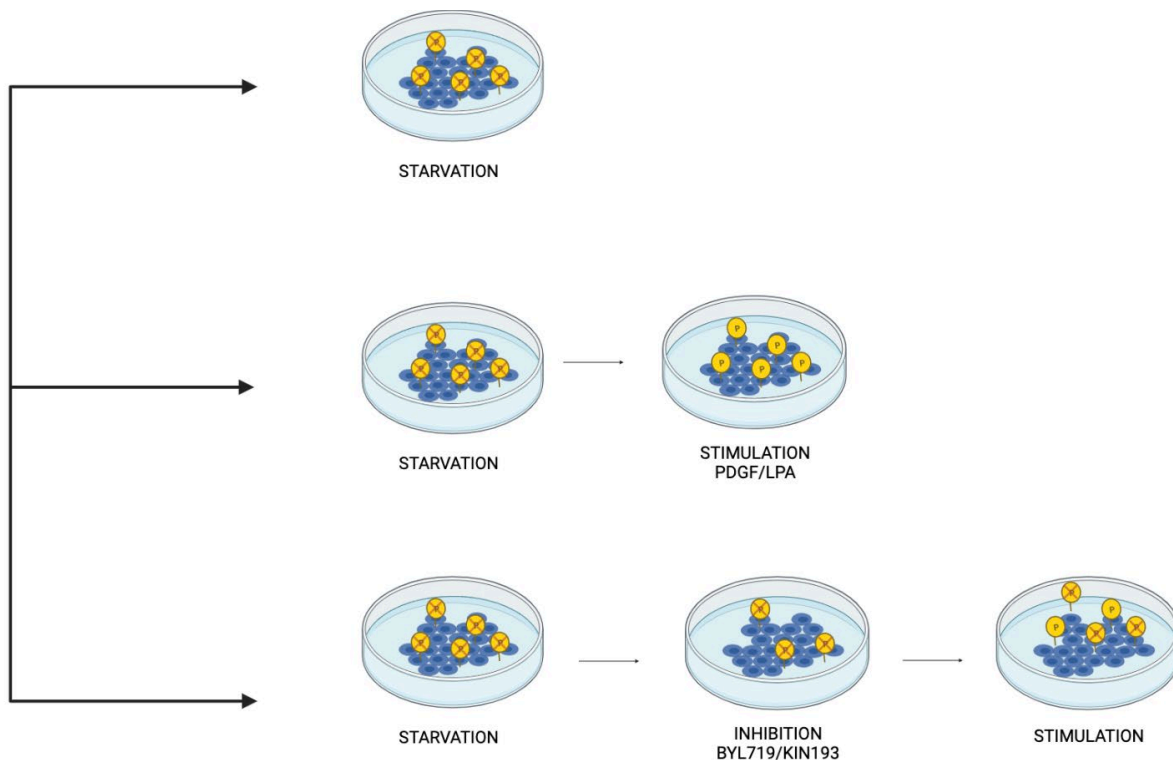
Figure 3.2 Percentage inhibition of crystal violet growth assay showing the dependence of DKO HA α and HA β on their respective isoforms p110 α and p110 β . Cells were treated with 2uM BYL719 and KIN193 for 5 days. 5000 cells seeded in a 6well plate. Each condition was seeded in triplicates of wells. Percentage inhibition was calculated in comparison to the DMSO control. n=1

3.3 Characterization of DKO HA-p110 α and DKO- p110 β MEfs by starvation stimulation and inhibition experiments

The cells are characterized for the establishment and their dependence on the PI3K pathway by a single expressed isoform of p110 α or p110 β .

DKO-HA p110 α and p110 β cells are serum starved for 4 hours in empty DMEM to remove all the phosphorylation signals, they are then stimulated with PI3K pathway stimulants, and then inhibited with isoform specific inhibitors, BYL719 or KIN193. Starvation acts like providing a clean slate by clearing the phosphorylations, so once the cells are stimulated with a PI3K pathway specific stimulant, phosphorylations that appear can be attributed to the PI3K pathway. When the isoform is inhibited after

starvation and the cells are then stimulated, the phosphorylations that disappear/decrease and don't reappear can be attributed to be dependent on the WT isoform expressed in the cells.



Created in **BioRender.com**

Schematic representation of the starvation stimulation inhibition experiment work flow

Figure 3.3 shows the establishment of the PI3K pathway in the isogenic immortalized DKO-p110 α and p110 β cells described in Section 3.1. Cells are verified for their isoformic dependence by checking for the downstream signalling phosphoproteins in the PI3K pathway. The p110 α isoform of PI3K appears to be responsible for the majority of PI3K signaling downstream of RTKs, while p110 β seems to preferentially signal downstream of GPCRs. Upon starvation, the p-Akt signal blunts out showing a starvation response and the phosphorylation gets restored upon stimulation with PDGF in both HA-p110 α and p110 β cell lines. PDGF can lead to similar activation levels for both p110 α and p110 β since the receptor it binds to (PDGFR) contains high affinity

p85 binding sites that can bind both p110 isoforms with the same efficiency. LPA surmounts a higher recovery of the p-Akt signals in HA- β cells because LPA mainly binds to GPCRs and is also a more potent stimulant thereby verifying the dependency of DKO-p110 β in the p110 β isoform (Figure 3.3A). Akt, ribosomal s6 and mtor, the downstream signalling components of the PI3K pathway also show regulation in their phosphorylation levels when the cells are starved, stimulated and inhibited. Akt is higher up in the PI3K pathway so it's one of the first cellular affectee in the pathway. In HA- α and HA- β cells, Akt, s6 and mtor all show decrease in phosphorylation levels when starved and treated with their isoform specific inhibitors. Pras40, which is further down in the signalling pathway does not observe the depletion of phosphorylation in the starved condition but the signal disappears when p110 α is inhibited. This may be because the starvation time of 4 hours might not be enough to get rid of the accumulated phosphorylations (Figure 3.3B). From these results, we establish the PI3K pathway by the isoform dependency in the isogenic immortalized MEF DKO-HA p110 α and HA-p110 β cells.

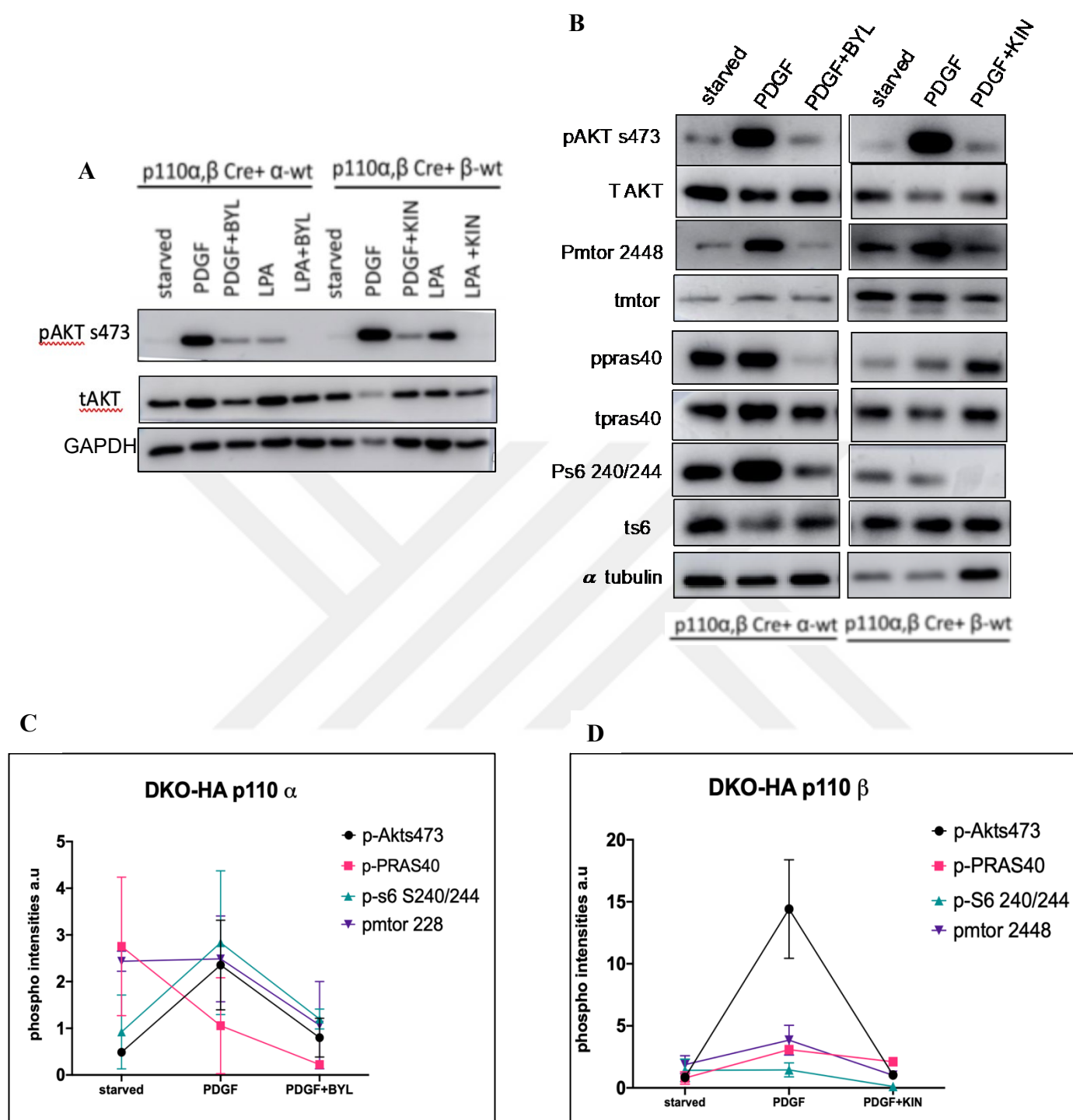


Figure 3.3 Characterization of DKO-HA p110 α and p110 β cells for the establishment of PI3K pathway in MEFs. Western blot analysis characterizing the DKO- HA α and HA β MEFs for downstream signalling phosphoproteins in the

PI3K pathway. **A)** p-Akt levels when the DKO-p110 α and p110 β cells are treated with isoform specific inhibitors BYL719, KIN193 and, PDGF and LPA as stimulants. **B)** Phosphorylation levels of downstream components of the PI3K pathway upon PDGF stimulation and isoform specific inhibition. **C, D)** Quantification of phosphorylation levels in A. SD plotted. 20ug of proteins were loaded. PDGF: 20ng/ml; LPA: 20uM, BYL-719, KIN-193: 0.5uM; n=2

3.4 Mass spectrometry analysis of HA- p110 α -wt and HA- p110 β -wt DKO MEF samples treated with growth factors and isoform-specific PI3K inhibitors

Phosphoproteomic analysis of the HA-p110 α and p110 β cells were carried out by our collaborating lab. Our replicates comprised of 3 biological replicates and 2 technical replicas for each biological replicate, totalling to 6 replicates. We saw high correlation within the replicates which averaged to Pearson correlations of 0.76 and 0.75 for HA- α and HA- β cells respectively (Figure 3.4).

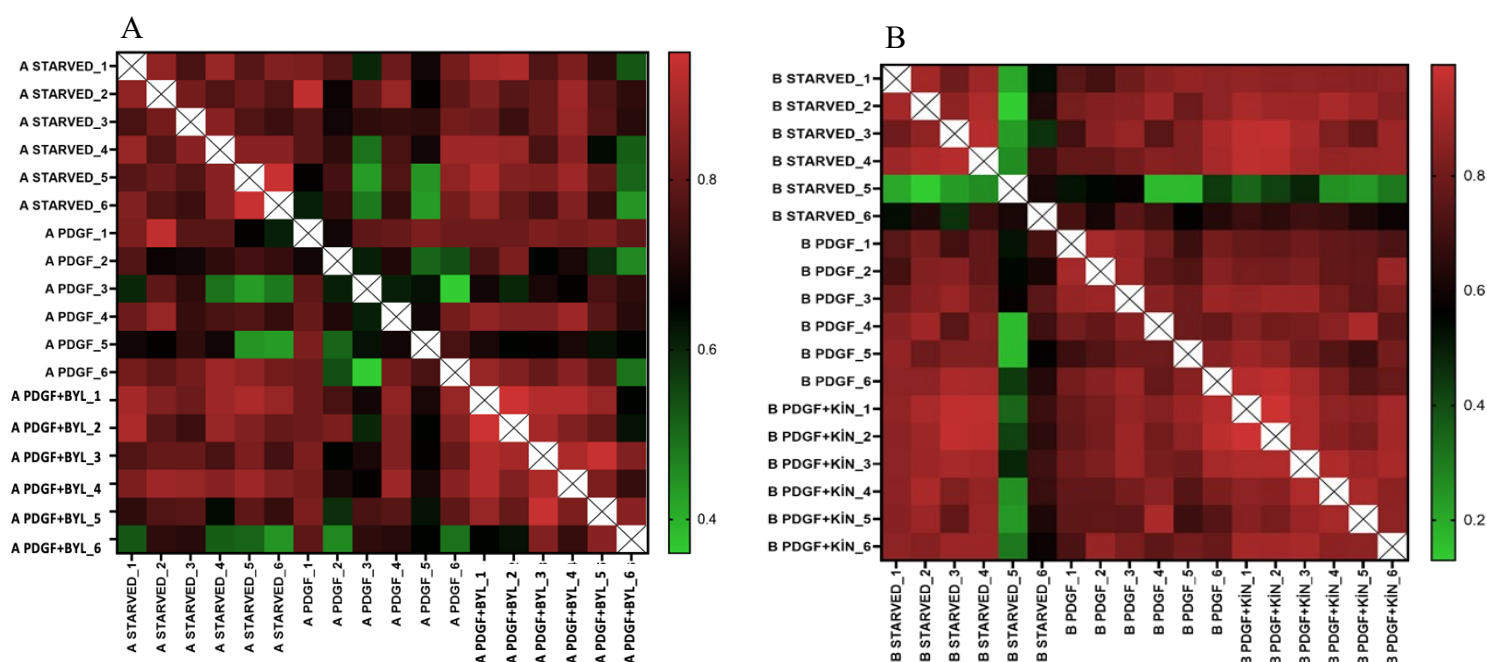


Figure 3.4: Heat map of Pearson correlation coefficients of the 6 replicates of each condition of HA α and HA β MEFs. A) Heat map of replicates of HA α cells that were starved, PDGF stimulated and inhibited by BYL719. B) Heat map of replicates of HA β cells that were starved, PDGF stimulated and inhibited by KIN19

As described in section 3.3, HA α and HA β cells were starved, PDGF stimulated and inhibited with BYL719 to investigate differentially phosphorylated proteins by the p110 α and p110 β isoforms. Our phosphoproteomic data yielded in total 1742 phosphorylated peptide residues for the p110 α dependent HA α MEFs and 1751 phosphorylated peptide residues for the p110 β dependent HA β MEFs. Out of those, 15 phosphopeptides were significantly differentially phosphorylated by the p110 α isoform and 16 phosphopeptides were significantly differentially phosphorylated by p110 β isoform. These phosphopeptides had a fold change greater than 2 when they were stimulated with PDGF, and showed a decrease of fold change greater than 1.5 when treated with the isoform specific inhibitor. There were three significant phosphopeptides that showed significant regulation with both the HA α and HA β isoform however they weren't differentially regulated by either isoform they were eliminated (Figure 3.5 A).

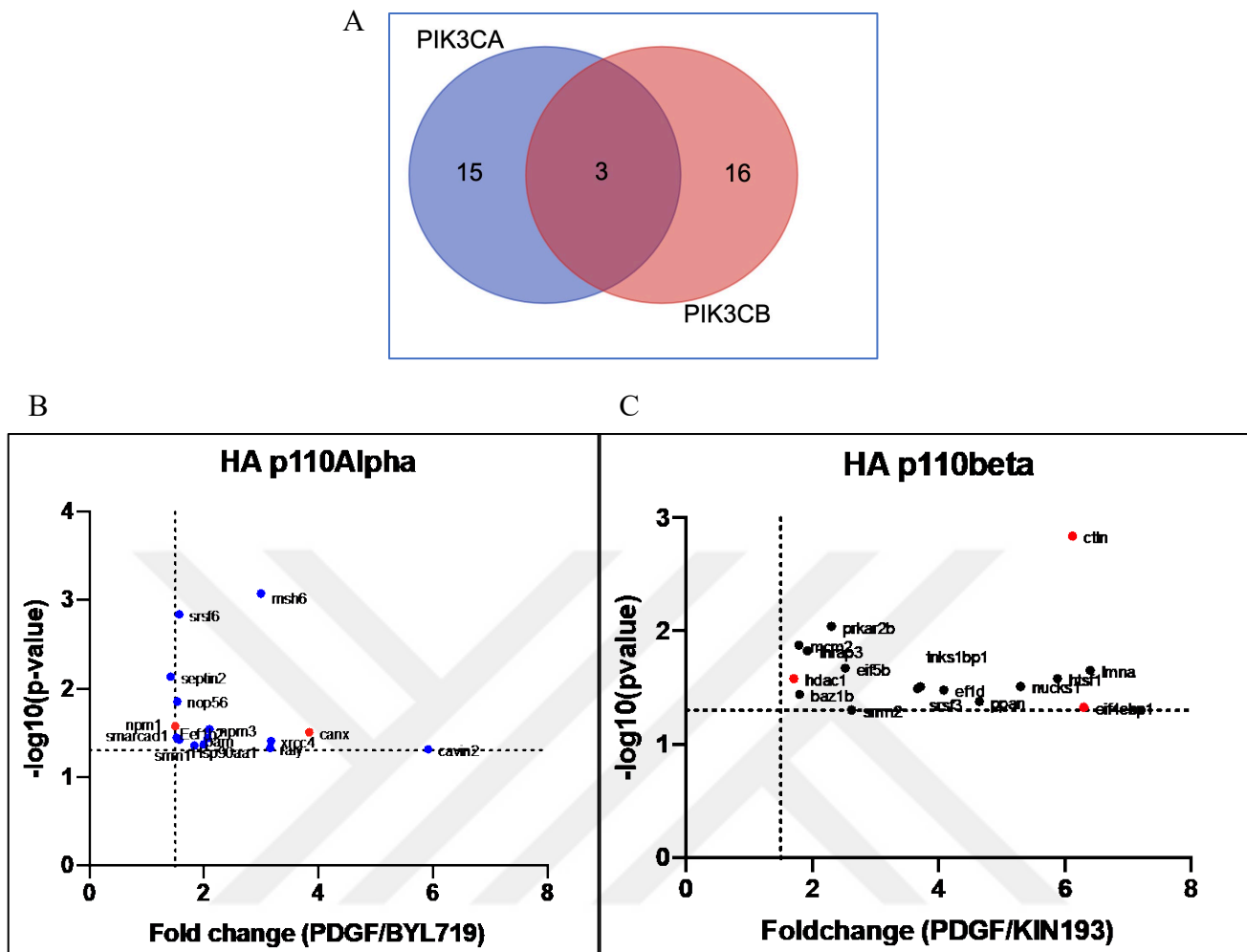


Figure 3.5 Differentially regulated phosphoproteins by the p110 α and the p110 β isoforms. Phosphoproteins that had a p-value less than 0.05 and fold change > 1.5 are shown. A) Venn Diagram summarizing the number of differentially and commonly regulated phosphoproteins by the p110 α and p110 β isoform. B) Volcano plot showing significant phosphoproteins ($p < 0.05$) with fold change > 1.5 upon isoform specific inhibition. Blue dots: PIK3CA regulated genes with fold change > 1.5 and $p < 0.05$; Black dots: PIK3CB regulated genes with fold change > 1.5 and $p < 0.05$; Red dots: shortlisted candidates for biochemical verification

3.5 Bioinformatics analysis and shortlisting of candidate proteins

Reactome, c-bioportal OncoPrint, cancer type, survival plot, pathway analysis and STRING analysis was performed to shortlist the candidate proteins for biochemical verification.

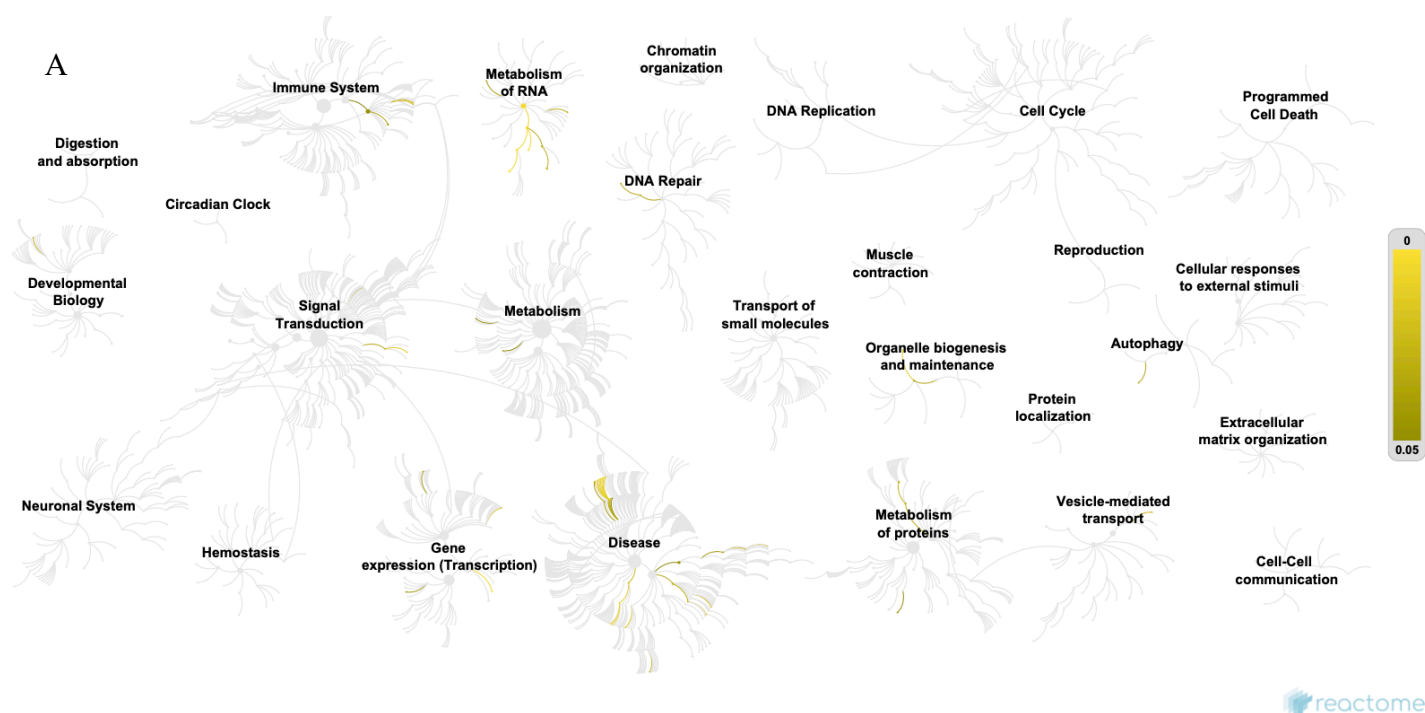
3.5.1 REACTOME Analysis

Phospho-proteins identified by the mass spectrometry analysis were subjected to Reactome analysis. Differentially regulated proteins by the p110 α isoform in the HA- α cells had strong enrichment for DNA repair pathways, metabolism of RNA, regulation in immune system, drug resistance of ERBB2 KD mutants, all of which are strongly implicated in cancer (Figure 3.6 A, B).

Contrary to the pathways enriched for p110 α , the pathways enriched for the p110 β isoform regulated proteins were mainly in the nucleolus. The enriched pathways included DNA replication pathways, cell cycle regulation, signal transduction and gene expression pathways (Figure 3.7A, B).

p110 β has a nuclear localization signal in the C2 domain that p110 α lacks because of which p110 β is found both in the cytoplasm and the nucleolus as opposed to p110 α which is mainly found in the cytosol. Because of its localization in the nucleus p110 β is able to undertake several roles in the nucleus as well such as those of cell cycle progression, DNA replication, and repair of DNA double-strand breaks. Our reactome analysis was in line with these previous reports of p110 α being mainly present in the

cytosol and implicated in cell survival and p110 β having a role in DNA synthesis or



B

Pathway name	Entities				Reactions	
	found	ratio	p-value	FDR*	found	ratio
mRNA 3'-end processing	3 / 58	0.004	5.65e-05	0.008	3 / 3	2.27e-04
RNA Polymerase II Transcription Termination	3 / 67	0.005	8.65e-05	0.008	1 / 4	3.02e-04
Transport of Mature mRNA derived from an Intron-Containing Transcript	3 / 81	0.006	1.51e-04	0.009	4 / 4	3.02e-04
Transport of Mature Transcript to Cytoplasm	3 / 90	0.006	2.06e-04	0.009	4 / 13	9.83e-04
Defective Mismatch Repair Associated With MSH6	1 / 2	1.36e-04	0.003	0.064	1 / 1	7.56e-05
Metabolism of RNA	5 / 782	0.053	0.003	0.064	28 / 187	0.014
Defective Mismatch Repair Associated With MSH2	1 / 3	2.04e-04	0.004	0.064	1 / 2	1.51e-04
Processing of Capped Intron-Containing Pre-mRNA	3 / 256	0.017	0.004	0.064	20 / 32	0.002
Resistance of ERBB2 KD mutants to AEE788	1 / 5	3.40e-04	0.006	0.064	1 / 1	7.56e-05

Figure 3.6 Reactome Pathway Analysis report for HA α MEFs. A) Genome-wide overview of the pathways enriched. Color code denotes overrepresentation of the pathway in the p110 α isoform regulated proteins. Light grey represents pathway that

are not significantly over-represented. B) The following table shows the top most relevant pathways sorted by p-value. FDR: False Discovery Rate

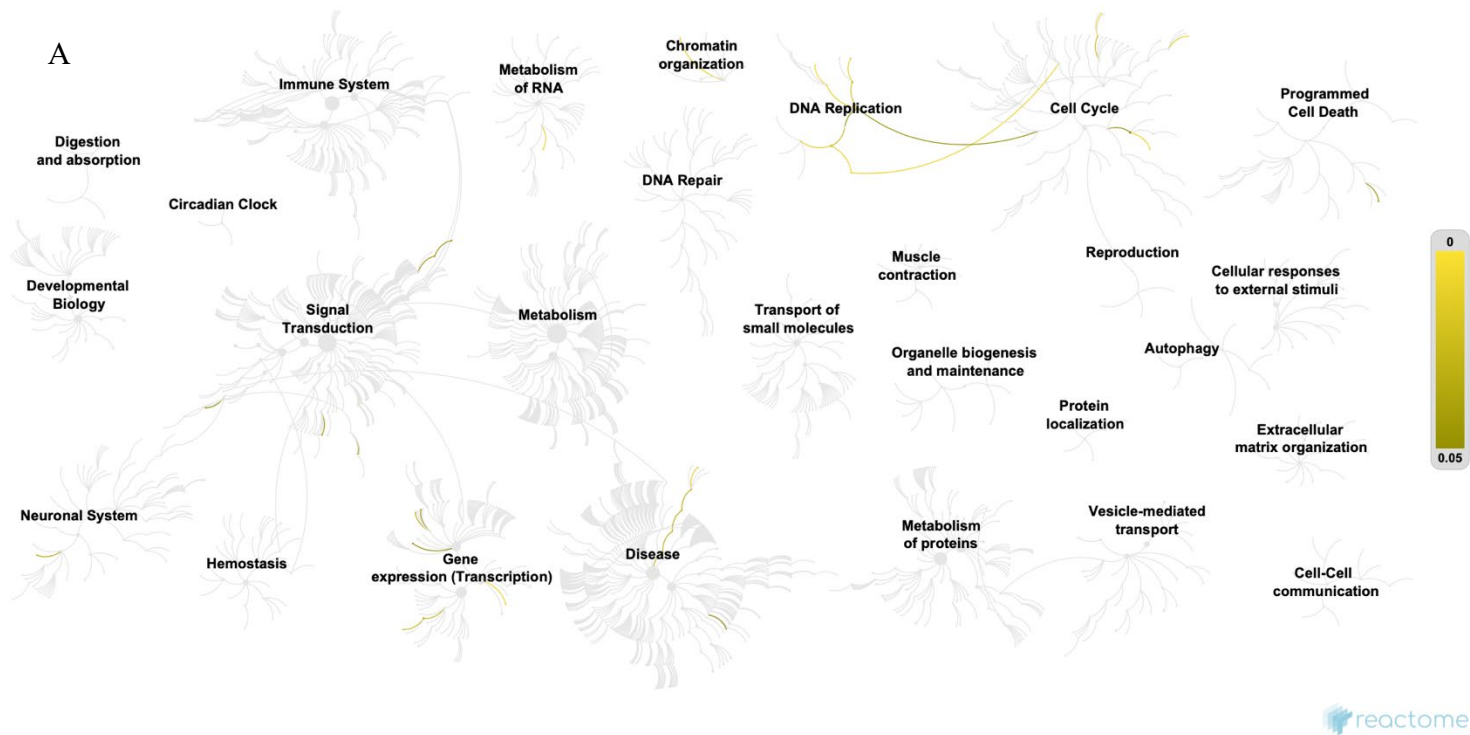


Figure 3.7 Reactome Pathway Analysis report for HA β MEFs. A) Genome-wide overview of the pathways enriched. Color code denotes overrepresentation of the pathway in the p110 β isoform regulated proteins. Light grey represents pathway that are not significantly over-represented. B) The following table shows the top most relevant pathways sorted by p-value. FDR: False Discovery Rate

3.5.2. STRING analysis

We performed a STRING analysis to see the predicted or known interactions of our differential p110 isoform regulated genes. With a medium confidence of 0.4 we see an interactome forming for PIK3CA and PIK3CB with the putatively regulated proteins by the respective isoforms.

B

Pathway name	Entities				Reactions	
	found	ratio	p-value	FDR*	found	ratio
p75NTR negatively regulates cell cycle via SC1	2 / 9	4.11e-04	1.18e-04	0.069	1 / 3	2.27e-04
Depolymerisation of the Nuclear Lamina	2 / 31	0.001	0.001	0.311	2 / 6	4.54e-04
Unwinding of DNA	2 / 44	0.002	0.003	0.311	2 / 4	3.02e-04
Activation of ATR in response to replication stress	2 / 63	0.003	0.005	0.311	2 / 9	6.80e-04
Activation of the pre-replicative complex	2 / 65	0.003	0.006	0.311	8 / 9	6.80e-04
RNA Polymerase II Transcription Termination	2 / 67	0.003	0.006	0.311	1 / 4	3.02e-04
mRNA 3'-end processing	2 / 71	0.003	0.007	0.311	3 / 3	2.27e-04
DNA strand elongation	2 / 73	0.003	0.007	0.311	2 / 15	0.001
HDACs deacetylate histones	2 / 77	0.004	0.008	0.311	2 / 5	3.78e-04
Orc1 removal from chromatin	2 / 77	0.004	0.008	0.311	1 / 4	3.02e-04
Initiation of Nuclear Envelope (NE) Reformation	3 / 78	0.004	0.008	0.311	3 / 7	5.29e-04
Loss of MECP2 binding ability to 5mC-DNA	1 / 5	2.28e-04	0.009	0.311	1 / 2	1.51e-04
Assembly of the pre-replicative complex	2 / 88	0.004	0.01	0.351	1 / 12	9.07e-04

Some noteworthy interactions in the interactome of PIK3CA and its regulated proteins are of NPM1, CANX, MSH6. The interactions seen in STRING have been

experimentally determined, have gene cooccurrence and gene fusions with their interactive partners in the interactome.

PIK3CB does not form a very large interactome but some interactions are observed especially between PIK3CB and Eif4ebp1. Eif4ebp1 is already a known substrate for mtor in the PI3K pathway. However, the putative phosphorylation sites that emerged in our mass spectrometry analysis are not the canonical phosphorylated sites of Eif4ebp1, which makes Eif4ebp1 a good candidate to be investigated for p110 β isoform specific regulation (Figure 3.8).

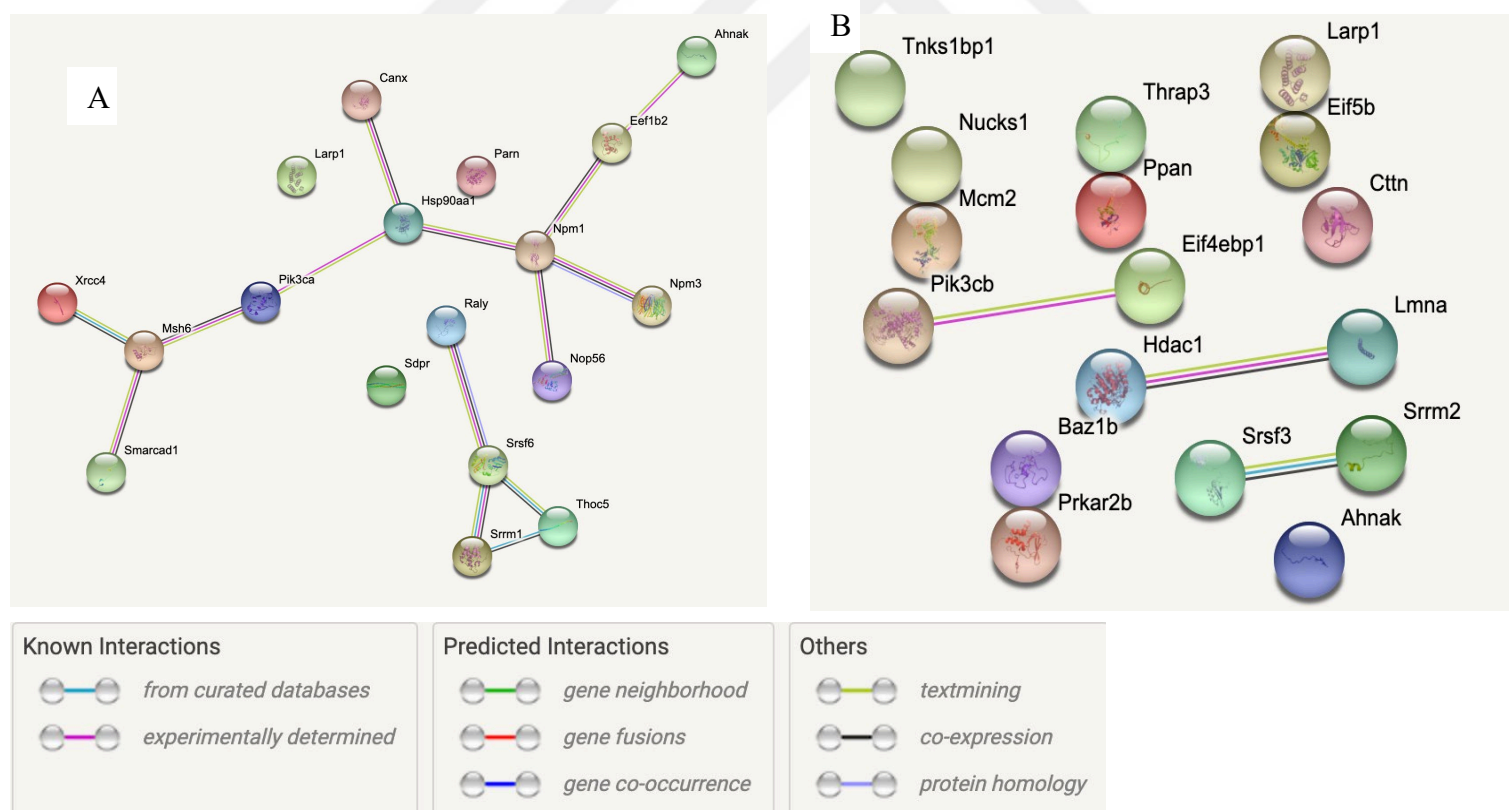


Figure 3.8: STRING interactions of proteins enriched with PIK3CA and PIK3CB isoform. A) Network was formed by querying PIK3CA with its identified candidate

genes. B) Network was formed by querying PIK3CA with its identified candidate genes **Medium confidence 0.4.**

3.5.3 c-Bioportal Cancer Genomics Analysis

Alteration frequencies

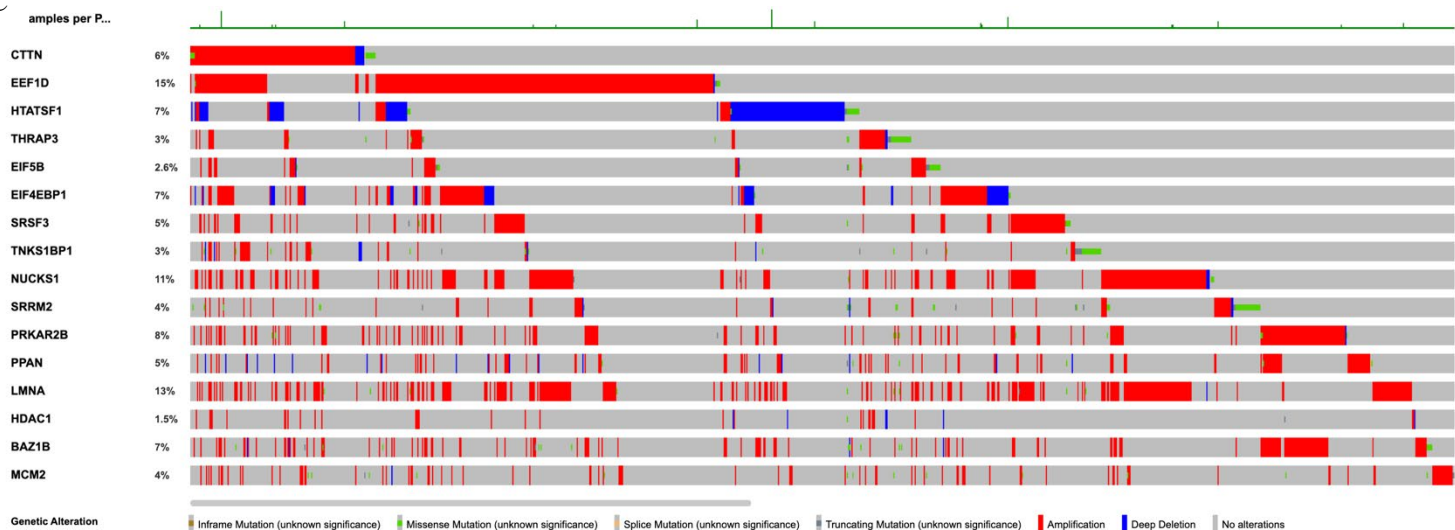
Cancer genome wide analysis was done on c-bioportal for the differentially regulated proteins we had identified for p110 α and p110 β isoforms. Figure 3.9A shows the percentage alteration of our identified p110 α regulated proteins in the Pan-cancer analysis of whole genomes (ICGC/TCGA, Nature 2020) database. 25% of the queried patients and queried samples had alterations from our list of p110 α regulated proteins. NPM1 and Calnexin had 5% and 4% of alterations in the database having amplifications mostly (Figure 3.9A). Analysis on PhosphositePlus revealed that NPM1 is a known substrate of Akt too which also suggests its interaction with the PI3K pathway. Our p110 α isoform regulated gene list also showed over 60% alteration frequencies of amplifications, mutations and deep deletions in several cancers, including colorectal cancer, ovarian cancer and breast cancer (Figure 3.9B).

Figure 3.9C shows the percentage alteration of our identified p110 β regulated proteins in the Pan-cancer analysis of whole genomes (ICGC/TCGA, Nature 2020) database. 44% of the queried patients and queried samples had alterations from our list of p110 β regulated proteins. Eif4ebp1 had 7% of genetic alterations, mainly amplifications. Our p110 β isoform regulated gene list also showed over 80% alteration frequencies of amplifications, mutations and deep deletions in different cancers, including endometrial cancers, breast cancer and lung cancer (Figure 3.8D). These results show that α and β isoform regulated gene list have some known implications in some cancers, however there might be isoform related relevance that still needs to be investigated and could be novel findings.

A



C



D

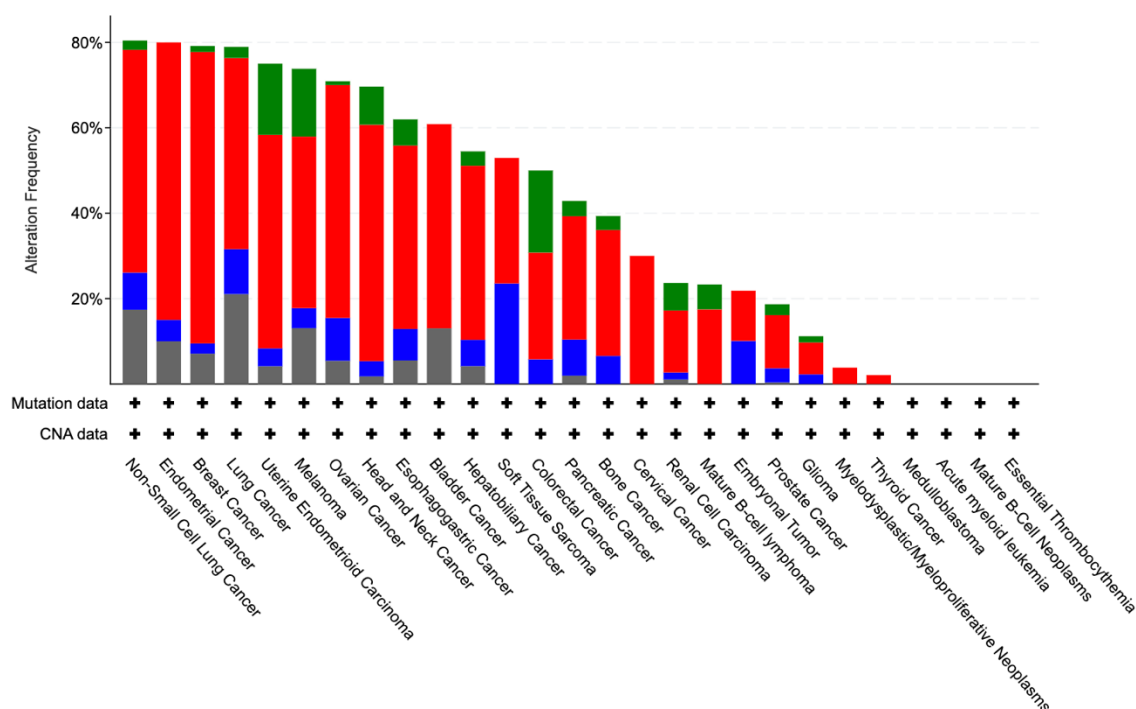


Figure 3.9 cBioPortal data on differential PIK3CA and PIK3CB regulated genes in Pan-cancer analysis of whole genomes (ICGC/TCGA, Nature 2020) database.

A) Genetic alteration frequencies for our mass spectrometry generated putative genes

for PIK3CA in the Pan-cancer database. B) Summary of alteration frequencies of PIK3CA regulated putative genes in different cancers. C) Genetic alteration frequencies for our mass spectrometry generated putative genes for PIK3CB in the Pan-cancer database. D) Summary of alteration frequencies of PIK3CB regulated putative genes in different cancers.

Overall survival and progression analysis:

We further analysed the overall survival and progression of cancer in our candidate genes. Figure 3.10 and 3.11 show the KM- survival plots of p110 α and p110 β isoform regulated proteins in Pan-Cancer TCGA database and invasive breast carcinoma Firehose RPPA study database. Figure 3.10 (A) shows a significant decrease in the overall survival of the entire PIK3CA regulated candidate gene list compared to the unaltered group. While the median survival of the unaltered group was 59.34 months, the median survival for the altered group was approximately 2 times less (34.18 months). This shows that the putative gene list regulated by PIK3CA is a potential list to be investigated because the alterations in them are strongly implicated for cancer survival. Figure 3.11 (A) investigates the overall survival median for PIK3CB regulated genes in the Pan Cancer data base. It also shows a significant and a much larger decrease in the overall survival compared to the unaltered group. The median months overall survival decreased approximately 3 times for the PIK3CB regulated genes as compared to the unaltered group. This shows a strong implication of these β isoform regulated genes in cancer.

Calnexin and NPM1 were further investigated in the Pan-Cancer TCGA and Invasive Breast Carcinoma FireHose RPPA database for their individual survival medians. In the TCGA Pan Cancer Data base, Calnexin and NPM1 showed significant decrease in survival compared to the unaltered group however, NPM1 showed a much larger

decrease of 7.5 times compared to the unaltered group. Both the proteins did not show any significant decrease in the Invasive Breast Carcinoma Firehose study (Figure 3.10 A, B, C)

For PIK3CB regulated potential genes, CTTN, Eif4EBP1 and HDAC1 were investigated in the Pan-Cancer TCGA and Invasive Breast Carcinoma FireHose RPPA database for their individual survival medians. For the Pan-Cancer TCGA database the overall survival medium dropped for all three genes significantly as compared to the unaltered group with HDAC1 having the least overall survival median of 6.74 as compared to the unaltered group (median overall survival: 94.70 months). Survival median of Ctnn and eif4ebp1 also considerably dropped 4 times and 2 times respectively. All three proteins did not show any significant change in the Invasive breast carcinoma firehouse study (Figure 3.11 A, B, C)

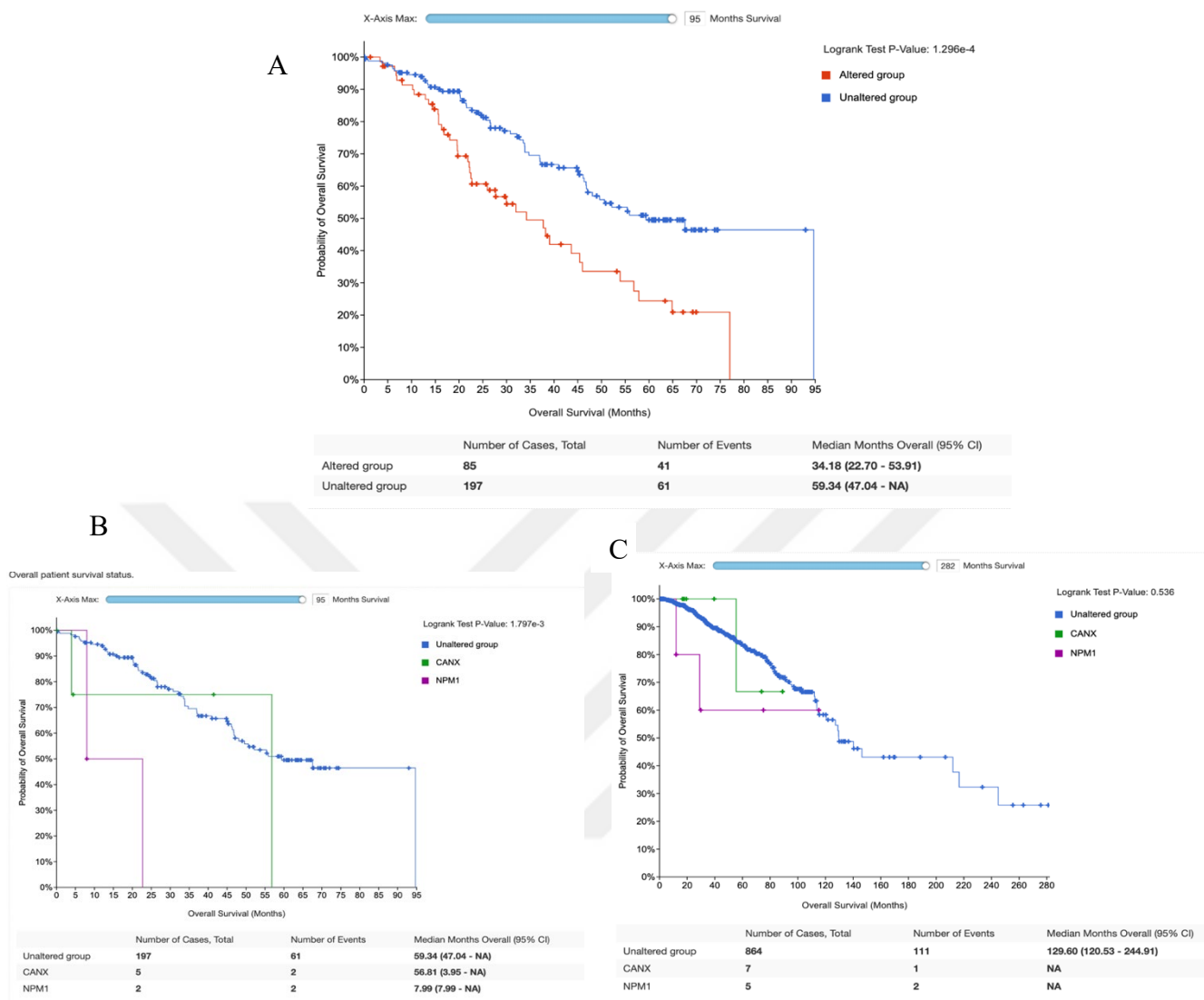
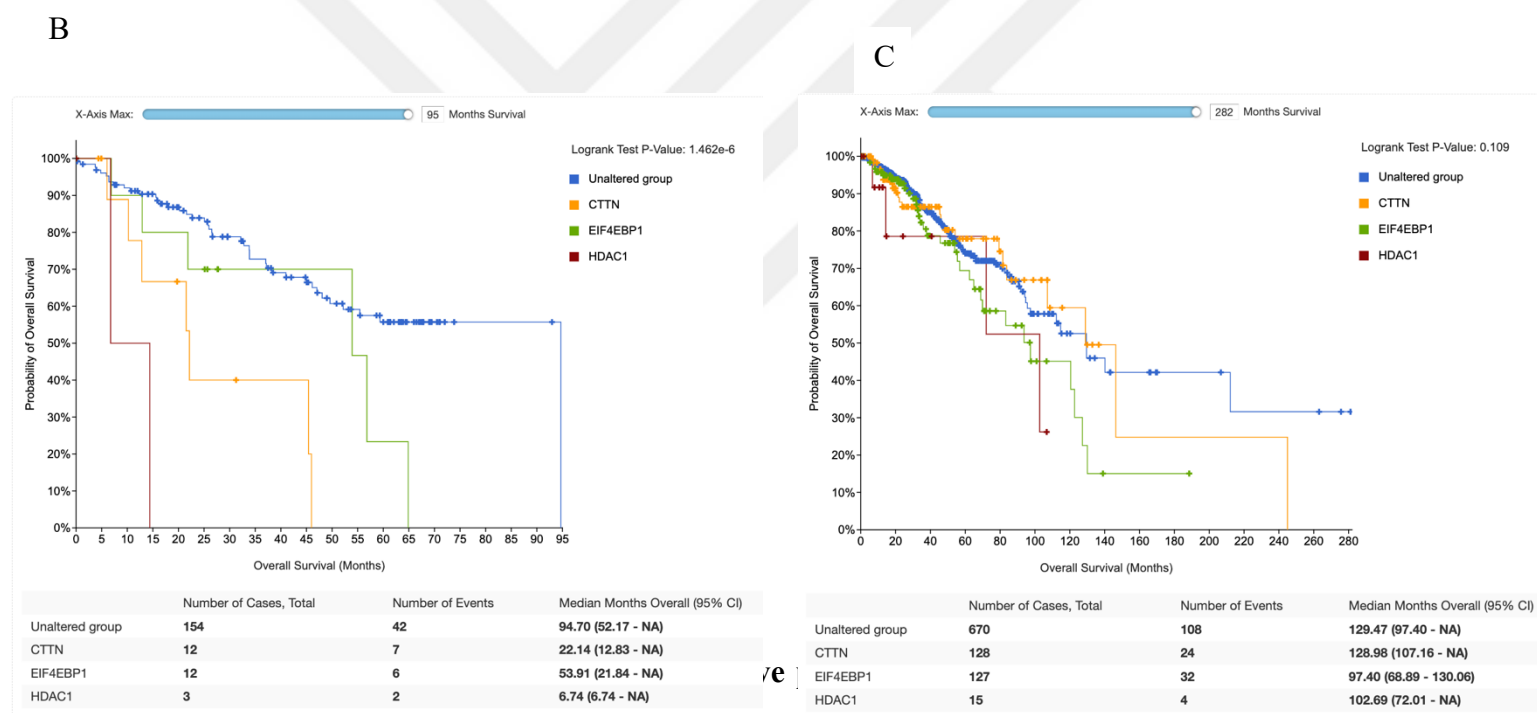
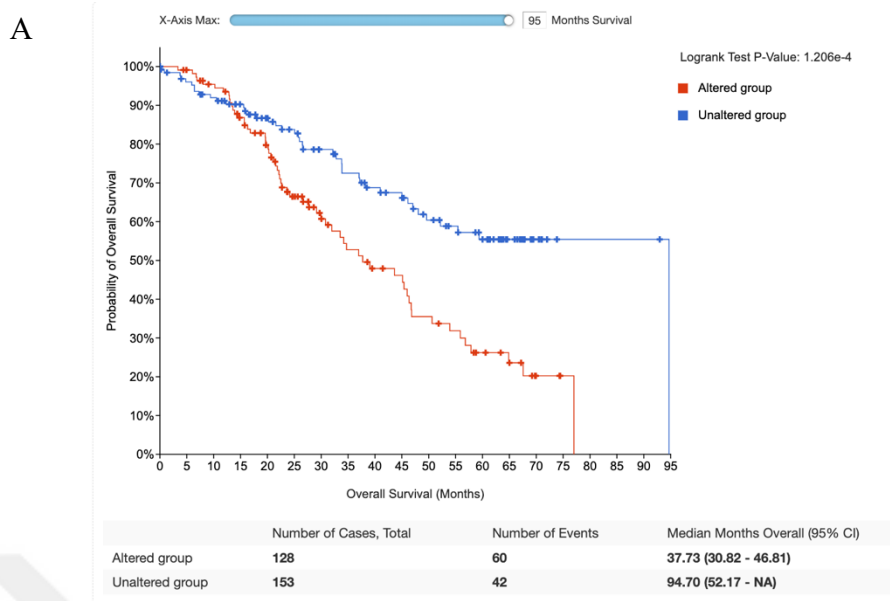


Figure 3.10 Survival plots of putative p110 α regulated genes and of shortlisted genes: NPM1 and CANX compared to the unaltered groups in A) Survival plot of p110 α regulated genes compared to the unaltered groups in Pan-cancer analysis of whole genomes (ICGC/TCGA, Nature 2020) database. P-value: 1.296e-4 B) Survival plot of NPM1 and CANX Pan-cancer analysis of whole genomes (ICGC/TCGA, Nature 2020) database. p-value 1.797e-3. C) in Breast Invasive Carcinoma (TCGA, Firehose Legacy) 0.536. p-value



genes: CTTN, Eif4ebp1, HDAC1 compared to the unaltered groups in A) Survival plot of p110 β regulated genes compared to the unaltered groups in Pan-cancer analysis of whole genomes (ICGC/TCGA, Nature 2020) database. P-value: 1.206e-4 B) Survival plot of CTTN and EIF4EBP1 Pan-cancer analysis of whole genomes (ICGC/TCGA, Nature 2020) database. p-value 1.462e-6. C) in Breast Invasive Carcinoma (TCGA, Firehose Legacy) p-value 0.109.

3.6 Shortlisting of candidate phosphoproteins for biochemical validation

For p110 α differentially regulated phosphoproteins, we shortlisted NPM1 and Calnexin to be biochemically validated invitro. For p110 β differentially regulated phosphoproteins, we shortlisted Eif4ebp1, Cortactin and HDAC1 to be biochemically validated invitro. We did this shortlisting based on the fold change they showed upon inhibition, their statistical significance, our bioinformatics analysis and the availability of the phosphor- specific antibody in the market. Table 3.1 and Table 3.2 shows the shortlisted candidate proteins, their fold changes, phosphopeptides and the residues.

Proteins	p-value	Fold Change PDGF/ BYL719	Phosphopeptides	Residues
NPM1	0.026454	1.504	HISGQHLVAVEEDAESEDEDEEDVKLLGMSG	125s
CANX	0.031305	3.844	EDSKPKAEDEILNRSPRNRKPRRE	582s

Table 1.1:Shortlisted Significant candidate phosphopeptides and their phosphorylated residues for p110 α isoform dependent cells (DKO-HA α)

Proteins	p-value	Fold Change PDGF/ KIN193	Phosphopeptides	residues
CTTN	0.001447	6.12	KLEEQARAKKQTTPASPSQPIEDRPPSSPI	364T, 368S, 370S
HDAC1	0.0263	1.707	SICSSDKRIACEEEFSDSDEEGEGGRKNSSN	421S,423S
EIF4EBP1	0.046781	6.3	TRIIYDRKFLMECRNSPVAKTPPKDLPAIPG	64S,69T

Table 3.2: Shortlisted significant candidate phosphopeptides and their phosphorylated residues for p110 β isoform dependent cells (DKO-HA β)

3.7 Biochemical Validation of Phospho-proteomic Data

Candidates were shortlisted from previous analysis and then subjected to growth factor signalling experiments, similar to previous ones in order to biochemically validate the mass spectrometry findings. Westernblot analysis was used to envisage the phosphorylation changes in the case where phospho-specific antibodies were available. With the aid of the phospho-specific antibodies, the isoform specific phosphorylation changes predicted by the mass spectrometry phosphoproteomic data can be detected and confirmed. For these experiments we used parental WT MEFs with the isoform specific inhibitors to create isoform dependency in the parental WT MEFs as well as DKO-HA α and DKO-HA β cells. For some phosphoproteomic candidate proteins we also tried to validate them in cancer model cells.

A. Characterization of PI3K pathway in Parental WT MEFs

Next, we validated the regulation of phosphorylation sites in the parental WT MEFs. As previously described, cells were starved for 4 hours, stimulated with 10% FBS for 15 minutes for more robust stimulation and then treated with a Pan-PI3K inhibitor and isoform specific inhibitors for 30 minutes to see isoform specific regulation. We first checked for the dependency of the PI3K pathway on its isoforms by treating it with a Pan- PI3K inhibitor GDC0941, BYL719 and KIN193 and checked for the AKT and s6 phosphorylation. In the presence of GDC the PI3K pathway is fully inhibited as the phosphorylation signal fully diminishes (Figure 3.12). When the p110 α isoform is inhibited the cells are dependent on p110 β and vice versa. The decrease in

phosphorylation when p110 β is inhibited is very less indicating that the cells are mostly dependent on the p110 α isoform as the more catalytically potent isoform.

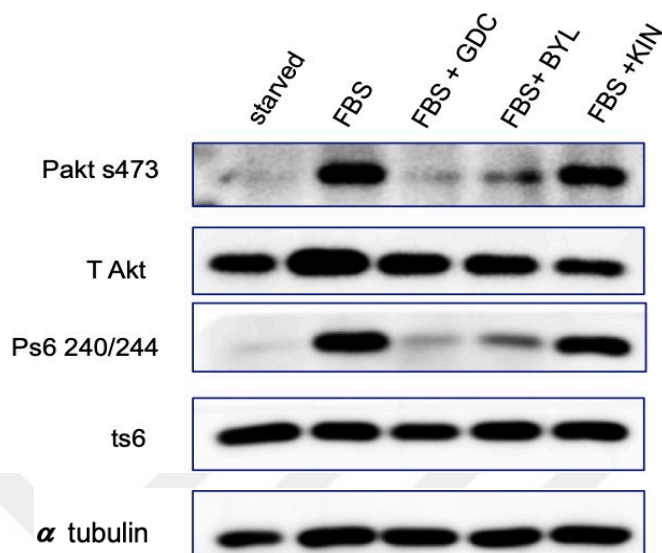


Figure 3.12 Western blot analysis with parental WT MEFs to characterize isoform dependency of PI3K pathway. Cells were starved for 4 hours, stimulated with 10% FBS for 15 minutes and inhibition treatment with GDC, BYL710, and KIN193 for 30 minutes. 2 μ M inhibitors concentrations. 20 μ g proteins. N=1

3.7.1 Biochemical Validation of PIK3CA candidates- p-NPM1 S-125 in MEFs and T47D cells

Regulation of NPM1 at the 125th Serine residue, was one of the phosphoprotein candidate we shortlisted for validation. In our phosphoproteomic data we saw that this site had a 3-fold increase change after stimulation with PDGF and a 1.5-fold decrease change when inhibited with p110 α specific isoform inhibitor BYL719. This made

NPM1 a good candidate to be biochemically investigated for any novel phosphorylation regulation by the p110 α isoform.

3.7.1A. Validation *p-NPM1* in Parental WT MEFs.

We performed immunoblot analysis with phosphor specific, anti phospho-NPM1 S125 antibody to see the regulation of the phosphorylation. A small hike in s125 phosphorylation was seen after stimulation with FBS whereas a 30% decrease was seen with both the inhibitions. Unfortunately, no isoform specific regulation was observed (Figure 3.13). We hypothesized that a change was not observable a) because western blot is less sensitive than high throughput mass spectrometry and so it gives an average of the changes happening in the protein lysate and b) NPM1 might be regulated much downstream of the pathway so it might need more time to dephosphorylate.

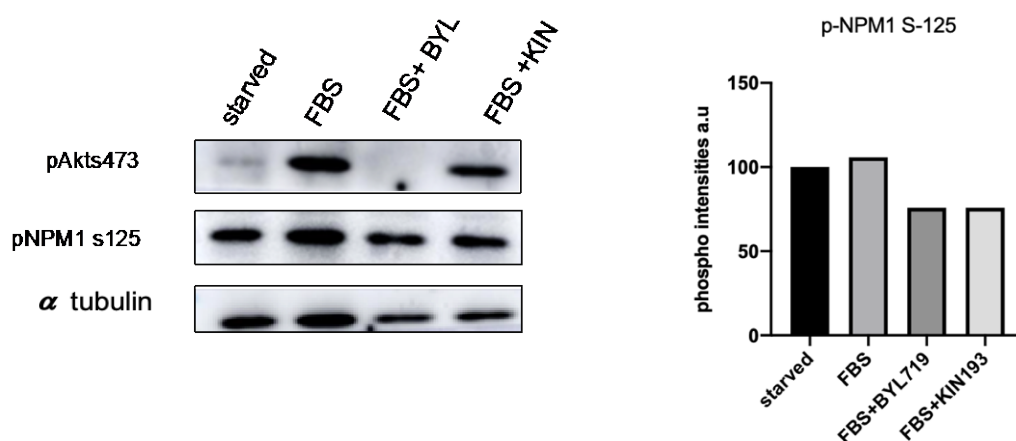


Figure 3.13 Validation *p-NPM1* S125 in Parental WT MEFs with short term inhibition. Immunoblot of p-NPM1 S-125 in WT MEFs upon starvation and short-term inhibition. WT MEFS Cells were starved for 4 hours then treated with 10% fbs

for 15 mins, and treated with 2uM inhibitors for half hour. Phosphorylation intensity normalized to starved condition. 20ug protein; N=1

We starved WT MEFs for 4 hours again and extended the stimulation time to 30 minutes, and extended the inhibition time to 6 hours. Upon extending the stimulation and inhibition time, the p-NPM1 signal increased upon FBS stimulation and a decrease with isoform specific inhibitor. The phosphorylation signal decreased more with the BYL719 inhibition as compared to beta isoformic inhibition (3.14). However, the difference was not paramount.

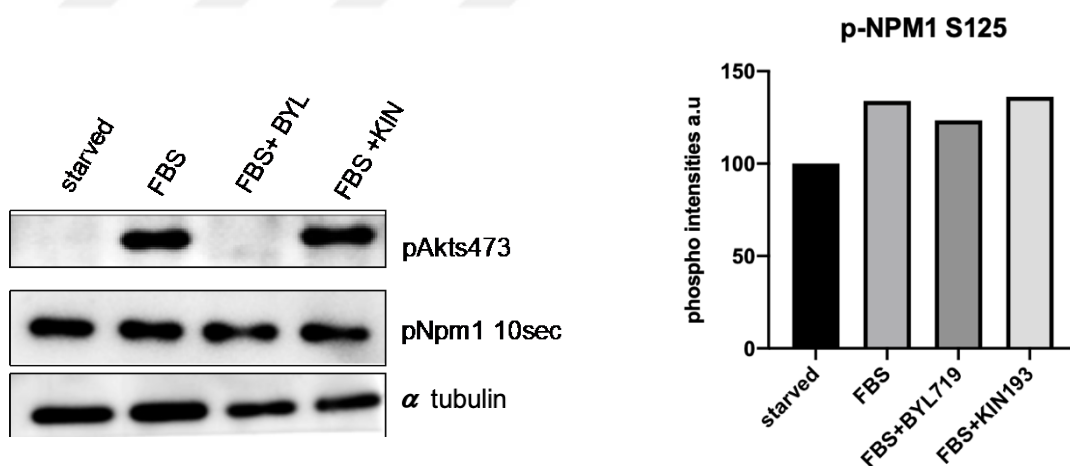


Figure 3.14 Validation p-NPM1 S125 in Parental WT MEFs with long term inhibition. Immunoblot of p-NPM1 S-125 in WT MEFs upon starvation and long-term inhibition. WT. MEFS Cells were starved for 4 hours then treated with 10% FBS for 30 mins, and treated with 2uM inhibitors for 6 hours. Phosphorylation intensity normalized to starved condition. 20ug protein; N=1

3.7.1B. Validation of p-NPM1 S125 in PIK3CA H1047R mutant

PIK3CA H1047R is a hotspot mutation in the PIK3CA protein's kinase catalytic domain that constitutively activates it. We used genetically modified DKO MEFs, with H1047R mutant PIK3CA ectopically expressed. PI3K pathway was constitutively activated in these cells when they were induced by doxycycline. Cells were treated with DMSO and BYL719 for 24 hours and checked for AKT phosphorylation and NPM1 phosphorylation. After 24 hours of inhibition, AKT phosphorylation had diminished indicating that the PI3K pathway was inactive in the mutant cells. NPM1 also showed 50% decrease upon inhibition when the p110 α isoform was inactive suggesting that p110 α isoform may have a role in regulating the NPM1 phosphorylation at S125 (Figure 3.15).

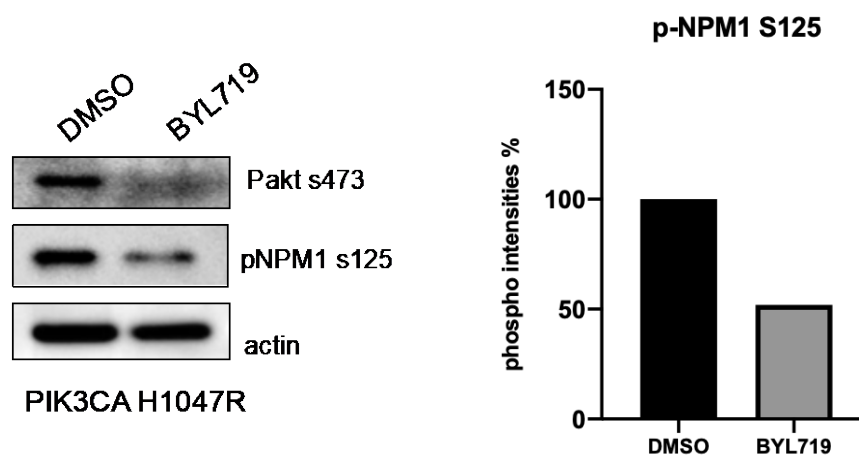


Figure 3.15: Validation of p-NPM1 S125 in PIK3CA H1047R mutant MEFs by western Blot analysis Cells were inhibited with 2uM BYL719 for 24 hours and the cells were then harvested and immunoblotting was performed. Mutant ectopic construct

induced with 0.25ug/ml doxycycline. Maintained with 50ug/ml NAT, 50ug/ml G418. Phosphorylation intensity normalized to DMSO control. 20ug protein ; N=1

3.7.1C Validation of p-NPM1 S125 in PIK3CB E1051K mutant

In order to control for the isoformic differential regulation of NPM1 phosphorylation at S-125 residue we used MEFs which expressed PIK3CB with constitutively activating E1051K mutation. We treated the cells similarly to the PIK3CA HR cells, with p110 β specific inhibitor KIN193, for 24 hours and then performed immunoblotting to detect the phosphorylation signals. E1051K mutation is very potent and over activates the PI3K pathway considerably more than the HR mutation. Despite a very high activation by the p110 β EK mutant, we observed only a 20% decrease in the phosphorylation signal of s-125 pNPM1 as compared to a 50% decrease with the p110 α HR mutant (Figure 3.16). These results could indicate that the NPM1 phosphorylation at S-125 may primarily be regulated by the p110 α isoform.

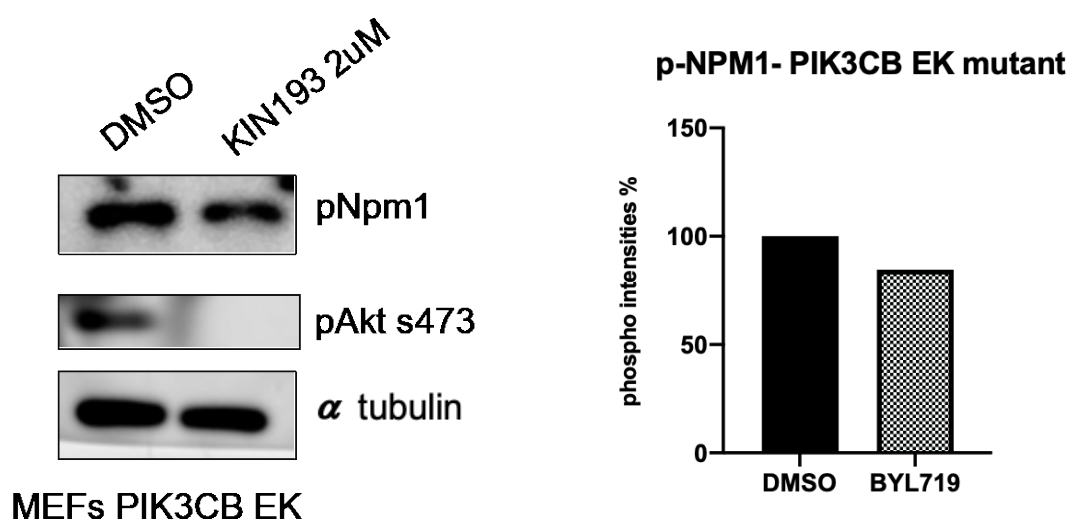


Figure 3.16: Validation of p-NPM1 S125 in PIK3CB E1051K mutant MEFs by western Blot analysis. Cells were inhibited with 2uM KIN193 for 24 hours and the cells were then harvested and immunoblotting was performed. Induced with 0.25ug/ml doxycycline. Maintained with 50ug/ml NAT, 50ug/ml G418. Phosphorylation intensity normalized to DMSO control. 20ug protein; N=1

3.7.1D Investigating p-NPM1 S125 phosphorylation as a downstream effector of PI3K-AKT-mTOR pathway

To investigate NPM1 phosphorylation as a downstream effector of the PI3K-AKT-mTOR axis, we cultured DKO HA α and HA β cells in maintenance media and treated them with p110 α and p110 β isoform specific inhibitor, BYL719 and KIN193 respectively, AKT inhibitor MK2006 and mTOR inhibitor Everolimus. We inhibited different components of the PI3K pathway to see which inhibition provided with a potent decrease in NPM1 phosphorylation and hence could be an upstream regulator of NPM1. We performed an immunoblot to control for the inhibited components of the PI3K pathway. p70s6Kinase is a downstream substrate of mTOR, upon treatment with everolimus we see a complete erasure of phosphorylation at p70s6kinase indicating successful inhibition of mTOR. Pras40 is a downstream substrate of AKT, so is used as a proxy to investigate the activity of AKT. Upon MK2006 inhibition we see complete eradication of p-pras40 signal, indicating that AKT is also successfully inhibited. Upon BYL719 and KIN193 inhibition we see a decrease in phosphorylation in all three downstream effectors of PI3K pathway, p70s6k, pras40 and Akt, indicating that the PI3K pathway is successfully inhibited with 24 hours of inhibition with isoform specific inhibitor. We checked the p-NPM1 s125 levels in these cells and we do not see any significant or conclusive differences between different inhibitions.

For HA α cells we see a slight increase in p-NPM1 levels with p110 α and AKT inhibition and no change in the levels when mtor is inhibited (Figure 3.17). This may be accounted by a negative feedback loop, because the cells are inhibited for 24 hours, prolonged inhibition of PI3K pathway maybe causing an increase in the NPM1 phosphorylation. As compared to HA- p110 α cells, we see a decrease in NPM1 s125 phosphorylation with different inhibitions, especially when mtor is inhibited. These results are not conclusive or suggestive of differential regulation. Much better insight could be gained if the same experiment is repeated with PIK3CA HR mutant cells, where the PI3K pathway is constitutively activated, and hence any regulation of NPM1 by the components of the PI3K pathway can be better elucidated.

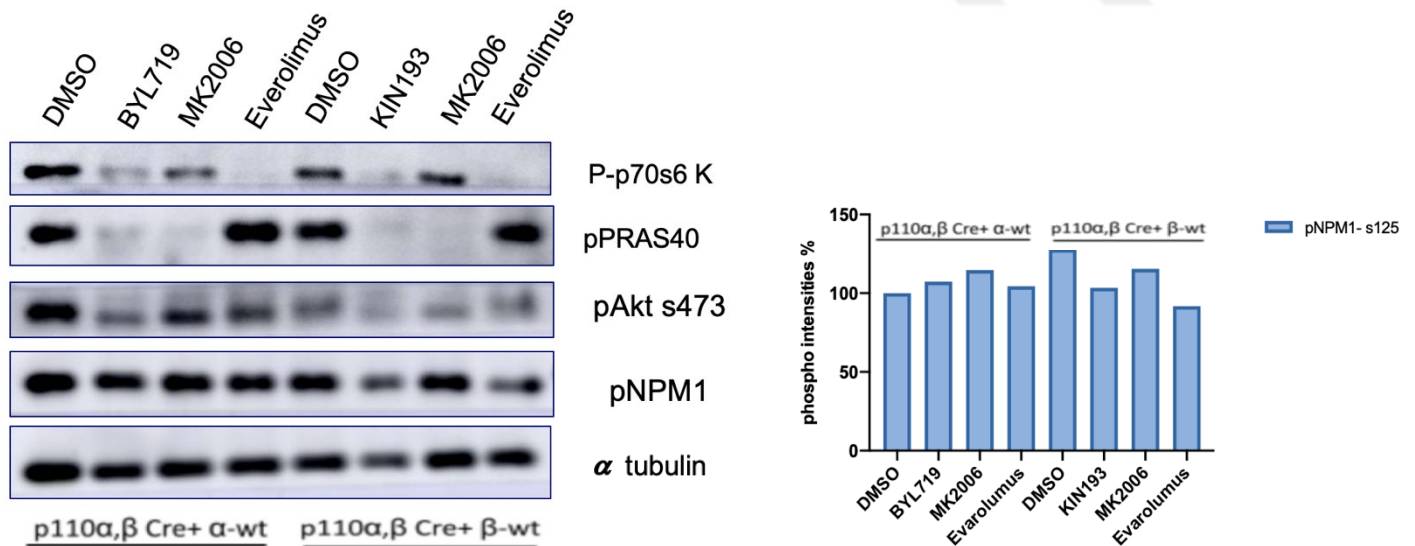


Figure 3.17 Investigating p-NPM1 S125 phosphorylation as a downstream effector of PI3K-AKT-mTOR pathway Immunoblot of p-NPM1 S-125 levels when p110 α /p110 β , AKT or mTOR are inhibited in the PI3K pathway. DKO HA α , HA β

MEFs were cultured in 8% FBS, and treated with BYL719, KIN193, MK2006 and Everolimus for 24 hour. BYL719, KIN193, MK2006: 2uM, Everolimus: 50nM. 25ug proteins; n=1. Phosphorylation levels of NPM1 are normalized to DMSO control of HA α .

3.7.1E Biochemical validation of p-NPM1 s125 in T47-D breast cancer cells.

T47D are breast cancer cells that have an activating mutation in the catalytic domain of PIK3CA which makes it a good cancer model to validate our candidate protein NPM1. We treated the cells with BYL719 for 24 hours and harvested them for immunoblotting. Upon inhibition the PI3K pathway was blocked as seen from the disappearance of the AKT signal. We observed approximately a 50% decrease in NPM1 phosphorylation at S-125 when the cells were treated with BYL719 similar to what we had seen with the PIK3CA HR cells (Figure 3.18). These results indicate that our phosphoproteomic candidate, NPM1 could be a suggestive differential downstream substrate for the p110 α isoform.

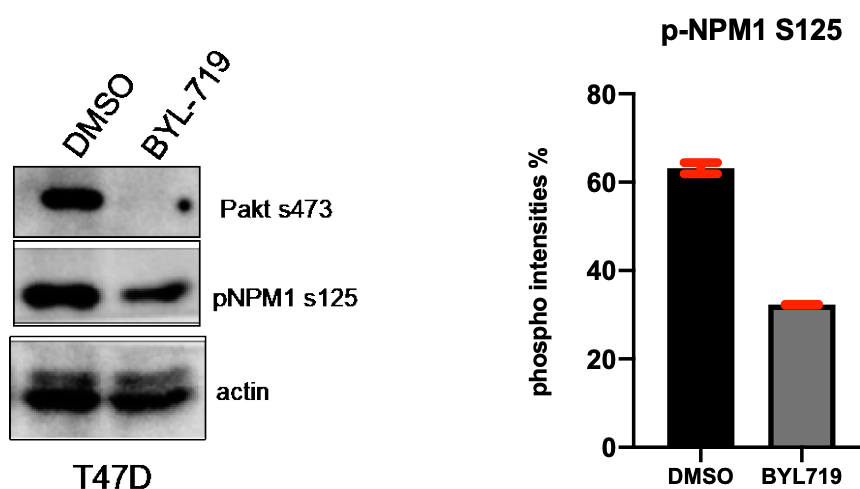


Figure 3.18 : Validation of pNPM1 S-125 in T47D breast cancer cell line; Cells were inhibited with 2uM BYL719 for 24 hours and the cells were then harvested and immunoblotting was performed. Phosphorylation intensity normalized to DMSO control. 20ug protein; N=2; SEM plotted.

3.7.2 Biochemical Validation of Phospho-proteomic PIK3CB candidates in MEFS – p-EIF4EBP1 S64, Thr69

Regulation of Eif4ebp1 at the 64th Serine residue and the 69th Threonine residue was one of the PIK3CB regulated candidate sites we predicted from the mass spectrometry data. However, from checking online databases we found s65 and T70 to be a known phosphorylation site for Eif4ebp1 also these were the sites with available antibodies that could be utilized. It is also one of the limitations of mass spectrometry that the detection of some residues might be displaced by one or two residue. Hence we investigated S-65 and T-70 residues instead of S-64 and T-69.

3.7.2A Validation of p4ebp1 s65, Thr70 in DKO- HA α and HA β cells

We starved, stimulated and treated DKO- HA α and HA β cells with PDGF and isoform specific inhibitors as previously described. We performed immunoblotting and observed the changes in the phosphorylation levels of Eif4ebp1 at S65 and T70. For S65, the HA α cells do not show a big change in phosphorylation levels when stimulated or inhibited however more than 6-fold decrease is observed when cells are inhibited with KIN193 after stimulation with PDGF. This suggests that p110 β might be exerting a greater effect on this phosphorylation site as compared to p110 α .

The Thr70 site seems to be differentially phosphorylated by the p110 β isoform. Upon immunoblotting we see a decrease in phosphorylation of the Thr70 signal when the p110 β isoform is inhibited as opposed to the signal in p110 α dependent cells where the phosphorylation increases upon inhibition suggesting that p110 α may be a negative regulator 4ebp1 at Th70 (Figure 3.19).

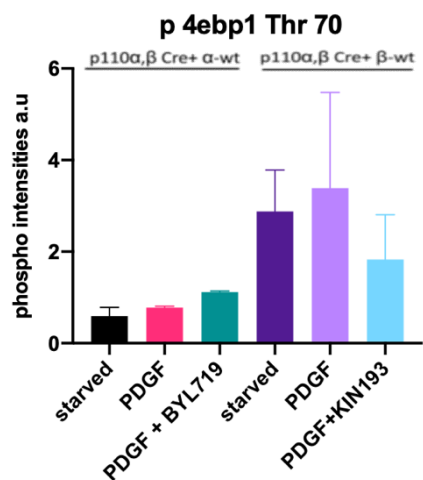
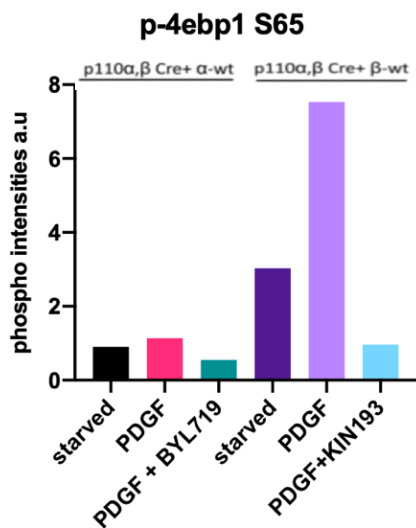
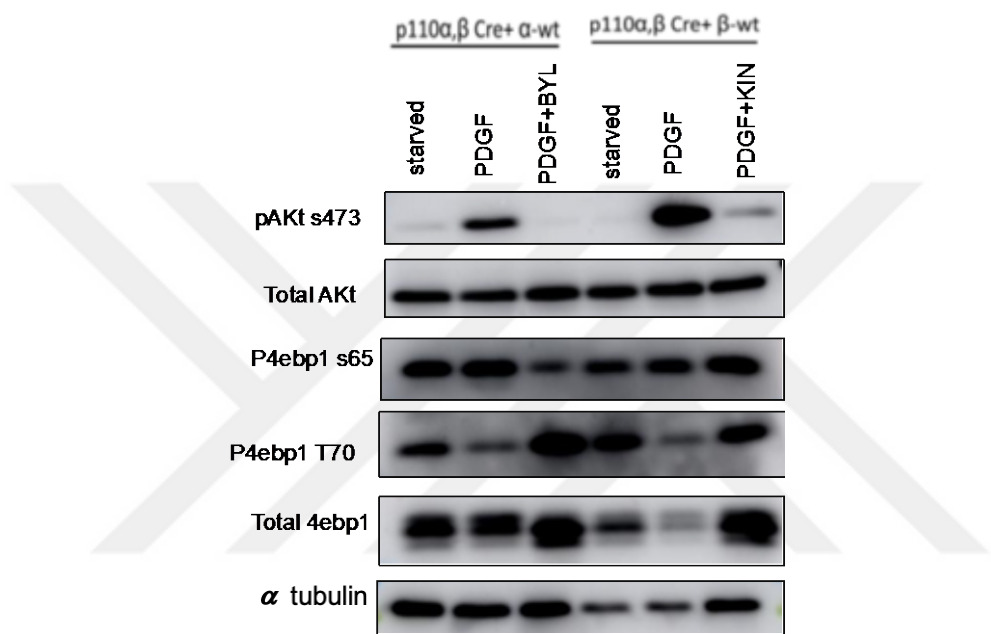


Figure 3.19 Validation of p-EIF4EBP1 in DKO HA, p110 α and p110 β cells.

Cells starved for 4 hours, 5 minutes stimulation and 30 minutes inhibition with p110 α and p110 β specific inhibitor. Cells were harvested and immunoblotting was performed. 0.5uM BYL719, KIN193 inhibitors. N=2 replicates for Th-70. SEM

3.7.2B Validation of p4ebp1 s65, Thr70 in parental WT MEFs

We also checked the canonical phosphorylation site of EIF4ebp1 T37/46 in parental WT MEFs which is downstream substrate of mtor. We see a similar phosphorylation pattern with a greater decrease when p110 α is inhibited showing that the canonical phosphorylation of 4ebp1 is reflecting the activation of PI3K pathway. We also saw a comparable migration difference in the banding of Total Eif4ebp1; the GDC treated band is shifted the most downward suggesting most dephosphorylation and hence the lightest. BYL719 treated band shows more shift downwards as compared to KIN193 suggesting there are more p110 α differentially regulated phosphorylation sites on Eif4ebp1 as compared to p110 β and T70 and S65 may be one of them (Figure 3.20).

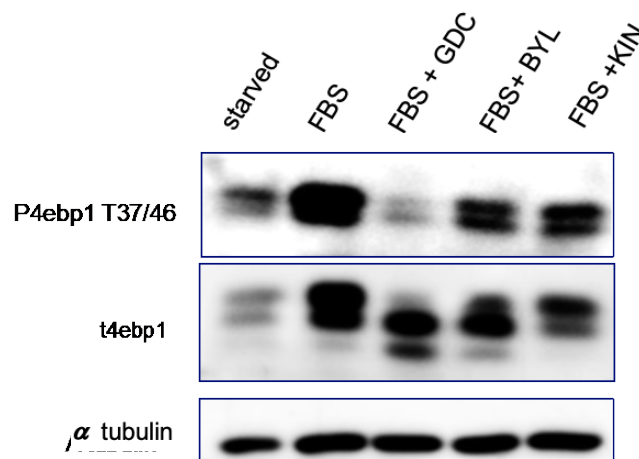


Figure 3.20 Western blot analysis with parental WT MEFS to check the canonical phosphorylation of Eif4ebp1. Cells were starved for 4 hours, stimulated with 10% FBS for 15 minutes and inhibition treatment with GDC, BYL710, and KIN193 for 30 minutes. 2uM inhibitors concentrations. 20ug proteins. N=1

Serine-65 residue and Thr-70 residue were then checked in parental WT MEFs by treating them with isoformic inhibitors. In parental WT MEFS as well, we see an increase in S-65 phosphorylation when the p110 α is inhibited with BYL719, in line with our previous results with DKO-HA α cells indicating negative regulation of s-65 residue by the p110 α isoform. The phosphorylation decreases slightly when cells are treated with p110 β inhibitor KIN193. In the lanes of total levels, the BYL719 treated lane is much lower than the KIN193 treated lane, indicating a lower molecular weight, which might occur due to lesser post translational modification. The total levels of Eif4ebp1 also indicate similar to previous results that the p110 α isoform may be regulating more phosphorylation sites on 4ebp1 as compared to p110 β such that when BYL719 is inhibited the post translational modifications decrease much more. However, our mass spectrometry had revealed Thr70 residue as p110 β regulated site (Figure 3.21). So, we next validated Thr70 in parental WT MEFs.

We starve, stimulate and inhibit the cells with isoformic inhibitors as previously described. Upon inhibition with BYL719, the phosphorylation increased slightly however decreased significantly when the p110 β isoform was inhibited with KIN193. This significant decrease upon the inhibition of p110 β suggests that Thr70 residue of Eif4ebp1 could be a differential substrate of the p110 β isoform whereas p110 α may be negatively regulating 4ebp1 phosphorylation (Figure 3.22).

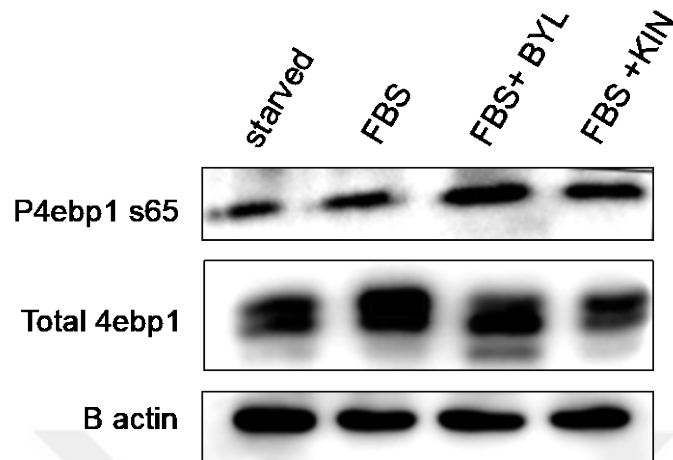


Figure 3.21 Validation of p4ebp1 s65 in parental WT MEFs Cells were starved for 4 hours then treated with 10% fbs for 15 mins, and treated with 2uM inhibitors for 30 minutes. Immunoblotting was performed to analyse the phosphorylation levels. 20ug protein. ; N=1

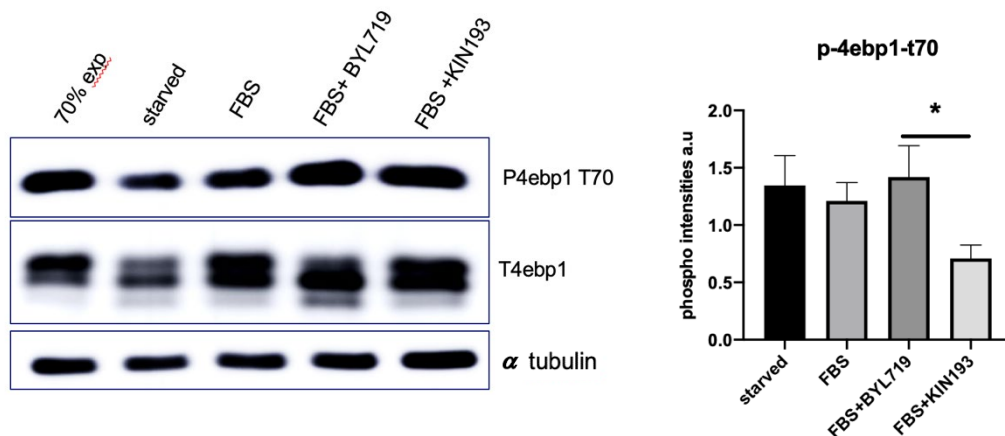


Figure 3.22 Validation of p4ebp1 T70 in parental WT MEFs

WT-MEFs Cells were starved for 4 hours then treated with 10% FBS for 15 mins, and treated with 2uM inhibitors for 30 minutes. Immunoblotting was performed to

analyse the phosphorylation levels. 28ug protein. N=3, SD error bars plotted.*

p<0.05

3.7.3 Biochemical Validation of Phospho-proteomic PIK3CB

candidates in MEFS – Cortactin 368S, 370S

We next wanted to validate Cortactin phosphorylation at 368S and 370S residue. A suitable phospho-specific antibody for the candidate phospho-protein Ctnn was not available so Cortactin was enriched by immunoprecipitation with anti-cortactin monoclonal mouse antibody and subjected to Western blot analysis with selective antibody specific for phosphoserine. With this method, it was anticipated that cortactin will be pulled down and the change in serine phosphorylation amounts for cortactin could be seen even though it would be total serine levels and not specific serine residues. For proteins that do not currently have phospho-specific antibodies at a western blot level, this method could be used as a suggestive method to derive preliminary data for the validation of the specific p-serine residues.

Santacruz's Cortactin Antibody (H-5): sc-55579 was used for this experiment, and the data sheet showed cortactin band appearing $\cong$ 90kDa, however before performing the immunoprecipitation, we first characterized the antibody in our WT MEFs and saw that the cortactin band appeared approximately at 55kDa (Figure 3.23).

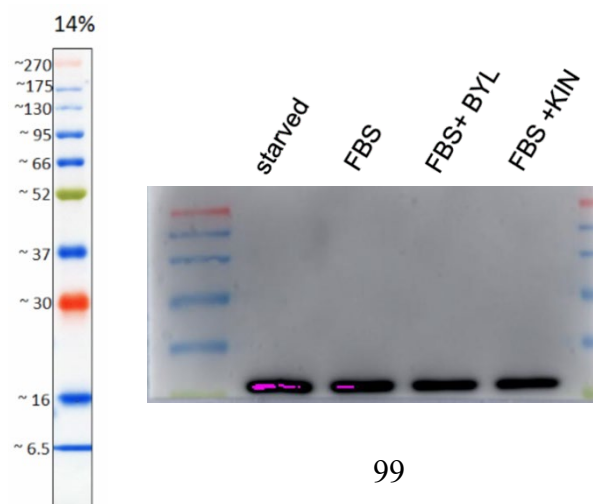


Figure 3.23 Characterization of Cortactin antibody in parental WT MEFs.

Cortactin band appears right above the 52kDa mark.

We performed immunoprecipitation (IP) of cortactin in whole cell lysates (WCL) of WT MEFs, which were starved, stimulated with 10% FBS and inhibited as previously described. We had a positive control with WCL and a negative control of our cortactin pull down with random rabbit IGG. 500ug of protein was pulled down using anti-cortactin mouse monoclonal antibody that detected mouse, rat and human cortactin. Immunoprecipitation was performed for each condition: starved, stimulation with FBS, p110 α isoform inhibition by BYL719 and p110 β isoform inhibition by KIN193. Western blot was then performed for the pull down in each condition having a positive control (WCL), negative control (Random Igg) and cortactin pull down (Cttn IP) lanes. The membrane was immunoblotted with anti-phospho Serine rabbit antibody and the signals were detected.

When we performed immunoblot for p-Serine with anti-rabbit secondary antibody we detected a thick band of rabbit Igg heavy chain chain in the random rabbit Igg lane at ~52 kDa which masked the p-serine enriched band on the cortactin IP pulldown down because of the high intensity of the heavy chain band. The predicted band site for cortactin from previous characterization is ~ 55kDa, which very close to the site of the rabbit heavy chain in the neighbouring lane therefore visualization of the enriched p-Serine band was hard to perform. We saw some faint bands in the Cttn IP lanes, but it was difficult to derive conclusions from them (Figure 3.24A). We stripped our membrane and checked for total cortactin levels by immunblotting cortactin with anti-mouse antibody. However, the bands could not be visualized because the mouse Igg heavy chains of cortactin antibody which had been used to pull down cortactin masked

the cortactin bands due to them being present at the same site. In the whole cell lysate lanes we saw faint cortactin bands, that were also masked by the neighbouring high intensity bands of mouse IgG heavy chains (Figure 3.24B).

This experiment can be repeated by separately using mouse random IgG rather than rabbit random IgGs. VeriBlot for IP Detection Reagent can also be used to detect total levels of cortactin pulled down. It recognizes primary antibodies only in their native form, avoiding heavy chain detection in IPs.

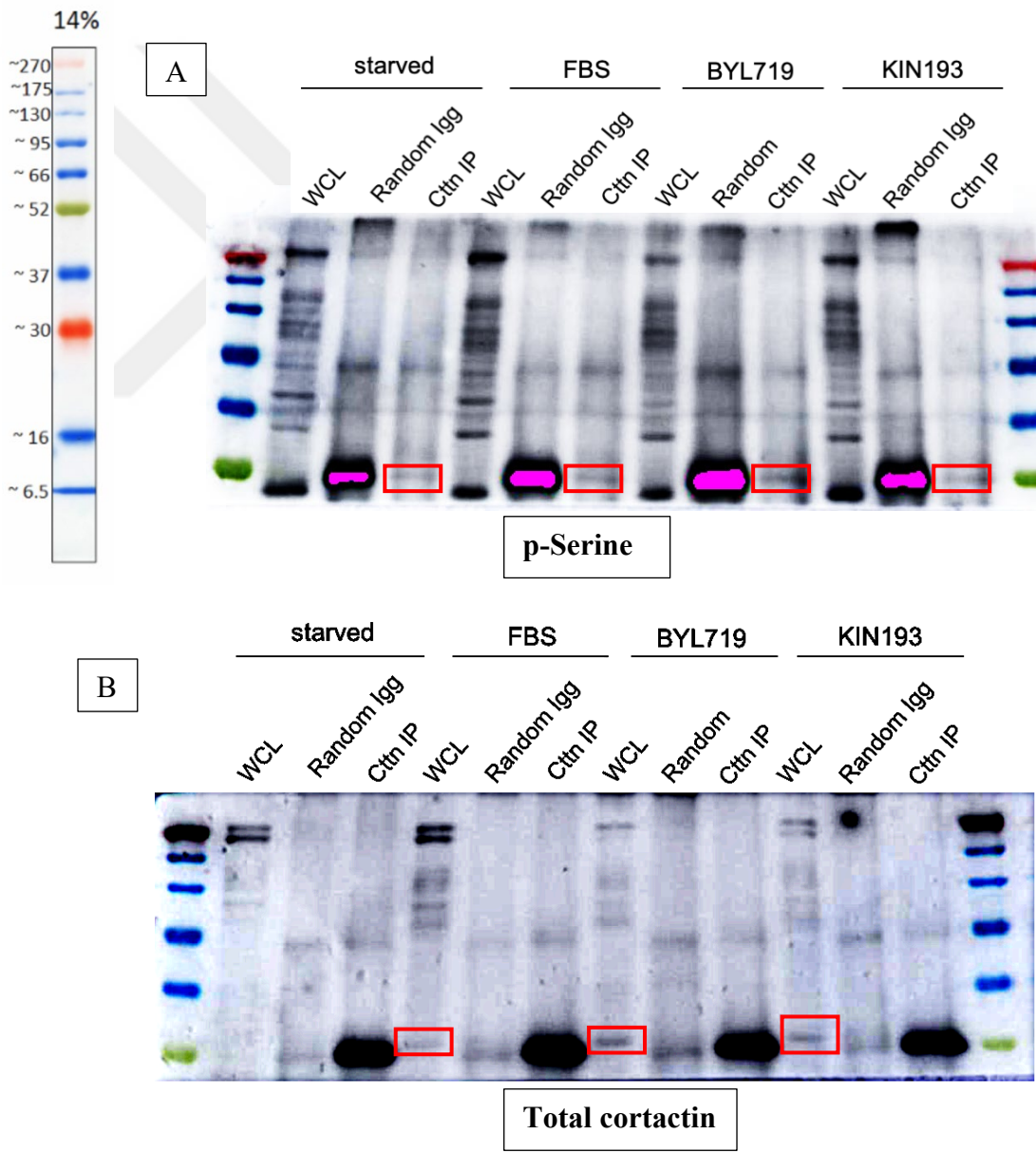


Figure 3.24 Enrichment of Cortactin by IP to detect p-Serine levels by immunoblotting A) immunoblot of p-Serine, red boxes show the potential phosphorylation of serine on cortactin. Pink bands in random rabbit Igg lane represent overexposed heavy chains of random rabbit Igg. B) immunoblot of total cortactin pulled down. Bands in the Cttn IP lane represent heavy chains of mouse Igg. Red boxes in WCL lane represent levels of cortactin pulled down. WCL: Whole cell lysate; Random Igg: rabbit random Igg pulldown; Cttn IP: cortactin immunoprecipitation



CHAPTER 4: DISCUSSION

While Phosphoinositide-3 Kinase (PI3K) signal transduction pathway is an essential pathway for several physiological processes such as metabolism, cell proliferation, differentiation, survival, and motility (Thorpe et al., 2015), it is also one of the most frequently dysregulated pathways in cancer development (Engelman, J.A, 2009).

Cancer genomics studies have revealed that the PIK3CA gene is one of the most frequently somatically mutated gene in cancers, right after TP53 (Karakas et al., 2006; Kandoth et al., 2013), whereas the PIK3CB gene often undergoes gene amplification in cancers. Clinical data has shown that several Pan-PI3K pharmacological inhibitors used in clinics have resulted in severe adverse effects such as hyperglycemia and liver toxicity (Fruman, D. A. et al, 2017). For this reason, several novel isoform-specific PI3K inhibitors have entered clinical trials (Rodon et al., 2013). However, preclinical studies with some of these drugs have revealed that alternative signaling mechanisms downstream in the pathway may get activated to compensate for the silenced component, leading to the problem of resistance to single-agent therapy (Thorpe et al., 2015). Circumventing this problem requires an understanding of the unique downstream targets of the PI3K isoforms, p110 α and p110 β , so that they may be targeted uniquely in individual cases.

In order to elucidate the phosphoproteome landscape regulated by the p110 α and p110 β differentially, we made use of isogenic MEFs, which had both PIK3CA and PIK3B gene knocked down, so as to eradicate any internal signalling by the endogenous expressing isoform. We exogenously expressed p110 α or p110 β isoform,

ensuring that the cells were either entirely dependent on the α or β isoform and hence their downstream signalling could be evaluated. In order to ensure their dependence on the respective isoforms, we stimulated them with both PDGF and LPA and saw their response by measuring the phosphorylation events of the downstream signalling molecules of the PI3K pathway. Because p110 β can act downstream of GPCRs unlike, p110 α isoform, we see the activation of the pathway only when DKO HA β cells are treated with the mitogenic stimulant, LPA (Figure 3.3A).

We characterised the signalling pathways downstream of those class IA PI3K based on earlier investigations conducted in our lab that showed various cell biological characteristics with p110 α or p110 β suppression or constitutive activation by myristoylation tags. From our western blot analysis of the downstream signalling components of the PI3K pathway we see that our single isoform dependent cells had the PI3K pathway configured. Our lab had previously tried to investigate the differential isoformic signalling using the same cell lines but by using ITRAQ labelling method which has many more limitations and biases as compared to the label-free method. One of the biggest limitations is of the number of samples that could be analysed, and because of that our experimental setup was limited to only PDGF and LPA stimulation in HA α and HA β cell lines without isoform specific inhibition or starvation.

To amend for the flaws of our previous experimental setup and to investigate the isoformic differential phosphorylation signature, we starved our cells first so as to clear all the phosphorylations and make space for the phosphorylations induced by the p110 isoforms upon stimulation, we then inhibited the isoforms and observed the absence

of the specific phosphorylation signals that had appeared on stimulation indicating that the specific phosphorylations were indeed regulated by the p110 α / β isoform. However, we made use of two different concentrations of inhibitors. For our mass spectrometry samples we used 0.5 μ M of BYL719 and KIN13 whereas for western blotting analysis we used 2 μ M of inhibitors. Mass spectrometry is a much sensitive method to detect the phosphorylation signals, hence any change brought about by the low dosage of inhibitors could be detected. However, western blot gives an average of signal and has low sensitivity, so in order to have a more pronounced effect we increased our dosage to 2 μ M. However, it should be kept into consideration that increasing the dosage of the inhibitors may affect its efficacy and there might be off-target inhibition of some kinases and any differences seen maybe due to the off-target effect. Another factor to keep into consideration is that one phosphosite may have more than one parent kinases, and any differences seen could be due to those effects too.

In contrast to the previously used ITRAQ method, we made use of label-free high throughput LC-MS method. It is challenging for the phosphoproteins and phosphopeptides to be identified using conventional non-targeted discovery MS methods due to their low prevalence in comparison to their non-phosphorylated equivalent. This is a limitation because only a small number of peptides may be chosen for fragmentation and analysis from a complicated sample mixture such as of WCL using a mass spectrometer [109]. Due to this reason phosphopeptides are enriched to enhance their detection and analysis. There are many ways by which this can be done such as that of IMAC (immobilised metal affinity chromatography). IMAC methods may favour proteins having a range of phosphorylations, but they do not make distinctions depending on the residue of amino acids with which the modification is

connected. However, IMAC enrichment may introduce a bias because of its preference for binding to amino acids with more than one phosphate [110]. To overcome this limitation the samples can be enriched with transition metal such as TiO_2 , which prefers singularly phosphorylated peptides. This is one limitation that should be kept in mind because of which our data might have a bias of generating candidate proteins that are abundantly phosphorylated and also missing out on candidate proteins that would lose phosphorylation after inhibitions and on starvation.

In order to increase the significance of our data we performed our experiment with 3 biological replicates, and each biological replicate having two technical replicates. Pearson correlation measured across three biological replicates were greater than 0.7 indicating good biological reproducibility (Figure 3.4).

Following quantification we devised a stringent pipeline for analysing the differential regulation. We first only accounted for the phosphopeptides that crossed a threshold of 2 fold change upon stimulation as compared to starvation. Keeping in mind that even a slight increase in phosphorylation can cause an effect on the localization or activity of that protein, this threshold is very stringent. Additionally protein had to cross another criteria of having a p-value < 0.05 to be sorted for the second pipe of analysis. Fold change of phosphorylation levels of the proteins which crossed these criterion were again analysed in comparison to when they were treated. If the phosphorylation levels decreased with a fold change of 1.5 or more and a p-value < 0.05 , then the phosphopeptides were finally shortlisted as final phosphocandidates. While it may be argued that these criteria are very stringent, we wanted to shortlist the phosphocandidates that were truly differentially regulated, however due to these stringent criterion it's possible that we would have increased false negatives but decreased false positives.

In our analysis we also saw some phosphorylation events that increased after the isoformic inhibition. These changes indicate that a feedback loop maybe in the working however we did not investigate these effects but could potentially be looked at. For example, phosphorylation at Th70 on 4ebp1 increased in DKO HA α cells when p110 α is inhibited, similariy the same pattern is seen in WT paternal MEFs where we see an increase in phosphorylation upon BYL719 treatment.

In our phosphoproteome data for PIK3CA we identified 90% serine residues and 10% Threonine resides, and for PIK3CB data we identified 89% serine residues and 11% Threonine residues which is in line with the relative abundance of serine:threonine:tyrosine (88:11:1) phosphorylation events in a cell [111].

Our bioinformatic analysis provided us with good foresight to shortlist the phosphocandidates to be validated. We also saw phosphosites from our label-free MS method that were also previously seen in our ITRAQ data as well. This increased our confidence level further in the shortlisted candidate phosphosites. However, we could only shortlist some depending on the phospho-specific antibodies available and also the financial restraints to buy those antibodies. In this thesis we could only attempt to biochemically validate NPM1, two phosphorylations sites of Eif4ebp1, and Ctnn even though we had shortlisted Calnexin and HDAC1 too. The calnexin antibody that we had was specific to the human homolog of calnexin and therefore it was not able to detect the protein. It was also not appropriate for performing an IP therefore it could not be enriched and checked for phosphorylations as Ctnn was done for. We had attempted to identify the phosphorylation differences of calnexin by observing the migration differences on a low percentage SDS gel. However, since our antibody was not able to detect the protein we were not able to validate it. HDAC1 did not have a

phosphospecific antibody either and so it was intended to enrich it by IP, however, the ordered HDAC1 antibody did not arrive on time therefore the protein could not be investigated.

Upon pathway analysis in the TCGA Pan- Cancer cohort we saw NPM1 to be significantly implicated in the Acute Myeloid Leukemia (AML) pathway. One of the most common genetic abnormality in the whole AML patient population is that of NPM1, present in about 40% of the patients. P110 α mutants E545K and H1047R were also found to phosphorylate p85 much more compared to p110 α - wild type and this was confirmed in AML cell lines as well [106] This also made NPM1 a good candidate to be investigated.

NPM1 is involved in a number of cellular functions, including as centrosome duplication, protein chaperoning, and cell growth. The encoded phosphoprotein transports ribosomal proteins and core histones from the nucleus to the cytoplasm while moving back and forth between the nucleolus, nucleus, and cytoplasm. The tumour suppressor ARF is known to be sequestered in the nucleolus by this protein, preventing its destruction until it is required. Previous studies had showed that NPM1 expression increases in PTEN null prostate cancer cells and when cells are treated with mTOR inhibitor rapamycin, NPM1 gets downregulated [108]. These reported findings indicated that NPM1 protein expression is regulated with the regulation of the PI3K pathway. Aurora Kinase A (AURKA) AND Aurora Kinase B, a mitotic kinase is known to be an upstream kinase responsible for the phosphorylation of NPM1 at our candidate site S125 site. During late cytokinesis, phosphorylated-S125-NPM1 (pS125-NPM1) localises to the midbody area where it colocalizes with Aurora B. Mitotic abnormalities and centrosome dysregulation were caused by the overexpression of the mutant (S125A) NPM1, which may have occurred as a result of cytokinesis failure.

NPM1 phosphorylated at Ser125 is also seen to be abundant in oral tumour tissues. [112]. Donella et al., reported incomplete inhibition of AURKA was a typical cause of therapeutic failure where drug resistance was linked to AURKA maintenance following drug therapy in breast cancer models, including t47D cells. Combinations of PI3K, AKT, or mTOR inhibitors with the AURKA inhibitor MLN8237 were highly synergistic and durably inhibited mTOR signaling, resulting in tumor regression in vivo. It is possible that this synergistic effect on NPM1 by Aurka and mtor may have some differential regulation by the p110 α isoform [107]. STRING analysis also showed that AKT1 directly interacts with NPM1, also making it a potential upstream regulator of NPM1.

Regulation of NPM1 was difficult to validate in the WT parental MEFs, this could be because NPM1 might be much lower in the pathway to get effected on short stimulation and inhibition intervals (Figure 3.13). When NPM1 regulation was checked in MEFs with constitutively activating mutations of PI3Kinase pathway and long term inhibitions we saw that the phosphorylations decreased (Figure 3.15). This may suggest that the short term stimulation in WT MEfs may not be enough to constitutively activate the PI3K pathway as it is activated in cancers and if phosphorylated the inhibition time might not be enough to dephosphorylate NPM1 which maybe effected much downstream in the pathway. We also did cross-species validation by investigating the phosphorylation in human T47D breast cancer cells (Figure 3.18). We saw quite a large decrease in the phosphorylation of S125 on NPM1 in the cancer cells also indicating its relevance in breast cancer model.

Phosphorylation of 4E-BP1 is complex: early investigations found at least five phosphorylation sites in 4E-BP1 that play different roles in modifying 4E-BP1 binding to eIF4E or allowing phosphorylation of other sites in the protein (i.e., 4E-BP1

phosphorylation is hierarchical) [113][114][115]. T70 is phosphorylated by two N-terminal canonical threonines phosphorylation (T37 and T46 in the human protein), which in turn phosphorylates S65. Phosphorylation at T70 appears to be crucial in the release of 4E-BP1 from eIF4E, whereas phosphorylation at S65 appears to impede the reassociation of eIF4E and 4E-BP1 [116][117]: 4E-BP1 phosphorylated at this location does not associate with eIF4E.[115]. S65 and T70 are located near the eIF4E-binding motif in the main sequence. Rapamycin inhibits phosphorylation of S65 and T70 in vivo. It has been shown that Phosphorylation at S65 and T70 residues has no effect on binding to free eIF4E but interferes with 4E-BPs' capacity to displace eIF4G from preexisting eIF4E-eIF4G complexes and suppress translation [118].

In summary, we managed to provide some evidence for the differential regulation of S125 NPM1 phosphorylation and 4ebp1 Th70 and S65 phosphorylation by the p110 α and p110 β isoform respectively which could be further investigated such as by functional studies.

CHAPTER 5: CONCLUSION AND FUTURE PERSPECTIVES

In this study we aimed to investigate the differential downstream phosphorylation signal of the p110 α and p110 β PI3Kinase isoforms.

Our experiment design could be made more elaborate by introducing LPA stimulation in both HA α and HA β cells. Since LPA only triggers the pathway downstream of p110 β , it would have been interesting to see three things: (1) Any phosphorylations seen due to LPA stimulation can be ascribed conveniently to the p110 β isoform (2) any phosphorylation seen downstream to p110 α would be a novel target to characterize (3) Any phosphorylation seen in p110 β cells that were present from LPA stimulation and not from PDGF stimulation could also be ascribed to be happening downstream of GPCRs only.

For this study most experiments have been performed once, to increase the statistical power and to render them significant, the experiments can be replicated two to three times more to see significant validation.

Our study lacked the investigation of the functional and physiological implications of the phosphorylation of these residues. This could be studied by introducing point site directed mutations by introducing plasmids that has a point mutation in the protein that prevents it from getting phosphorylated at our candidate residues. Effect on migration of these cells can be investigated by using scratch assays, proliferation using growth assays and also interaction with other proteins can be investigated by doing IPs and Co-IPs. These candidates can also be further validated in different cancer models to see their physiological significance and also be seen if they synergize with any other components of the PI3K pathway. It can also be investigated whether these phosphorylations are activatory or inhibitory in the physiological processes that they effect.

From our Pan-Cancer TCGA data, we saw that the PIK3CA regulated genes were seen to be mostly altered in colorectal and ovarian cancers. It might be beneficial to see the differential regulation of these genes in the cell lines from these cancer models.

Upstream kinases can be investigated of the candidate phosphoproteins from different databses such as phosphosite plus, GPS and scansite. Upon finding that, these kinases maybe inhibited by specific inhibitors and their effect on our candidate phosphorylation can be observed.

Other phosphor candidates in our list that we did not shortlist for this study can also be investigated and novel substrates can be found that can further elucidate the mechanics and differential signalling of the PI3K isoforms.

In the long term, upon proper characterization of these candidates they can be used as markers to identify patients who will benefit from PI3K-focused therapies and to understand the modalities of resistance that may develop against pathway inhibition.

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7.APPENDIX

7.1 COPYRIGHTS AND PERMISSION

FOR FIGURE 1.1 AND FIGURE 1.2

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