INVESTIGATION OF PI3K FUNCTIONAL COMPENSATION VIA ACTIVATED TYROSINE KINASES

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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

INVESTIGATION OF PI3K FUNCTIONAL COMPENSATION VIA ACTIVATED TYROSINE KINASES

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Protein tyrosine kinases and serine-threonine kinases have crucial functions in cell signaling, differentiation, motility, and proliferation. PI3K is the most deregulated pathway in human cancers and an essential regulator of cellular proliferation. PI3K pathway is activated via oncogenic Ras/receptor tyrosine kinases (RTKs), PTEN loss, or activating mutations in PI3Ks. Moreover, PI3K is one of the most promising pathways for targeted therapies. Thus, many serine-threonine or tyrosine kinases contribute to drug resistance elicited by PI3K inhibition. In order to identify an individual tyrosine kinase that contributes to PI3K functional compensation, the activated tyrosine kinase library was screened and found out that ZAP70 can compensate growth upon PI3K abrogation. This study suggests a mechanism of activated ZAP70 mediated partial resistance in MEFs lines. Moreover, we demonstrated the role of activated tyrosine kinase, ZAP70, in cancer cells as a tumor-initiating factor. Activated ZAP70 is able to enhance the growth ability of untransformed cells. Also in these cells, activated ZAP70 can develop partial resistance to PI3K inhibition. This resistance occurs via activated downstream targets of tyrosine kinase signaling such as STAT3/MAPK axis. Furthermore, we showed that activated ZAP70 has a high transformation capability associated with the formation of malignant phenotype in untransformed cells. Overall, ZAP70 may be a potent driver of proliferation and transformation in untransformed cells as well as in cancer cells.

Keyword: PI3K, tyrosine kinases, drug resistance, cell signaling

ÖZET

AKTİVE EDİLMİŞ TİROZİN KİNAZ ARACILI PI3K FONKSİYONEL KOMPENSASYONUNUN ARAŞTIRILMASI

Melike Demir

Moleküler Biyoloji ve Genetik, Yüksek Lisans

Tez danışmanı: Onur Çizmecioğlu

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Protein tirozin ve serin treonin kinazlar hücre sinyalizasyonunda, büyümede, çoğalmada, farklılaşmada ve motilitede önemli rol oynarlar. PI3K insan kanserlerinde en fazla deregülasyona uğrayan sinyal yolağıdır ve hücre proliferasyonunda önemli bir düzenleyicidir. PI3K yolağı, onkojenik Ras/ reseptör tirozin kinazların aktivasyonuyla, PTEN kaybıyla ya da PI3K'lardaki aktive edici mutasyonlarla aktive olur. Buna ek olarak, PI3K hedeflenmiş terapiler için umut verici bir yolaktır. Buna ek olarak, PI3K inhibisyonunun neden olduğu ilaç direnci geliştirmede birçok serin treonin ve tirozin kinazın katkısı vardır. PI3K fonksiyonel kompensasyonuna katkı sağlayan ayrı bir tirozin kinaz tanımlamak için, tirozin kinaz kütüphanesi tarandı ve ZAP70'in PI3K iptaline karşı büyümeyi kompense ettiği gösterildi. Bu çalışma, MEF hatlarında aktive ZAP70 aracılı kısmi direnç mekanizmasını önermektedir. Ayrıca, aktive tirozin kinaz ZAP70'in kanser hücrelerinde tümör başlatan bir faktör olarak rolünü gösterdik. Aktive edilmiş ZAP70 transforme olmayan hücrelerde büyüme yeteneğini artırabilir. Aynı zamanda, aktive edilmiş ZAP70 PI3K inhibisyonuna bu hücrelerde kısmi direnç mekanizması geliştirebilir. Bu direnç, STAT3 / MAPK ekseni gibi tirozin kinaz sinyal yolağının aktive edilmiş aşağı akış hedefleri aracılığıyla oluşur. Ayrıca, aktive edilmiş ZAP70'in dönüştürülmemiş hücrelerde kötü huylu fenotip oluşumu ile ilişkili yüksek bir dönüştürme kabiliyetine sahip olduğunu gösterdik. Genel olarak ZAP70, transforme olmayan hücrelerde ve ayrıca kanser hücrelerinde proliferasyon ve transformasyon için güçlü bir etken olabilir.

Anahtar kelimeler: PI3K, tirozin kinazlar, ilaç direnci, hücre sinyalizasyonu

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CHAPTER 1

1. INTRODUCTION

1.1 Cell Signaling by Protein Tyrosine Kinases

Protein kinases (PKs) are enzymes that transfer the phosphoryl group from ATP (adenosine triphosphate) into an acceptor protein [1]. PKs are classified by substrate specificity and amino acid sequences of catalytic domains [2]. Protein phosphorylation causes activation of signal transduction pathways, which are crucial for many biological processes [3]. The most well- characterized protein kinases in the human genome are protein serine/threonine kinases (STKs) and protein tyrosine kinases (PTKs) [1]. Protein tyrosine kinases have been linked to the development of many human disease states such as cancer. In addition to this, tyrosine kinases play a role in regulation of cell growth, metabolism, differentiation, motility, and death [4]. PTKs are divided into two main groups; receptor and non-receptor tyrosine kinases (TKs). Receptor TKs (RTKs) are transmembrane proteins, that have catalytic intracellular kinase domain and ligand-binding domain. Non-receptor tyrosine kinases (NRTKs) are intracellular cytoplasmic proteins with no transmembrane domains. According to enzymatic activities of both TKs, high levels of tyrosine phosphorylation corresponding to enhanced proliferative state [5]. The first idenditified NRTK is Src family. Src is a proto-oncogene that contributes to cancer progression [6, 7]. Most of NRTKs have N-terminal Src-homology (SH) domains along with C-terminal kinase domain [8] (Figure 1.1). The SH2 domain enables phosphorylated tyrosine binding and SH3 domain enables protein-protein interaction [6, 7]. The tyrosine kinases activate via autophosphorylation of tyrosine residues. However, this activation is firmly regulated. Uncontrolled activation of RTKs leads to defective cell proliferation and cancer [9]. RTKs are activated via dimerization of receptors which is mediated by ligands and leads to trans-phosphorylation of tyrosine in kinase domain [10].

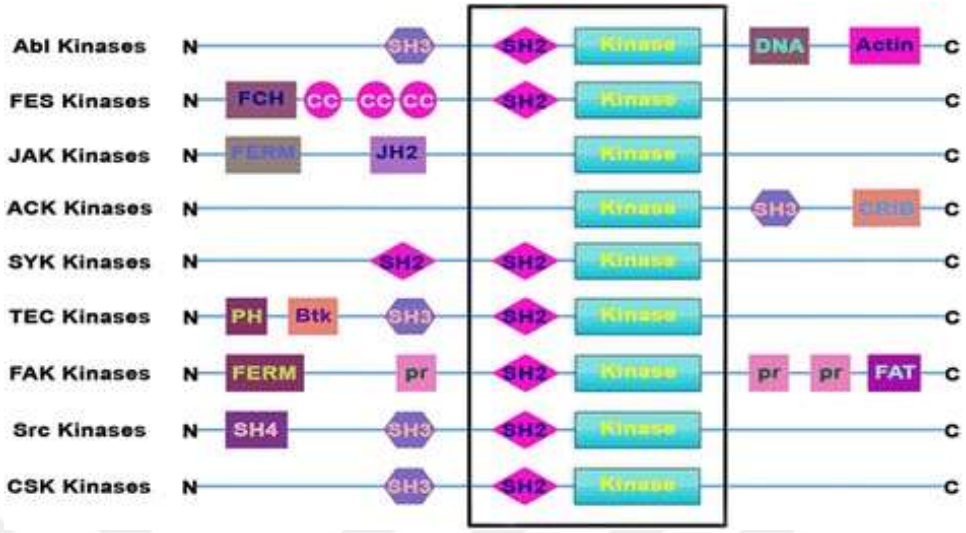


Figure 1.1: Non-receptor tyrosine kinase family domain structures. (The figure is adopted from Siveen et al., 2018)

N: Amino terminus, SH3: SRC Homology 3 domain, SH2: SRC Homology 2 domain, Kinase: Catalytic kinase domain, DNA: DNA binding domain, Actin: Actin binding domain, FCH: Fes/Fer/Cdc-42-Interacting Protein homology domain, CC: Coiled coil motif, FERM: Four-point-one, ezrin, radixin, moesin domain, JH2: Janus homology domain 2, CRIB: Cdc42/Rac-interactive domain, PH: Pleckstrin homology domain, Btk: Btk-type zinc finger motif, pr: Proline rich region, FAT: Focal-adhesion targeting domain, SH4: SRC Homology 4 domain, C: Carboxy terminus

1.2 Relevance of PI3K Signaling Pathway in Human Cancers

Phosphatidylinositol 3-kinase (PI3K) pathway is one of the most activated signaling pathways in human cancer. Several studies have shown that, PI3K deregulation leads to tumor progression of human cancer [11]. This pathway is also crucial regulator of cell proliferation,survival and motility [12]. PI3K harbors lipid kinase activity and is consisted of a regulatory and catalytic subunits which have heterodimeric structure [12].

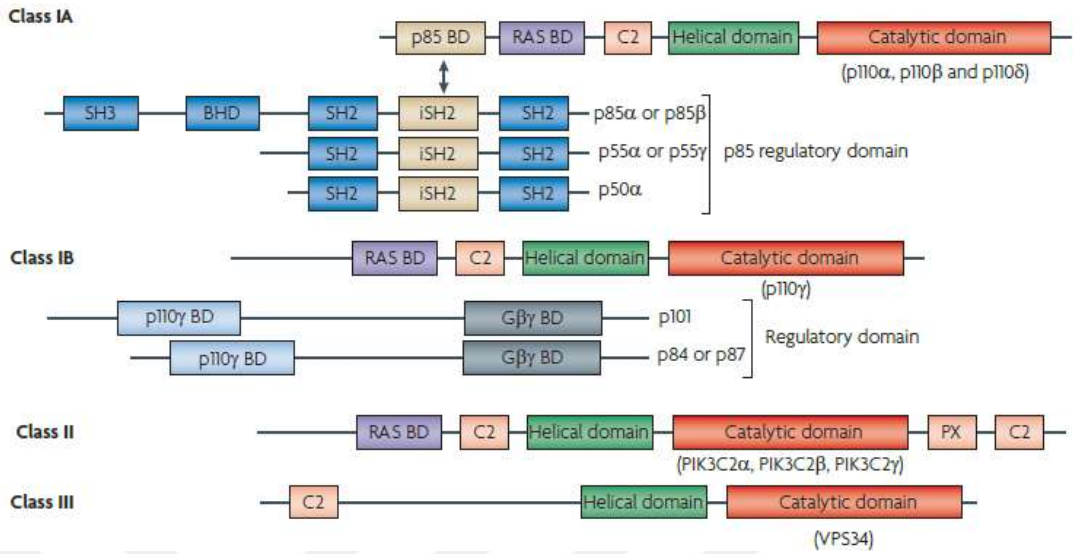


Figure 1.2: PI3K family domain structures (the figure adopted from Liu et al., 2008)

PI3Ks are classified into three classes; Class I, Class II and Class III PI3Ks. Class I PI3Ks are composed of class IA and class IB enzymes [13]. Activation of class IA PI3Ks occur via receptor tyrosine kinases (RTKs) or G-protein-coupled receptors (GPCRs) [14]. The class IA PI3Ks contain p110 catalytic subunit and a p85 regulatory subunit. PIK3CA, PIK3CB, PIK3CD genes encode p110α, p110β and p110δ catalytic subunit isoforms. PIK3R1, PIK3R2, PIK3R3 encode p85α, p85β and p55γ regulatory subunit isoforms. Also, PIK3R1 encodes alternative splice variants, p55α and p50α. The class IA catalytic subunits have an amino-terminal p85-binding domain (p85 BD), RAS-binding domain (RAS BD), Putative membrane-binding domain; C2, helical domain and carboxyl-terminal kinase catalytic domain. The class IA regulatory subunits have p110 binding domain which is also called inter-Src homology (iSH2) domain in between two Src homology 2 (SH2) domains, Src-homology 3 (SH3) domain and a BCR homology (BH) domain (Figure 1.2). Whenever p85 binds to p110 via iSH2 domain, catalytic activity of p110 is inhibited. In normal cells, p110α and p110β ubiquitously expressed whereas p110δ is mostly expressed in immune cells [14].

Upon activation of RTKs, recruitment of PI3K to the cell membrane occurs via coupling SH2 domains of p85 with tyrosine phosphorylated residues of activated

receptors. The second messenger phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P3) is synthesized from phosphatidylinositol- 4,5-bisphosphate (PI-4,5-P2) via activated PI3K [14].

The class IB PI3Ks are mostly regulated by GPCRs. Class IB PI3Ks contain p110γ catalytic subunit and p101, p87 and p84 regulatory subunit [14]. Class II and Class III PI3Ks are less studied classes of this pathway. Class II PI3Ks generate PI-3-P and phosphatidylinositol-3,4-biphosphate (PI-3,4-P2) and regulated by receptor tyrosine kinases and G-protein coupled receptors. Class III PI3Ks generate phosphatidylinositol-3-phosphate (PI-3-P) from phosphatidylinositol (PI) and have crucial role in membarne trafficking and cell growth [13] [14].

Phosphatase and tensin homologue (PTEN) and AKT (PKB) are two essential components of PI3K pathway. PTEN is a tumor suppressor and negatively regulates PI3K pathway by converting PtdIns(3,4,5)P_3 to PtdIns(3,4,5)P_2 via lipid phosphatase activity. Loss of PTEN leads to aberrant PI3K signalling mediated by p110β, and may lead to cancer [14] [15]. AKT is a serine/threonine protein kinase and composed of three domains including N-terminal pleckstrin homology (PH) domain, serine-threonine catalytic domain, C-terminal regulatory domain. The AKT has three isoforms, AKT1, AKT2 AND AKT3 which are encoded by different genes. AKT is activated via direct interaction of its PH domain with PtdIns(3,4,5)P_3 on plasma membrane. This is the first step in its activation, dual phosphorylation of AKT is more crucial for its maximal activation. Phosphoinositide-dependent kinase-1 (PDK1) phosphorylates AKT at Thr308. Mammalian target of rapamycin complex-2 (mTORC2) phosphorylates AKT at Ser473. Activated AKT regulates various cellular processes including cell survival, proliferation, metabolism and motility by phosphorylation of downstream proteins [16] [17].

PI3K components are mutated, amplified or deleted in a wide range of human cancer (Figure 1.3) [12]. The class IA PI3Ks are commonly mutated and these mutations occur in the catalytic subunits, p110α, as well as regulatory subunit, p85α. Ras is a primarily identified effector of PI3K [18]. Upon activation of RTKs, recruitment of PI3K to the cell membrane occurs via coupling SH2 domains of p85 with activated tyrosine phosphorylated residues receptors [12]. Oncogenic Ras and RTKs also cause

PI3K activation. These findings support the idea that targeting PI3K pathway is one of the most promising approaches for cancer therapy.

Table 1 | **Evidence of PI3K-signalling deregulation in human malignancies**

Cancer type	Type of alteration	References
Glioblastoma	*PTEN* mutation	133
Ovarian	Allelic imbalance and mutations of *PTEN* gene	134
	Elevated AKT1 kinase activity	135
	AKT2 amplification and overexpression	71
	PI3K *p110α* amplification	70
	PI3K *p85α* mutation	74
Breast	Elevated AKT1 kinase activity	135
	AKT2 amplification and overexpression	71
	RSK amplification and overexpression	78,79
	Loss of heterozygosity at *PTEN* locus	136
	PI3K and AKT2 overactivation	137
Endometrial	*PTEN* mutation	138
	PTEN silencing	139
Hepatocellular carcinoma	*PTEN* mutation	140
Melanoma	*PTEN* mutation	141
	PTEN silencing	142
Digestive tract	Aberrant *PTEN* transcripts	143
	PI3K *p85α* mutation	74
Lung	PTEN inactivation	144
Renal-cell carcinoma	*PTEN* mutations	145
Thyroid	*PTEN* mutations	146–148
	AKT overexpression and overactivation	149
Lymphoid	*PTEN* mutations	150,151
	p85–EPH fusion (only one case reported)	75

EPH, ephrin; PI3K, phosphatidylinositol 3-kinase.

Figure 1.3: Alterations of components of PI3K in human malignancies. (Figure is adopted from Vivanco et al., 2002)

Inhibiton of class I PI3K activity by using small molecule inhibitors leads to retardation of cell proliferation rather than induce apoptosis [19]. Isoform specific PI3K inhibitors are in clinic development. Studies suggest that p110α specific inhibitors, such as Alpelisib (BYL719) might be effective for suppressing PI3K pathway in cancer. Alpelisib is clinically approved by FDA for treatment of a subgroup of breast cancers [20]. For example, in breast cancer harboring RTK amplification or oncogenic PIK3CA mutated tumors are sensitive to p110α inhibiton. Also this inhibition can be bypassed via loss of PTEN and activated RTK oncogene. Otherwise p110β specific inhibitors, such as AZD6482 (KIN-193) might be effective for PTEN deficient tumors [21].

1.3 PI3K Resistance Mechanisms

Activation of the Phosphatidylinositol-3-kinase (PI3K) /AKT/mammalian target of rapamycin (mTOR) signaling axis is important for resistance mechanisms of clinical therapies against human cancers [22]. Previous studies have identified that activated tyrosine kinases or serine/threonine kinases contribute to growth compensation upon PI3K inhibition [23] [24] [25]. In addition to this, p110α is a crucial mediator of RTK signaling. Pharmacological inhibition of the PI3K pathway in cancer cells leads to upregulation of RTKs through feedback loops that circumvent baseline level of PI3K inhibition. These compensatory mechanisms contains FOXO transcription factor along with mitogen-activated protein kinase (MAPK) dependent pathway [26] [27].

By using isoform specific PI3K inhibitors or pan-PI3K inhibitors to mimic catalytically inactive versions of p110α/p110β causes growth retardation which is compensated by activation of compensatory mechanisms [23]. BYL719 which is a p110 alpha inhibitor is approved by the FDA (Food and Drug Administration). On the other hand, AZD6482 which is a p110 beta inhibitor is in preclinical evaluation [28]. For that reason, to explicate the primary mechanisms of resistance might be a significant therapeutic approach for oncogenesis [22].

Overexpression of PIM (Proviral Integration site for Moloney murine leukemic virus) protein kinases are related to therapy resistance in hematological malignancies as well as solid tumors by regulating activation of signaling pathways [29] [30] [31] [32]. PIM kinases are constitutively active serine/threonine kinases that consists of three isoforms, PIM1, PIM2 and PIM3. The PIM1 has identified as a proto-oncogene and induced tumor progression by enhancing cell proliferation/differentiation and inhibiting apoptosis [29] [30] [31] [33]. Previous studies identified that overexpression of PIM1 induce tumorigenesis along with elevated MYC levels [29] [30] [31] [33]. Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway is the main regulators for PIM1 gene expression by modulating transcription factors [30] [33]. In conclusion, oncogenic PIM1 regulates cell proliferation and cell survival that might be leading reduction of therapeutic drug efficacy by promoting JAK/STAT signaling pathway in MYC driven cancers [31] [34] [35].

In addition to this, amplification/overexpression of HER2 in breast cancer cell lines can compensate PI3K/mTOR pathway inhibition via activated MAPK pathway [25].

In literature, activation of non-receptor tyrosine kinases such as focal adhesion kinase (FAK), Src family of tyrosine kinases (SFKs) develop drug resistance to tyrosine kinase specific inhibitors and chemotherapeutics in haematological malignancies and solid tumors [8] [36] [37].

1.4 Zap70 Gene

The T-Cell receptor (TCR) ζ-associated protein of 70 kDa, ZAP70 is a cytoplasmic tyrosine phosphoprotein. It belongs to spleen tyrosine kinase (SyK) family non-receptor tyrosine kinase [38]. ZAP70 is mainly expressed in T-cell and natural killer (NK) cells as a vital player of adaptive immunity. Moreover, ZAP70 has a crucial role in TCR signaling and activation, T-cell development [39].

Domain structure of ZAP70 composed of three domains that containing N-terminal two Src homology (SH2) domains, C-terminal kinase domain. In addition to this, interdomain A and interdomain B are present in this structure as a linker residue of SH2 and kinase domains respectively [40] (Figure 1.4). Based on structural studies, upon T-cells activation by antigens resulting Lck phosphorylation, SH2 domains of ZAP70 bind to tyrosine phosphorylated immunoreceptor tyrosine-based activation motifs (ITAMs) then recruited to the membrane [41]. Activation of ZAP70 leads to phosphorylation of linker of activated T-cells (LAT) transmembrane protein [41]. The phosphorylation residue of Tyr315 and Tyr319 on interdomain B participate an autoinhibitory effect of ZAP70 that regulates catalytic activity [40].

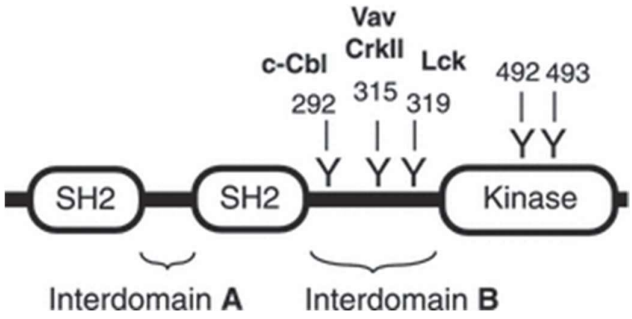


Figure 1.4: ZAP-70 domain structure (Figure is adopted from Weiss et al., 2009)

Loss-of-function mutations in the ZAP70 gene that impair normal T-cell and B-cell development have been linked to Severe combined immunodeficiency (SCID) and other immunodeficiency phenotypes [42]. Gain-of-function mutations in the ZAP70 gene has been observed in several human cancer types in chronic lymphocytic leukemia (CLL) [42].

Based on previous studies non-immunological functions of ZAP70 have identified in solid tumors. ZAP70 promotes cell migration and invasion of prostate cancer cell lines [43]. Moreover, in mammalian oocytes and embryonic stem cells (ESCs) ZAP70 has a role in maintaining stemness and differentiation by regulating Jak/Stat3/c-Myc signaling axis [44]. Additionally, ZAP70 has identified as a prognostic marker in colorectal cancer in response to radiation [45].

1.5 Aim of the study

High levels of tyrosine phosphorylation are associated with the proliferative state in cells and PI3K signaling is the crucial regulator of cellular proliferation. Thus, our first aim in this thesis, to identify potential tyrosine kinases that contributes to generating resistance to PI3K inhibition. Our second aim is to elucidate the potential mechanism of activated ZAP70 mediated resistance to PI3K targeted therapies. In addition to this, we wanted to understand the potential role of activated ZAP70 in untransformed and cancer cells.

CHAPTER 2

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Buffers & Solutions

Fixation Solution	10% Acetic acid, 10% Ethanol, 80% double distilled water
Crystal Violet Solution	0.4% crystal violet powder, 20% Ethanol, filled with double distilled water
Destaining Solution	10% Acetic acid, 90% double distilled water
Lysis Buffer	RIPA Lysis Buffer; 1M DTT; Protease inhibitor 25X; Phosphatase inhibitor 100X; 100mM Na3VO4
10% Separating (Resolving) Gel	4.1ml ddH2O; 3.3ml %30 acrylamide/bis; 2.5ml Tris pH 8.8 resolving buffer; 5ul TEMED, 50ul 10% APS
4% Stacking Gel	3ml ddH2O; 0.66ml %30 acrylamide/bis; 1.26ml Tris pH 6.8 resolving buffer; 5ul TEMED, 25ul 10% APS
Resolving Buffer (1.5 M Tris-HCl pH:8.8)	187gr Trizma base, up to 1L ddH2O, adjust pH to 8.8
Stacking Buffer (0.5 M Tris-HCl pH:6.8)	60.5gr Trizma base, up to 1L ddH2O, adjust pH to 6.8
10X Transfer Buffer	30.3gr Trizma Base, 144.1gr Glycine, up to 1L ddH2O
10X Running Buffer	30gr Trizma Base, 144gr Glycine, 10gr SDS, up to 1L ddH2O
10X TBS	80gr NaCl, 2gr KCl,30gr Trizma Base, up to 800 ml ddH2O, adjust pH to 7.4 with HCl
1X TBS-T	1L 1X TBS, 1 ml Tween
50X TAE	242gr Trizma base, 57.1ml acetic acid, 100ml 0.5M EDTA (disodium EDTA: 18.61gr)
CCMB80 (250 ml)	2.95 gr CaCl2.2H2O, 0.81 gr MnCl2.22H2O, 0.5 gr MgCl2.6H2O, 0.245 gr KOAc, 2.94 ml Glycerol, Add up to sterile ddH2O

2.1.2 Cell Culture Reagents/Media

Catalog No	Name	Company
D6429	DMEM High Glucose	Sigma Aldrich, USA
52400-025	RPMI-1640	Gibco,USA
11320-074	DMEM/F-12	Gibco,USA
25300-054	Trypsin-EDTA	Gibco,USA
59202C	L-glutamine	Sigma Aldrich, USA
15140-122	Pen-Strep	Gibco, USA
S1810-500	Fetal Bovine Serum (FBS)	Biowest, France
M7145	MEM Non-essential Amino acid solution	Sigma Aldrich, USA
12800-017	DMEM powder	Gibco, USA
50101	SeaPlaque Low melting temperature agarose	Lonza, Switzerland
A11138-03	Puromycin	Gibco, USA
47995.01	G418 Solution	Serva, Germany
2004955	Lipofectamine 2000	Invitrogen, USA
2106984	Lipofectamine 3000	Invitrogen, USA
S2814	Alpelisib (BYL719)	Selleckchem, USA
S1462	AZD6482 (KIN193)	Selleckchem, USA
A3602-100	PBS Tablets	Biomatik, USA

2.1.3 Cell Lines & Growth Medium

Cell Line	Growth Medium
$p110\alpha^{flox/flox}$; $p110\beta^{flox/flox}$ MEFs	High Glucose (4.5g/L) DMEM; 8% FBS; 1% Pen/Strep
HEK293T	High Glucose (4.5g/L) DMEM; 8% FBS; 1% Pen/Strep
RPE1-hTERT	DMEM/F-12; 8% FBS; 1% Pen/Strep; 1% L-Glutamine
MCF10A	DMEM/F-12; 4% FBS; 1% Pen/Strep; 1% L-Glutamine; EGF (10ng/ml); Insulin (10ug/ml); Hydrocortisone (0.5ug/ml)
T47D	RPMI-1640; 8% FBS; 1% Pen/Strep;
DU145	RPMI-1640; 8% FBS; 1% Pen/Strep
LNCaP	RPMI-1640; 10% FBS; 1% Pen/Strep; 1% Non-essential aminoacid
MCF7	RPMI-1640; 10% FBS; 1% Pen/Strep
HMEC	DMEM/F-12; 4% FBS; 1% Pen/Strep; 1% L-Glutamine; EGF (10ng/ml); Insulin (10ug/ml); Hydrocortisone (0.5ug/ml)
A498	High Glucose (4.5g/L) DMEM; 10% FBS; 1% Pen/Strep; 1% Non-Essential Amino Acids

2.1.4 Routinely Used Kits/ Reagents/ Enzymes

Catalog No	Product Name	Company
34075	SuperSignal West Dura Extended Duration Substrate	Thermo Fisher Scientific, USA
23225	Pierce BCA Protein Assay Kit	Thermo Fisher Scientific, USA
RPN2209	ECL Western Blotting Detection Reagents	Amersham Pharmacia Biotech, UK
48501.01	LB Medium, Powder	Serva, Germany
1610373	Precision Plus Protein All Blue Standards	Bio-Rad,USA
P0758S	Sodium Orthovanadate	New England Biolabs, USA
B7204S	10X Cut Smart Buffer	New England Biolabs, USA
11836170001	cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail	Merck, Germany
39055.01	Phosphatase Inhibitor Mix II, Solution	Serva, Germany

10688.01	Acrylamide/Bis Solution	Serva, Germany
11930.03	Albumin bovine Fraction V, pH 7	Serva, Germany
161-0747	4X Laemmli Sample Buffer	Bio-rad, USA
23391.02	Glycine	Serva, Germany
10835269001	Ampicillin	Roche (USA)
B0202S	10X T4 DNA Ligase Buffer	New England Biolabs, USA
M0202S	T4 DNA Ligase	New England Biolabs, USA
R3136S	BamHI-HF Restriction enzyme	New England Biolabs, USA
R3138S	SalI-HF Restriction enzyme	New England Biolabs, USA
RIPA-100	ClearBand RIPA Lysis buffer	EcoTech Biotechnology, Turkey
D2500-01	E.Z.N.A. Gel Extraction Kit	Omega bio-tek, USA
D6943-01	E.Z.N.A. Plasmid DNA Mini Kit	Omega bio-tek, USA
D6904	E.Z.N.A. Plasmid DNA Midi Kit	Omega bio-tek, USA
T1503	Trizma Base (Tris)	Sigma Aldrich, USA
M6250	2-mercaptoethanol	Sigma Aldrich, USA
A3672	Dimethyl sulfoxide (DMSO)	Applichem, Germany
822184	Tween 20	Merck, Germany

2.1.5 Antibodies

Catalog No	**Name**	**Company**
7076S	Anti-mouse IgG	Cell Signaling Technology, USA
7074S	Anti-rabbit IgG	Cell Signaling Technology, USA
9131S	Phospho-STAT3(Tyr705)	Cell Signaling Technology, USA
5605S	c-Myc (D84C12)	Cell Signaling Technology, USA
5726S	Phospho-p44/42 MAPK (Erk1)(Tyr204)/(Erk2)(Tyr187)	Cell Signaling Technology, USA
9271S	phospho-Akt (Ser473)	Cell Signaling Technology, USA
9234S	Phospho-p70 S6 Kinase (Thr389)	Cell Signaling Technology, USA
2118	GAPDH	Cell Signaling Technology, USA
2317S	S6 Ribosomal protein	Cell Signaling Technology, USA

9139S	STAT3	Cell Signaling Technology, USA
4249S	PI3 Kinase p110alpha (C73F8)	Cell Signaling Technology, USA
sc-376641	PI3 Kinase p110beta (C-8)	Santa Cruz Biotechnology, USA
9101	Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	Cell Signaling Technology, USA
3584T	Phospho-LAT (Tyr191)	Cell Signaling Technology, USA
2704T	Phospho-ZAP70 (Tyr493 / Syk (Tyr526)	Cell Signaling Technology, USA
sc-32760	ZAP70 (1E7.2)	Santa Cruz Biotechnology, USA
13038S	phospho-Akt (Thr308) (D25E6)	Cell Signaling Technology, USA
-	Phospho-Tyrosine 4G10	-

2.1.6 Consumables

Product Name	**Company**
5mL serological pipets	Greiner-BioOne, Germany
10mL serological pipets	Greiner-BioOne, Germany
25mL serological pipets	Greiner-BioOne, Germany
1000uL tips	Greiner-BioOne, Germany
200uL tips	Greiner-BioOne, Germany
10uL tips	Greiner-BioOne, Germany
1.5mL Tubes (w/lock)	Isolab, Germany
1.5mL Reaction Tubes	Isolab, Germany
15 ml falcon tubes	Isolab, Germany
50 ml falcon tubes	Isolab, Germany
Cryovials	Greiner-BioOne, Germany
Cell lifter	Isolab, Germany
PCR Tubes	Axygen, Corning, USA
100 mm dishes	Greiner-BioOne, Germany
60 mm dishes	Greiner-BioOne, Germany
12-well plates	Greiner-BioOne, Germany
6-well plates	Greiner-BioOne, Germany
96-well plates	Greiner-BioOne, Germany
Nitrocellulose western blotting membrane	Amersham Life Science, UK

2.2 Methods

2.2.1 Maintenance of Cell Lines

Cells were subcultured when they reached 70-80% confluency with a split ratio of at least 1/6. Cell dishes are taken from incubator and old growth medium is discarded. Cells are washed with PBS. Trypsin/EDTA is added into plates and incubate the cells at 37°C and 5% CO_2 for 3-5 minutes. After cells are detached from surface of the dish, fresh growth medium is added into cell suspension. Then cell suspension is seeded into new dish and incubate them at 37°C and 5% CO_2.

2.2.2 Thawing Cells

Cells are stored inside sterile cryovials in liquid nitrogen or at -80°C. Cryovials are taken from liquid nitrogen tank or -80°C and thawed in 37°C water bath. Then melted cell-medium suspension is collected into 15 ml sterile falcon tubes containing proper growth medium. Afterwards falcon tubes are centrifuged at 1500 rpm for 3-4 minutes at RT (room temperature). Aspirate the supernatant of the falcon tube and cell pellet is resuspended with growth medium and transferred to one of the well of 6-well dish. Then dishes are maintained in incubator.

2.2.3 Cryopreservation of Cells

Culture medium of the cells is discarded and cells are washed with PBS. After that, cells are trypsinized until they detached from the surface. Cells-trypsin mixture is resuspended with growth medium and collected into 15 ml facon tube. Then centrifuged at 1300 rpm for 4 minutes. After centrifugation supernatant of the falcon tube is discarded, cell pellet is resuspended in freezing medium and added into cryovials. Cryovials are placed to the -20°C freezer for overnight. Thereafter vials are placed into -80°C for further uses. To preserved cells for a long time period vials are placed into liquid nitrogen tank.

2.2.4 Plasmid DNA/ Retroviral Transduction of Cell Lines

For 6 well plates, HEK293T cells were seeded 200.000 cells/well as triplicates one day before. For each wells, 250 ul serum free DMEM, 2 ug packaging plasmids (1 ug pCMV-vsvg + 1 ug gag-pol) and 2 ug of constructs DNA is added to DNA tube to 1.5 ml eppendorf. Cells are transfected with 4 ug DNA in total. The tubes are mixed thoroughly. Furthermore, 6 ul of Lipofectamine-2000 is added into 250 ul serum free DMEM to lipofectamine tube then the tube is mixed thoroughly and incubated at RT for 10 mins. After that, DNA mix is added into Lipofectamine tube and mixed. The DNA+Lipofectamine mix tube is incubated at RT for 15 mins. In addition to this, 500 ul final mixture is added on top of the 6-well plate of HEK293T cells as dropwise. After 24 hours of transfection incubation, medium is changed with the proper growth medium to induce virus production. The day after changing media, first supernatant which is compromised virus supplements is collected from the wells into a 15 ml sterile falcons. Falcons are stored at +4°C and wrapped with aluminium foil. For a second round of virus production, proper growth medium is added to wells. On the next day, second supernatant is collected and loaded over the first one. The supernatant complex is filtered by using 0.45 microne filter. Then, filtered supernatant complex is applied to the recipient cells that previously seeded. Polybrene is used at a final concentration of 10 ug/ml to increase transduction ability. The other day, medium is changed with proper growth medium and selection marker is added accordingly.

2.2.5 Transient Retroviral Transductions

For 6-well plate, HEK293T cells were seeded 200.000 cells/well as triplicates the day before of transducing protocol. Lipofectamine 3000 reagent is diluted in 125 ul of empty DMEM and mixed by pipetting. For each wells, 6 ul of Lipofectamine 3000 reagent is used. Later, DNA mastermixes are prepared by diluting 2500 ng DNA in 125 ul of empty DMEM. Then, for each wells, 5 ul of P3000 reagent is added into DNA mastermix. After that, DNA mix is added on top of Lipofectamine 3000+DMEM mix, incubate it at RT for 15 minutes. Afterwards, the DNA-Lipid complex is added into previously seeded cells, respectively. The transduced cells were incubated at 37°C and 5% CO_2 for 2-4 days.

2.2.6 Inhibitor Treatment and Cell Viability Assay

The cells are counted and seeded into 12-well or 6-well plates to be 15-20% confluent the next day. Seeding density changes upon each cell lines. The day after cell seeding, cells are treated with respective concentrations of p110α inhibitor BYL719 (Alpelisib), p110β inhibitor KIN193 (AZD6482) for approximately 7-10 days. Inhibitors are supplemented in reduced serum media. When, DMSO control treated cells reached 100% confluency, cells are fixed in 10% acetic acid and 10% ethanol. Plates are incubated in fixation solution for at least 24 hours at RT. After that, cells are washed with 1X non-sterile PBS and stained with 0.4% crystal violet and 20% ethanol for 1 hour at RT. Then, cells are washed with distilled water at least four times and plates are allowed to air-dry. Next, DNA intercalating dye is removed with 1 ml 10% acetic acid. Plates are incubated in destaining solution for 1 hour at RT. Then, 200 ul of solution is taken out from each wells and loaded into 96-well plate. Optical density (OD) of 96-well plate is measured at 595 nm on Synergy HT microplate reader. OD values are normalized to the OD of destaining solution to substract the background measurement.

2.2.7 Adenovirus-Cre Recombinase Transduction of Mouse Embryonic Fibroblasts (MEFs) Cells

MEFs constructs are seeded to 6 well plates as duplicate; one of the well for control and the second one is for treatment and the seeding density of MEFs is 100.000 cells per well to reached the confluency around 20%-25% the next day. After that day, 5 ul of Adenovirus/Cre recombinase is applied to the cells with 2% FBS containing serum media for each wells. For this, minimum amount of media is used as 1 ml per well. After 7 hrs of incubation, the media is removed from the wells and replaced it with proper growth medium. Adenovirus/Cre recombinase induction is repeated for 5 times. Then the cells are seeded into 12-well plates to be 2000 cells per well as triplicate and incubated at 37°C and 5% CO_2 for 6 days. Later on, cells are fixed in 10% acetic acid and 10% ethanol. Plates are incubated in fixation solution for at least 24 hours at RT. After that, cells are washed with 1X non-sterile PBS and stained with 0.4% crystal violet and 20% ethanol for 1 hour at RT. Then, cells are washed with distilled water at least four times and plates are allowed to air-dry. Next, DNA intercalating dye is removed with 1 ml 10% acetic acid. Plates are incubated in destaining solution for 1

hour at RT. Then, 200 ul of solution is taken out from each wells and loaded into 96-well plate. Optical density (OD) of 96-well plate is measured at 595 nm on SynergyHT microplate reader. OD values are normalized to the OD of destaining solution to substract the background measurement.

2.2.8 Soft Agar Assay

For soft agar assay, first of all 2.25% low melting agarose (SeaPlaqueTM Agarose, Lonza) is prepared by measuring of 2.25 gr agarose in ddH2O, then it is autoclaved for sterilization. Also 2X DMEM is prepared from 1 pocket of powdered medium which is soluble in 500 ml of ddH2O and supplemented with 29.3 ml of sodium bicarbonate then pH is adjusted to 7. Next, 2X medium is filtered by using 0.2 microne filter and 2% Pen-strep is added to media. Afterward, bottom agar layer is set up with 2.25% low melting agarose, 2X DMEM and ddH2O. The mixture is added into 6-well plates, as 2.5 ml per well and let it be solidified at RT for at least 30 minutes. In the meanwhile, top agar solution is prepared with 2X DMEM and ddH2O. Subsequently, 200.000 cells per well of a 6-well plate in at least triplicates, are resuspended in 9 ml top agar solution and 3.2 ml of 2.25% low melting agarose is added into this solution. The mixture is mixed properly and distributed as 2 ml per well. Then, let top agar be solidified at RT for 1 hour. After incubation, cells are feed with 2 ml proper growth medium and grown for 4 weeks at 37°C and 5% CO_2, until colonies are formed in 17tandarts. Within this period, medium of cells are refreshed every 4 – 5 days. At the end of the incubation time, the colonies are stained with 0.005% crystal violet and 10% ethanol solution. Then colonies are counted by eyes.

2.2.9 Preparation of Chemically Competent *Escherichia coli* C3040I

The stock *E.coli* C3040I cells are grown in LB plate without ampicillin to formed colonies the day before. Then, a colony is picked and inoculated in 3 ml LB medium for overnight at 37°C shaker. Also, CCMB80 solution is prepared and filtreted by using 0.2 microne filter and store it at +4°C. The next day, 2x25 ml LB with 1 ml overnight culture is grown at 37°C shaker for 2 hours. After incubation, the optical density (OD) is measured, the OD [550] value have to be 0.3 or 0.4. The overnight culture solution is transferred to 4x12.5 ml falcons and chilled on ice for 20 minutes. The solutions are centrifuged at 3000 rpm for 10 minutes at +4°C. Moreover, the

supernatant is removed. The pellets are dispersed in 12.5 ml cold CCMB80 solution by gently swirling. When the pellet is suspended 37.5 ml of CCMB80 solution is added. After that, the falcon tubes are incubated on ice for 20 minutes and centrifuged at 3000 rpm for 10 minutes at +4°C. Furthermore, after removed supernatant, the pellet is resuspended into 500 ul CCMB80 solution by gently swirling. Last, the suspension is aliquoted into eppendorfs for 50 ul each. Store them at -80°C after snap freezing.

2.2.10 Plasmid Cloning and Bacterial Transformation

The pBabe puro H-Ras G12V and pBabe hygro plasmids DNA are double digested with BamHI and SalI restriction enzymes which is initiated by 10X Cut Smart Buffer. The double digestion reaction are incubated at 37°C for 2.5 hours. The digested samples are running on 1% agarose gel and the gel is cut from appropriate size of digested constructs. Then, E.Z.N.A gel extraction kit protocol (Omega bio-tek, USA) is obtained for these samples. The DNA concentration of constructs are determined via NanoDrop measurement. Furthermore, ligation reaction is conducted with digested vector and insert, which is catalyzed by T4 DNA Ligase and T4 DNA Ligase Buffer at RT for 2.5 hours. The vector and insert concentrations are calculated by using NEBioCalculator for ligation reaction. For bacterial transformation, 20 ul of competent cells mixed with 2 ul of plasmid DNA as a 5:1 ratio. Then, the tubes are incubated on ice for 30 minutes. Next, heat shock of transformation mix is conducted at 42°C waterbath for 30 seconds by following incubation on ice for 5 minutes. Afterwards, 800 ul of LB broth medium is added into the tube and let it grown in 37°C shaking incubator for 1 hour. After outgrowth of plasmids, the LB medium plasmid mix is plated on LB agar plates and incubated at 37°C overnight. Previously transformed colonies are picked from LB agar plate as single colony and inoculated in 4 ml of LB broth medium for overnight into 50 ml falcon. Furthermore, the tube is centrifuged at 4000 rpm for 5 minutes to obtain bacterial pellet. Then, DNA is isolated by using E.Z.N.A Plasmid DNA Mini Kit protocol (Omega bio-tek, USA). Next, isolated DNA concentration is determined by NanoDrop and store at -80°C.

2.2.11 Harvesting Cells

The old medium is removed and cells are washed with ice cold 1X PBS. The plates are put on ice. Then, 7 ml 1X ice cold PBS is added to plates and cells are scraped with

cell scraper and collected into a 15 ml falcon tube. The tubes are centrifuged at 1500 rpm for 5 minutes at +4°C. After centrifugation, cell pellets are snap freeze in liquid nitrogen and stored them at -80°C.

2.2.12 Cell Lysis

The pellets are taken out from -80°C and put on ice. The pellets are resuspended in freshly prepared lysis buffer (formula is depicted in materials section) by pipetting. Later, the suspensions are shaken every 5 minutes while 30 minutes of ice incubation. The suspensions are centrifuged at 13000 rpm for 15 minutes at +4°C. After cell debris seperation by centrifugation, the supernatant that having proteins are transferred to another eppendorf tube and stored at -80°C after snap-freezing.

2.2.13 Determination of Protein Concentration

For determination of protein concentration, BCA Protein Assay Kit (Thermo Scientific, USA) is used according to the manufacturer's instructions. BCA working reagent solution is prepared by mixing 1 ml of Reagent A with 20 ul of Reagent B and distributed 200 ul into each well of 96-well plates. After that, the standarts are prepared according to Table-1 as triplicates in 96-well plates. 1 ul of unknown samples is added into wells with 24 ul ddH2O. The plate is incubated at 37°C for 30 minutes. After incubation, the absorbance is measured at 562 nm on Synergy HT microplate reader. The protein concentrations are calculated by using standard curve that is produced by protein standards.

Table-2.1: Preparation of protein standarts

Concentration of Standards (ug/ul)	Bovine Serum Albumin (BSA, 2ug/ul)	ddH2O
0	0 ul	25 ul
3	1.5 ul	23.5 ul
5	2.5 ul	22.5 ul
7	3.5 ul	21.5 ul
10	5 ul	20 ul
13	6.5 ul	18.5 ul
15	7.5 ul	17.5 ul
20	10 ul	15 ul

2.2.14 SDS-PAGE/ Western Blot

Gel casting system is assembled. Resolving and stacking gels are prepared based on the formula depicted in materials section. Gels are run at 100V for 90 minutes in 1X running buffer solution (formula is depicted in materials section). When loading dye reached the end of the gel, the run is stopped. For wet transfer of proteins to nitrocellulose membrane, sponges and whatman papers are soaked in 1X transfer buffer solution (formula is depicted in materials section). The transfer sandwich is set up accordingly; Anode (black side of the transfer casette), sponge, 2 layers whatman papers, gel, nitrocellulose membrane, 2 layers whatman papers, sponge, cathode. The sandwich casette is placed in the tank and filled with 1X Transfer Buffer. The proteins are transferred to membrane at 250 mA for 150 minutes. After transfer, the membranes are blocked in 5% TBS-T BSA or TBS-T milk for 1hour at +4°C. Furthermore, membranes are incubated in primary antibody which is prepared in 3% TBS-T BSA or milk for 48 hours at +4°C shaker. After that, for secondary primary incubation membranes are washed three times in 1X TBS-T for 10 minutes and then incubated with secondary antibody for 1 hour at RT. Moreover, membranes are washed again three times in 1X TBS-T for 10 minutes. Last, the signal is detected by using ECL system.

2.2.15 Bioinformatics Analysis

To draw Kaplan-Meier curves in GEPIA2 survival analysis is conducted by selecting disease free survival as a median %50 cutoff in individual TCGA cancer studies for our gene of interest. In addition to this, survival curves are drawn by using KM plotter by restricting the analysis by grades and chemotherapy treatment in progression free survival. In GEPIA2, the mRNA expression levels are detected by comparing TCGA normal and GTEx Datasets in individual cancer studies for selected gene. In UALCAN database depicting the comparison of mRNA expression levels in between tumor and normal TCGA studies. The whole statistical analyses are performed in GraphPad Prism 8.0 software.

CHAPTER 3

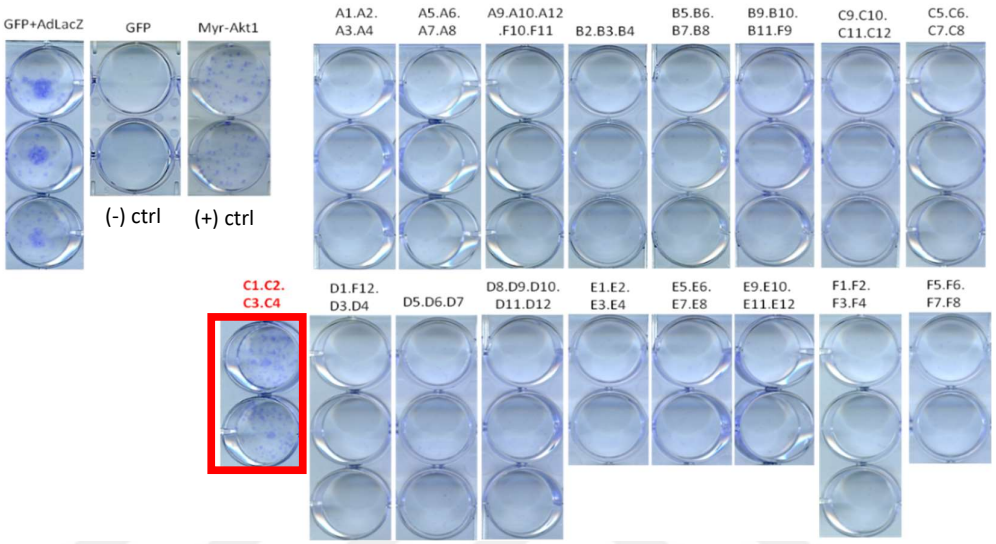
3. RESULTS

3.1 Library screening for identifying factors generating resistance to PI3K inhibition

To study the resistance mechanism of PI3K via activation of TKs, we wanted to evaluate compensatory potential of individual TKs in reponse to PI3K abrogation. For this purpose an activated tyrosine kinase library has been screened. Our tyrosine kinase library consists of 73 open reading frames (ORFs) of tyrosine kinases. This pooled tyrosine kinase library contains modified receptor as well as non-receptor tyrosine kinases. These constructs have a C-terminal dimerization tag, TEL, which is derived from a transcription factor (Kuno et al. 2001) [46]. The TEL transcription factor, allows their constitutive activation via trans-phosphorylation. In order to select a pool of TKs that might be contributing to cellular growth compensation imposed by p110α and p110β deficiency, tyrosine kinase pools were stably expressed in Mouse embryonic fibroblasts (MEFs). The immortalized $p110\alpha^{flox/flox}$; $p110\beta^{flox/flox}$ MEFs were established from compound homozygous embryos (Cizmecioglu et al. 2016) [47]. MEFs are genetically engineered with loxP regions, inserted into first exons of PIK3CA and PIK3CB. These regions are recognized by the Adenovirus / Cre recombinase (Ad/Cre) enzyme. After expressing tyrosine kinase pools, MEFs treated with Ad/Cre to excise out of the first exons of p110α and p110β. Consequently, the proliferation capability of cells was significantly abrogated. Any potential growth promoter is expected to compensate for this growth deficit overcoming complete abrogation of class IA PI3Ks. As a result of our screen, crystal violet growth assay analysis revealed that the C1-C4 pool, which is consisted of PTK2B, PTK6, ZAP70 and NTRK3, partially rescued growth deficits elicited by p110α/β loss (Figure 3.1). Nevertheless, growth data showed that C1-C4 pool expressing MEFs were able to grow efficiently upon Ad/Cre treatment along with active ZAP70 expressing MEFs in comparison to WT MEFs (Figure 3.1B). However, other tyrosine kinase pools did not yield any growing colonies. Therefore, it is concluded that ZAP70 is crucial for establishing PI3K inhibition resistance within the entire tyrosine kinase library. The Tyrosine Kinase Library screen was repeated twice with the same results. The GFP

expressing MEFs were used as a negative control. Myr-Akt1 expressing MEFs were used as a positive control. Also, GFP+LacZ expressing MEFs were used as a control for Ad/Cre treatment

A.



B.

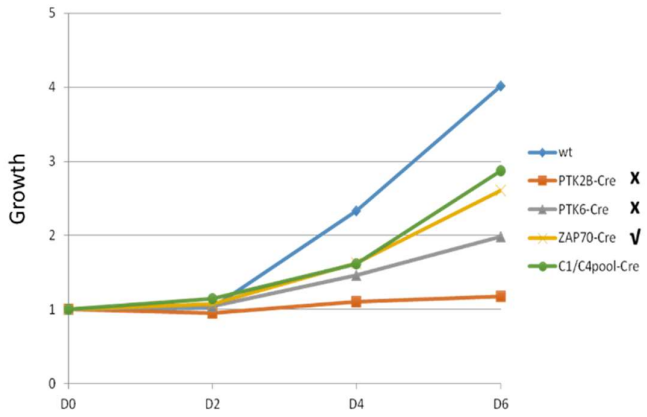
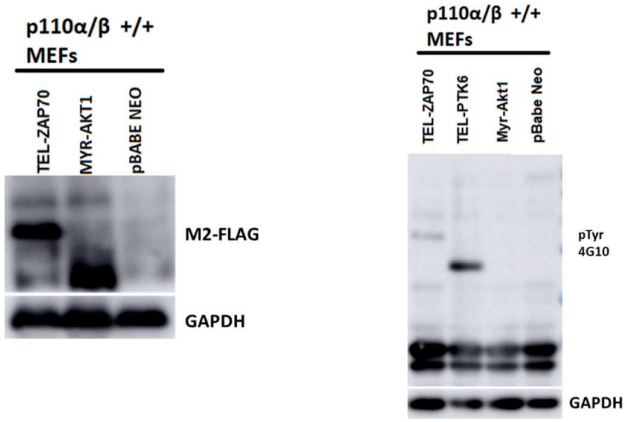


Figure 3.1. Screening of tyrosine kinase library. MEFs are transfected with GFP as a negative ctrl and Myr-Akt1 as a positive ctrl. The activated tyrosine kinases are expressed in MEFs. **A.** To check cell proliferation, crystal violet cell viability assay was obtained. **B.** The graph showing cellular growth in active tyrosine kinases expressing MEFs.

3.2 The role of TEL-ZAP70 in Mouse Embryonic Fibroblasts (MEFs) cell proliferation

In order to understand the role of TEL-ZAP70 in MEFs, we have stably transfected the MEFs with activated ZAP70 and PTK6, alongside of Myr-Akt1 for the positive control and also the empty vector pBabe Neo for the negative control (Figure 3.2A). Tyrosine phosphorylation on certain residues enhanced in active ZAP70 and PTK6 expressing MEFs (Figure 3.2A). Myr-Akt1 was tagged with myristoylation sequence which allows for AKT1 localize in cell membrane and become constitutively activated by PDK1 phosphorylation. Thus, myristoylated AKT has minimum dependency on PI3K to phosphorylate the downstream targets. We wanted to investigate whether activated ZAP70 was able to enhance cellular proliferation in MEFs. For this purpose, cells were seeded into 12-well plates and supplemented with 4% FBS DMEM. The cells were grown in reduced serum containing medium for reducing competition between serum proteins with cellular protein products as well as to synchronized the cells. Then, the cells were grown for 7 days and stained with crystal violet dye that intercalates the DNA of proliferative cells to check cellular proliferation. As a result, TEL-ZAP70 expressing MEFs had the highest growth rate in comparison to TEL-PTK6 as well as empty vector expressing controls (Figure 3.2B). Hence, overexpressed ZAP70 conferred significant proliferative ability to untransformed MEF in comparison to positive control Myr-Ak1 MEF.

A.



B.

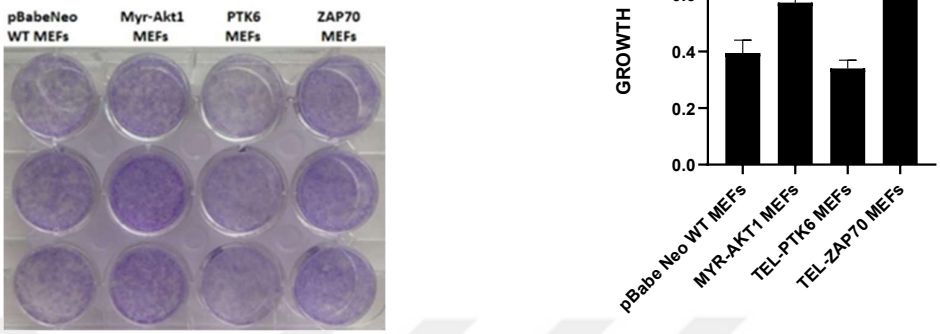


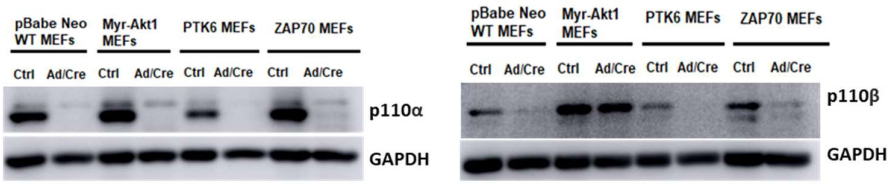
Figure 3.2. Impact of activated ZAP70 in MEF proliferation. A. To confirm the protein expression of activated ZAP70 in MEFs, western blot analysis was conducted. These vectors were tagged with FLAG sequence and the tag was able to detected by using M2-FLAG primary antibody. GAPDH was used as a loading control. **B.** Crystal violet growth assay analysis showing growth rate of MEFs constructs. Data shown as mean, SD, N of triplicate wells and displays of one experiment and analysed by using 2way ANOVA; ****$p<0.0001$

3.3 Investigation of growth compensatory potential of activated ZAP70 upon PI3K abrogation

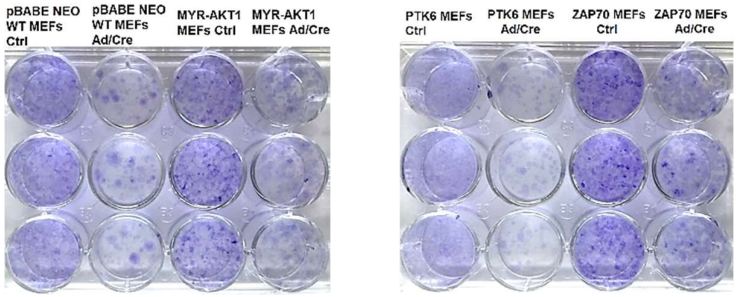
In order to confirm the tyrosine kinase library screening data which indicates activated ZAP70 was able to compensate growth upon p110α/β loss, we have conducted two different approaches to inhibit the PI3K pathway signaling in the MEF system. One of them takes advantage of molecular genetics and induce genetic abrogation of PIK3CA and PIK3CB in MEFs in and the other is pharmacological inhibition of p110α and p110β by isoform specific inhibitors. For molecular genetics system, we have used genetically engineered MEFs. The genetically engineered MEFs have loxP sites that recognized by Adenovirus/Cre recombinase to flox out the first exons of PIK3CA and PIK3CB. In this experiment, we have used stably expressed activated tyrosine kinases; TEL-ZAP70 nad TEL-PTK6 MEFs. Additionally, we have also used our positive control Myr-Akt1 MEFs and as well as negative control pBabe Neo WT MEFs. With this design, MEFs lines were seeded into 12-well plates as triplicates and treated with

5ul Adenovirus/Cre recombinase for 5 times. To calculate the the transduction efficiency, multiplicity of infection (MOI) should be determined for minimum cell death. MOI is depicting the number of viral particles per cell. In our assay, we have used 5 ul of Ad/Cre which corresponds to a MOI around 75. The Cre recombinase treatment is expected to ablate p110α/β expression. To demonstrate this ablation, we have conducted immuno blot analysis with Ad/Cre treated vs control MEFs constructs by using anti-p110α and anti-p110β primary antibodies. According to our results, p110α and p110β are efficiently knocked out (Figure 3.3A). The knock out of p110α/β transcripts leads to growth retardation in MEFs constructs (Figure 3.3B). Nevertheless, crystal violet growth assay analysis revealed that activated ZAP70 expressing MEFs have partially compensated cellular growth elicited by p110α/β knock out. The TEL-ZAP70 MEFs were able to grow as well as the Myr-Akt1 MEFs which were used as our positive control. However, PTK6 expressing MEFs were not able to compensate growth upon PI3K abrogation along with negative control pBabe Neo WT MEFs (Figure 3.3B)

A.



B.



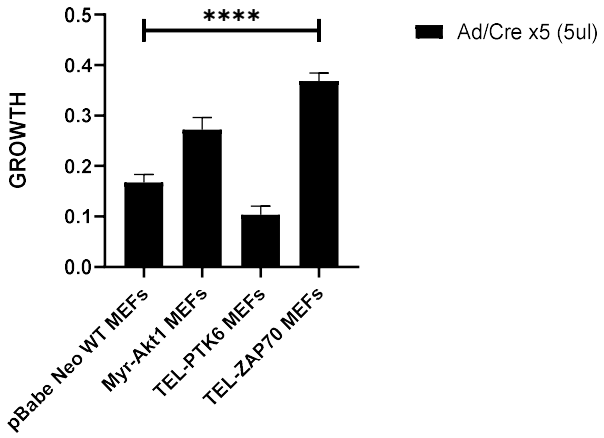


Figure 3.3. Ad/Cre treatment leads to ablation of p110α and p110β in MEFs. A. Western blot analysis showing p110α/β knock out efficiency in MEFs constructs. To detect protein levels, antibodies against p110α, p110β and GAPDH (loading control) was used. **B.** Crystal violet cell viability assay analysis showing TEL-ZAP70 MEFs have ability to recovery of growth upon p110α/β loss. Data shown as mean, SD, N of triplicate wells and displays of one experiment and analysed by using 2way ANOVA; ****$p<0.0001$

For pharmacological inhibitor approach, we have used FDA approved PI3K-p110α small molecule inhibitor, Alpelisib (BYL719) and PI3K-p110β small molecule inhibitor, KIN193 (AZD6482) to specifically inhibit kinase activities of PIK3CA and PIK3CB respectively. For this experiment, activated ZAP70 expressing MEFs along with positive control Myr-Akt1 and negative control pBabe Neo empty vector expressing MEFs were used. MEF lines were seeded into 12-well plates as triplicates. Then, the cells were treated with various concentrations of single or combined small molecule inhibitors and incubated for 7 days. As a result of crystal violet growth assay analysis, In KIN193 1uM inhibitor treatment, pBabeNeo WT MEFs were inhibited %41 but TEL-ZAP70 MEFs were inhibited %21. In BYL+KIN 0.3uM inhibitor treatment, pBabeNeo WT MEFs were inhibited %68 but TEL-ZAP70 MEFs were inhibited %49. Overall, we observed a trend towards decreased inhibition in response to BYL719+KIN193 0.3 uM treatment in between activated ZAP70 MEFs and pBabe Neo WT MEFs. Additionally, in BYL719+KIN193 0.5 uM and BYL719+KIN193 0.3 uM treatments, activated ZAP70 expressing MEFs were found to compensate the growth defects associated with PI3K inhibition, which is exhibited in our negative

controls (Figure 3.4). Hence, activated ZAP70 has a compensatory effect on cellular proliferation upon catalytic inhibition of p110α and p110β. Overall, these results suggest that, overexpression of ZAP70 induce partial resistance to either genetic or pharmacological inhibition of p110α/β.

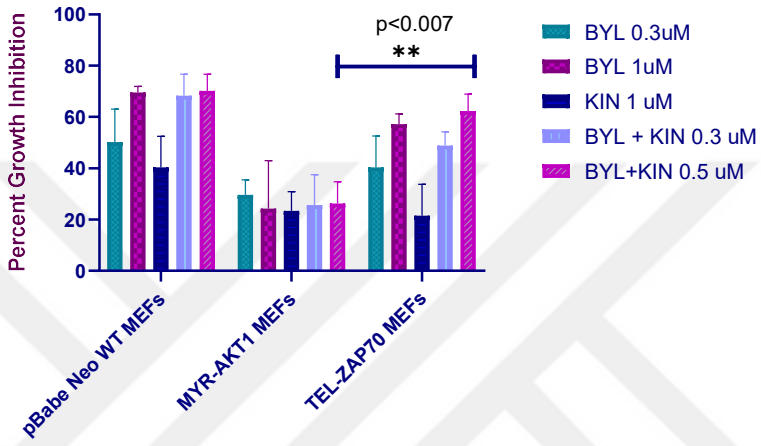


Figure 3.4. Pharmacological inhibitor mediated PI3K abolishment in MEFs. Crystal violet cell viability assay performed in stably expressing activated ZAP70 MEFs as well as controls. Representative graph showing cellular proliferation upon small molecule inhibitor treatments. The MEFs constructs treated with BYL719 and KIN193. Data shown as mean, SD, N of triplicate wells and displays of one experiment and analysed by using 2way ANOVA.

3.4 Biochemical analysis of MEFs constructs

The receptor tyrosine kinases and non-receptor tyrosine kinases might activate alternative signaling pathways besides PI3K via phosphorylation of downstream targets (Bennasroune et al., 2004, Gocek et al., 2014) [9] [6]. ZAP70 induced partial resistance might be accompanied by the activation of JAK/STAT and MAPK pathways. To elucidate the molecular mechanisms that contribute to the partial compensation of growth upon PI3K abrogation in TEL-ZAP70 MEFs, we have checked several downstream components of JAK/STAT and MAPK pathway. In immunoblot analysis, we have noticed that TEL-ZAP70 expressing MEFs have high levels of STAT3 phosphorylation. Moreover, the ERK1/2 phosphorylation levels were increased as well (Figure 3.5). ERK1/2 kinase activation occurs via dual

phosphorylation of different tyrosine residues; Tyr204 for ERK1, Tyr187 for ERK2. Hence, the two bands of ERK1/2 in blot represent dual phosphorylation of these residues. In addition to this, to investigate mTOR signaling axis, S6 Kinase phosphorylation levels were analyzed but we did not observe a difference in p-S6 in TEL-ZAP70 MEFs (Figure 3.5). These results implicate that, activated ZAP70 mediated growth compensation might occur via phosphorylation/activation of JAK/STAT or MAPK signaling pathways.

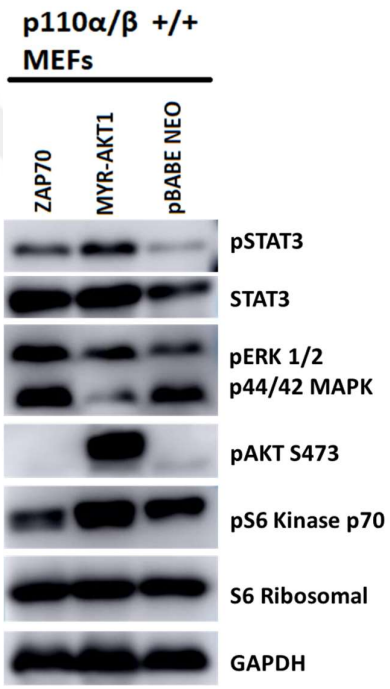
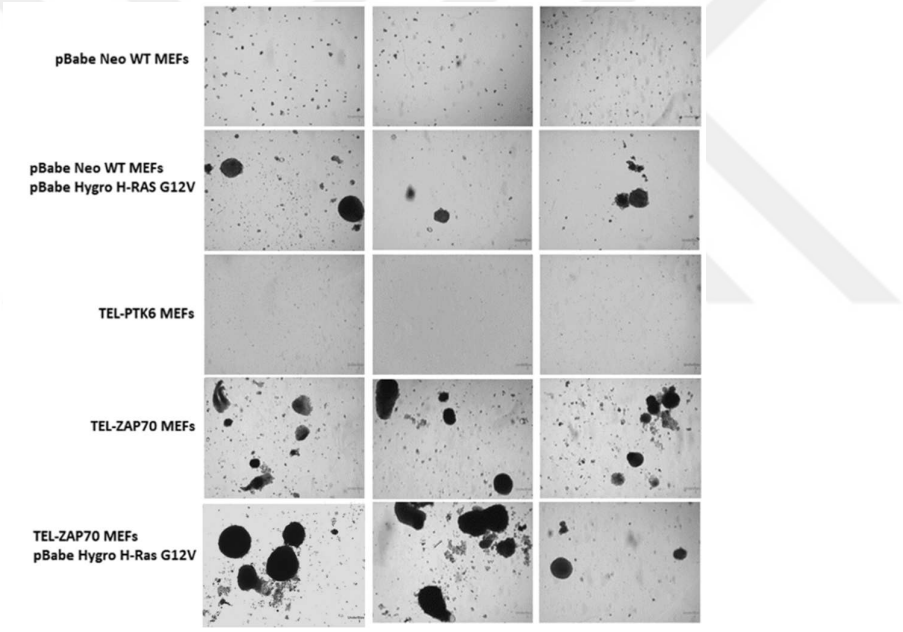


Figure 3.5. Detection of activated signaling pathways in ZAP70 overexpressed MEFs. Western blot analysis showing protein expression levels.

3.5 The role of TEL-ZAP70 in MEFs anchorage independent growth

In order to understand whether activated ZAP70 has a transformation ability, we conducted soft agar assays with our MEFs lines expressing activated ZAP70 as well as H-Ras G12V expressing MEFs for positive controls. The H-Ras gene is a proto-oncogene that belongs to small G-protein family. Moreover, G12V mutation constitutively activates H-Ras and enhances its transforming ability. Wild Type MEFs are not able to form colonies in soft agar. Therefore, we stably expressed H-Ras G12V in MEFs to obtain transforming capability. In this assay, we have used TEL-PTK6

MEFs as negative control together with pBabe Neo WT MEFs. In agreement with our clonogenic assays, we found out that activated ZAP70 expressing MEFs were able to grow on soft agar. Likewise, H-Ras G12V expression provide transformation ability in MEFs. However, activated PTK6 and pBabe Neo empty vector expressing MEFs were not be able to form colonies in soft agar. Otherwise, H-Ras G12V expression provide transformation ability in MEFs. Moreover, ZAP70 overexpression along with H-ras G12V was able to enhance colony formation on soft agar, implicating and additive effect of anchorage independent growth ability of ZAP70 alongside withactivated MAPK pathway (Figure 3.6).



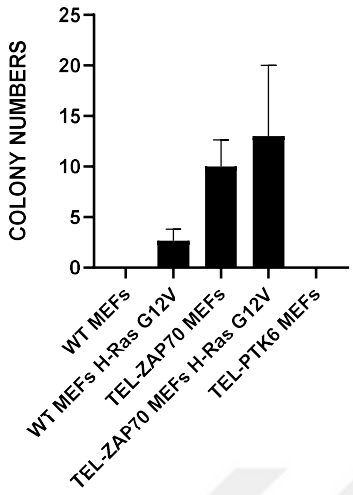


Figure 3.6. Additive effect of TEL-ZAP70 with H-RAS G12V in MEFs anchorage independent growth. Representative images showing formed colonies in soft agar as triplicates. We assessed the colony numbers by counting the colonies that consist of more than 50 cells.

3.6 Endogenous ZAP70 expression in different cell lines

We wanted to know whether endogenous ZAP70 is represented in different cell lines. To detect the protein expression level of ZAP70, we conducted western blot analysis. The blot showed that, except for p110α/β +/+ WT MEFs, all cell lines checked, expressed endogenous ZAP70 to different extent. We used Jurkat CD4+ T-Cell and TEL-ZAP70 expressing T47D as positive controls. The size difference in between positive controls is caused by TEL tag of ZAP70 expression in T47D (Figure 3.7A). In addition to that, we have checked the levels of tyrosine phosphorylations in these cells and the panel demonstrates immunoblots showing various tyrosines were phosphorylated (Figure 3.7B). To check if endogenously expressed ZAP70 is active and also able to activate downstream targets, we have checked phosphorylation of LAT and ZAP70. LCK phosphorylates and activates of ZAP70. Then, active ZAP70 phosphorylates LAT on several tyrosine residues. According to phospho-ZAP70 blot, besides of positive controls, HEK293T and MCF7 cells have high levels of active ZAP70 (Figure 3.7C). Since the bands of positive controls were overexposed we were cut the membrane and removed positive controls. The cut membrane shows that p-ZAP70 levels are increased in MCF10A and MEFs as well which indicates activation

of ZAP70 (Figure 3.7C-upper panel). Furthermore, in phospho-LAT blot, p110α/β +/+ WT MEFs and positive controls have high levels of LAT phosphorylation (Figure 3.7C-bottom panel).

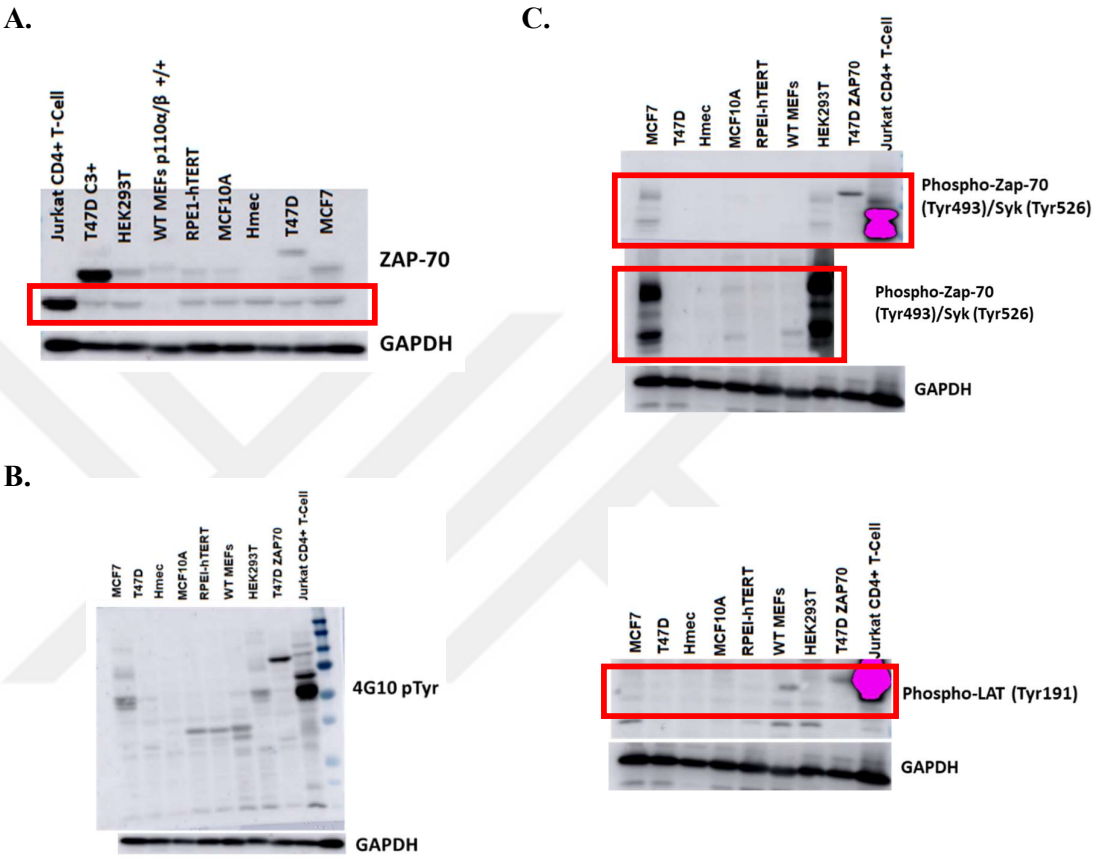
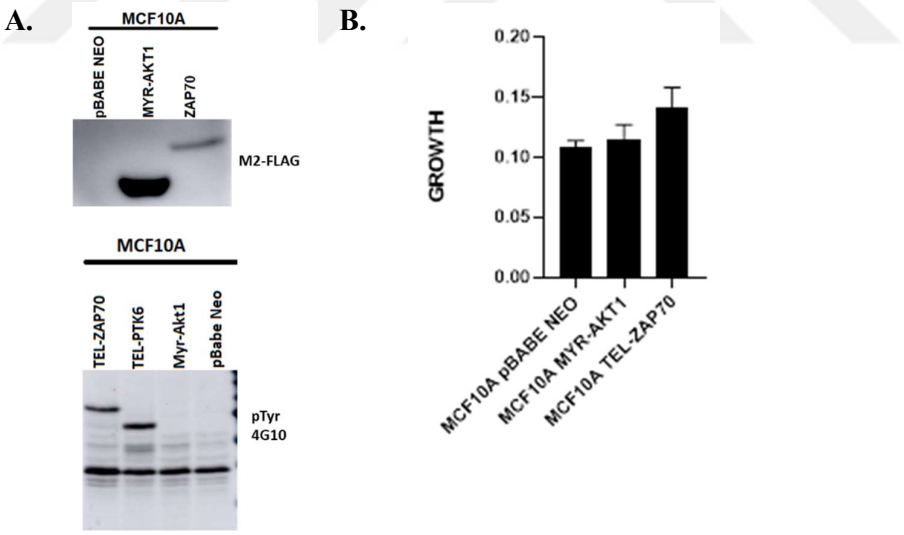


Figure 3.7. Detection of ZAP70 expression in cell lines. Western blot analysis showing **A.** endogenous ZAP70 expression. **B.** Tyrosine phosphorylation **C.** ZAP70 and LAT phosphorylation in different cell lines. The red boxes on blots indicate the size of antibodies.

3.7 Functional analysis of TEL-ZAP70 expression in non-transformed cells

In order to investigate the function of ZAP70 in non-transformed cells, we have used MCF10A and RPE1-hTERT (retinal pigment epithelium) cells. The morphological characteristics of MEFs are different from epithelial cells as they rather represent clls of mesenchymal origin. Most of the carcinomas are derived from an epithelial origin via epithelial-mesenchymal transition. Thus, we have used epithelial cells to

understand the impact of activated ZAP70 initiation of carcinogenesis. MCF10A is characterized as immortalized and non-tumorigenic human breast epithelial cell lines. RPE1-hTERT cells were immortalized by human telomerase reverse transcriptase subunit (hTERT) and derived from retinal tissue. For this purpose, we have generated stably expressing activated tyrosine kinase ZAP70 in MCF10A and RPE1-hTERT cells along with positive (Myr-Akt1) and negative (pBabe Neo) controls (Figure 3.8A – Figure 3.9B). Crystal violet growth assay revealed that activated ZAP70 expression enhance cellular proliferation in MCF10A (Figure 3.8B). Furthermore, to reveal the signaling pathway in MCF10A that contributes to the cellular proliferation capability of ZAP70, we conducted western blot analysis by checking downstream of tyrosine kinase signaling pathway components. According to our immunoblots, STAT3 phosphorylation level increased, although pS6K or pERK levels did not change. These data indicate that overexpression of ZAP70 enhance growth via activating JAK/STAT pathway in MCF10A.



C.

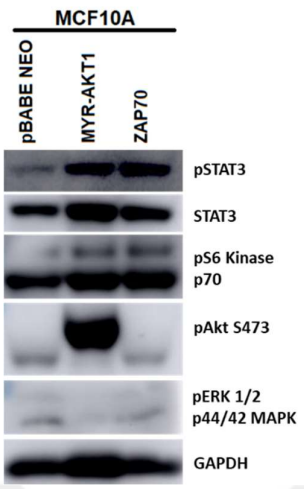
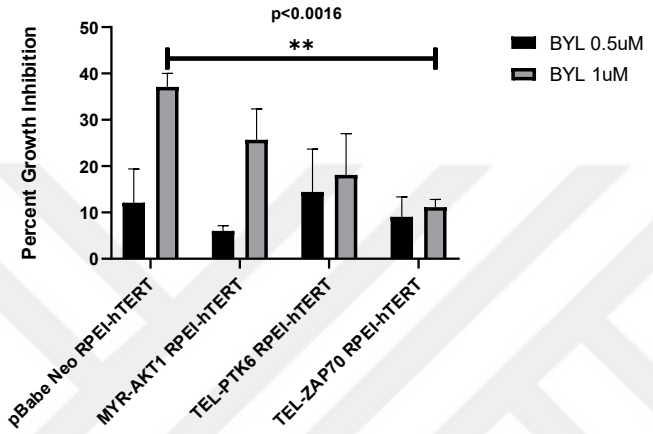


Figure 3.8. Investigation the function of TEL-ZAP70 in non-transformed cell lines. A. The blots showing TEL-ZAP70 expression in MCF10A by using M2-FLAG antibody. **B.** Crystal violet cell viability assay conducted to check cellular proliferation. The graph showing growth data of MCF10A constructs. **C.** Detection of activated signaling pathways by checking downstream targets by using western blot analysis.

To elucidate the effects of activated ZAP70 on cell viability upon PI3K abrogation in RPE1-hTERT, we have treated the cells with PI3K-p110α small molecule inhibitor, Alpelisib (BYL719) and PI3K-p110β small molecule inhibitor, KIN193 (AZD6482). For this purpose, we have used stably expressing activated ZAP70 RPE1 along with positive control Myr-Akt1 RPE1 and negative control pBabe Neo RPE1 cells. The crystal violet growth assay analysis indicate that TEL-ZAP70 expressing RPE1-hTERT cells could partially compensate growth upon 1uM BYL719 treatment. The percent growth inhibiton of TEL-ZAP70 RPE1 cells is less than pBabe Neo RPE1 cells upon 1uM of BYL719 treatment and the difference is statistically significant (Figure 3.9A). To demonstrate activation of downstream pathways that promotes to proliferation apart from PI3K signaling pathway, we have checked downstream targets of these signaling pathways. For this purpose, immunoblot analysis were performed. The first panel showing the protein expression of TEL-ZAP70 together with positive and negative controls by using M2-FLAG antibody. Also, tyrosine phosphorylations of activated tyrosine kinases depicted in pTyr immunoblot (Figure 3.9B). In addition to this, STAT3 phosphorylation levels dramatically increased in TEL-ZAP70 RPE1 cells (Figure 3.9B). This enhancement of pSTAT3 level may indicate JAK/STAT signaling pathway activation. Moreover, in TEL-ZAP70 RPE1 the phosphorylation

levels of ERK1/2 and S6 Kinase are decreased (Figure 3.9B). These result imply that, mTOR and MAPK pathway might not be effected by ZAP70 activation, however JAK/STAT signaling pathway appears to be persistently upregulated by active ZAP70.

A.



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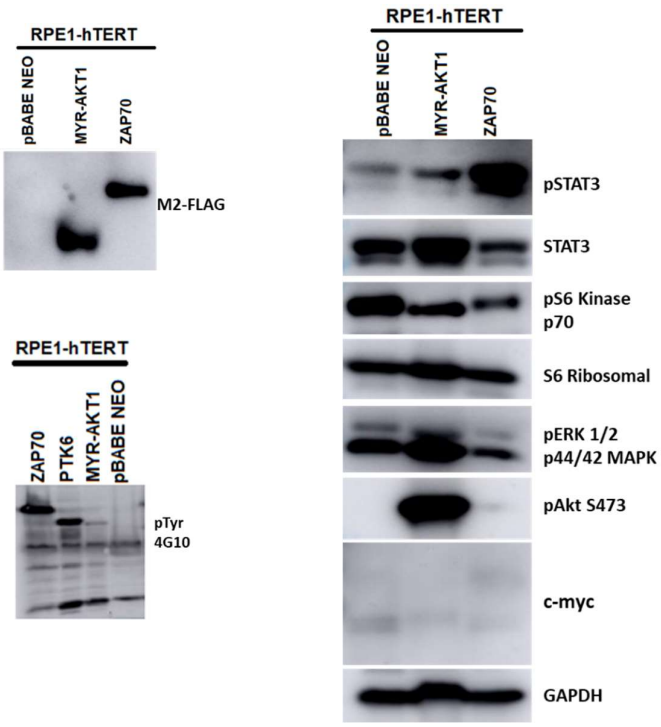
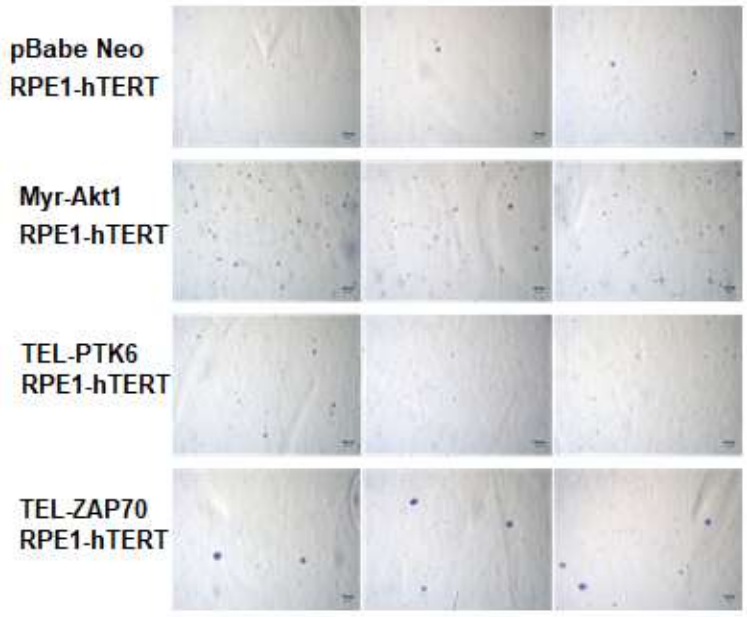


Figure 3.9. Functional characterization of TEL-ZAP70 in RPE1-hTERT cells. A. Crystal violet growth assay analysis of small molecule inhibitor treated RPE1-hTERT cells. The graph showing percent growth inhibition of RPE1-hTERT constructs upon PI3K abrogation. **B.** The western blot membranes showing TEL-ZAP70 expression and tyrosine phosphorylation pattern in RPE1-hTERT cells by using M2-FLAG antibody. Also, the big panel represent active signaling pathways.

After determining the importance of activated ZAP70 for cellular proliferation in RPE1-hTERT cells, we wanted to see if TEL-ZAP70 has the capability of forming colonies in anchorage independent growth assays. For this purpose, we have performed soft agar assay in RPE1-hTERT cell lines. In this assay, we have used stably expressing activated ZAP70 RPE1 along with positive control Myr-Akt1 RPE1 and negative control pBabe Neo RPE1 and TEL-PTK6 RPE1 cells. Our results of soft agar growth assays implicated that, TEL-ZAP70 expressing RPE1-hTERT was able to form colonies, although controls were not able to form as many colonies as activated ZAP70 construct in soft agar (Figure 3.10). Activated ZAP70 expressing RPE1 cells have transforming ability.



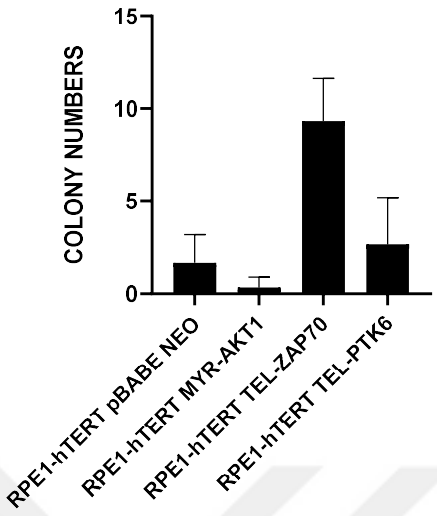


Figure 3.10. Determination of the potential of activated ZAP70 RPE1-hTERT cells in anchorage independent growth. The representative images showing soft agar results and the graph showing formed colony numbers. We studied as triplicates in this experiment.

3.8 Analysis of ZAP70 alterations in cancer studies *in silico*

In terms of bioinformatics analyses by using cBioPortal [48] platform combination of curated set of non-redundant studies with NCI-60 cell lines study (NCI cancer res., 2012), it was revealed that, ZAP70 is highly altered in renal cell carcinomas. And these alterations mostly occur in the form of gene amplification (Figure 3.11).

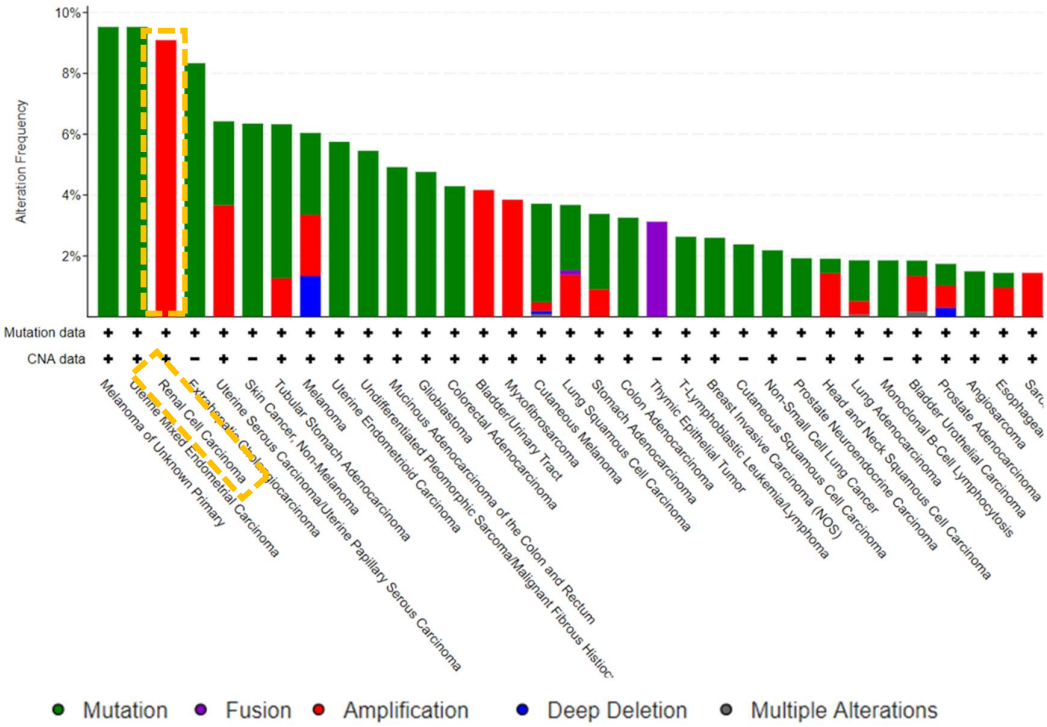
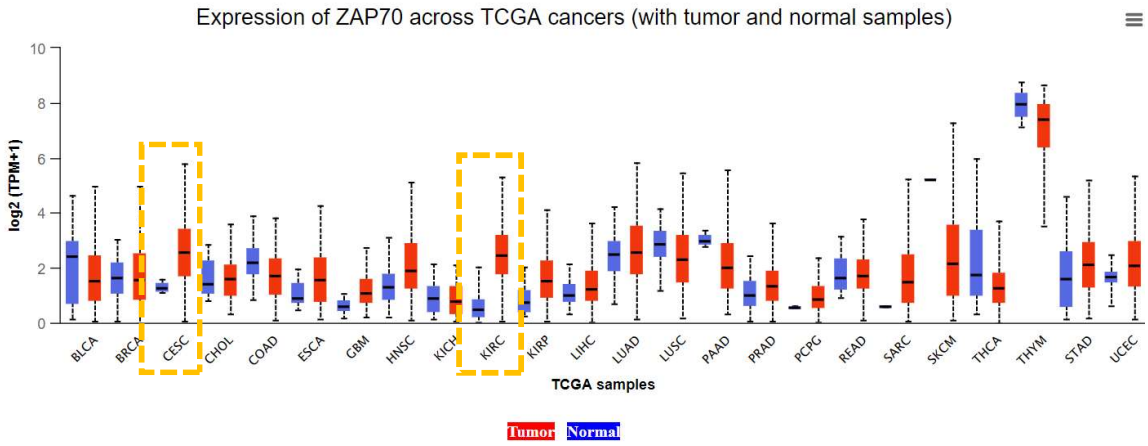


Figure 3.11. ZAP70 alterations in cBioPortal cancer studies. Based on the studies, ZAP70 amplification occurs highly in renal cell carcinomas.

Moreover, we investigated whether ZAP70 mRNA expression changes in between tumor vs normal tissue in various cancer types. For this purpose, we have used UALCAN [49] web portal and it was revealed that in Kidney renal clear cell carcinoma (KIRC) and Cervical squamous cell carcinoma (CESC) ZAP70 mRNA levels are higher in tumor tissues in contrast to normal tissues (Figure 3.12A). In addition to this analysis, we have used GEPIA2 [50] in order to compare ZAP70 mRNA level in tumor vs normal tissues in KIRC tumor vs normal tissues (Figure 3.12B).

A.



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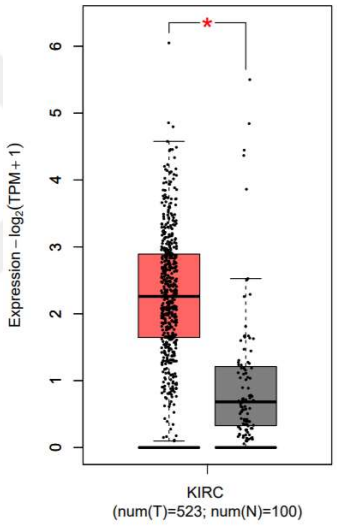


Figure 3.12. ZAP70 mRNA expression in different cancer types. A. According to UALCAN database, ZAP70 mRNA levels are higher in KIRC and CESC tumor tissues. **B.** The boxplots represents ZAP70 mRNA levels in KIRC to compare tumor vs normal tissues. We have used GEPIA2 bioinformatics tool for this analysis. *p<0.01

Furthermore, we analysed, Cervical Squamous Cell Carcinoma and Endocervical Adenocarcinoma (CESC) where ZAP70 expression is elevated. Datasets in GEPIA2 [50] database revealed that, ZAP70 mRNA expression level is significantly higher in TCGA CESC primary tumor samples in comparison to normal samples (Figure 3.13A). Additionally, Kaplan-Meier survival curves were plotted by using GEPIA2 [50]. These curves revealed that, high ZAP70 expression correlated with better survival in CESC for both overall and disease-free survival data (Figure 3.13B).

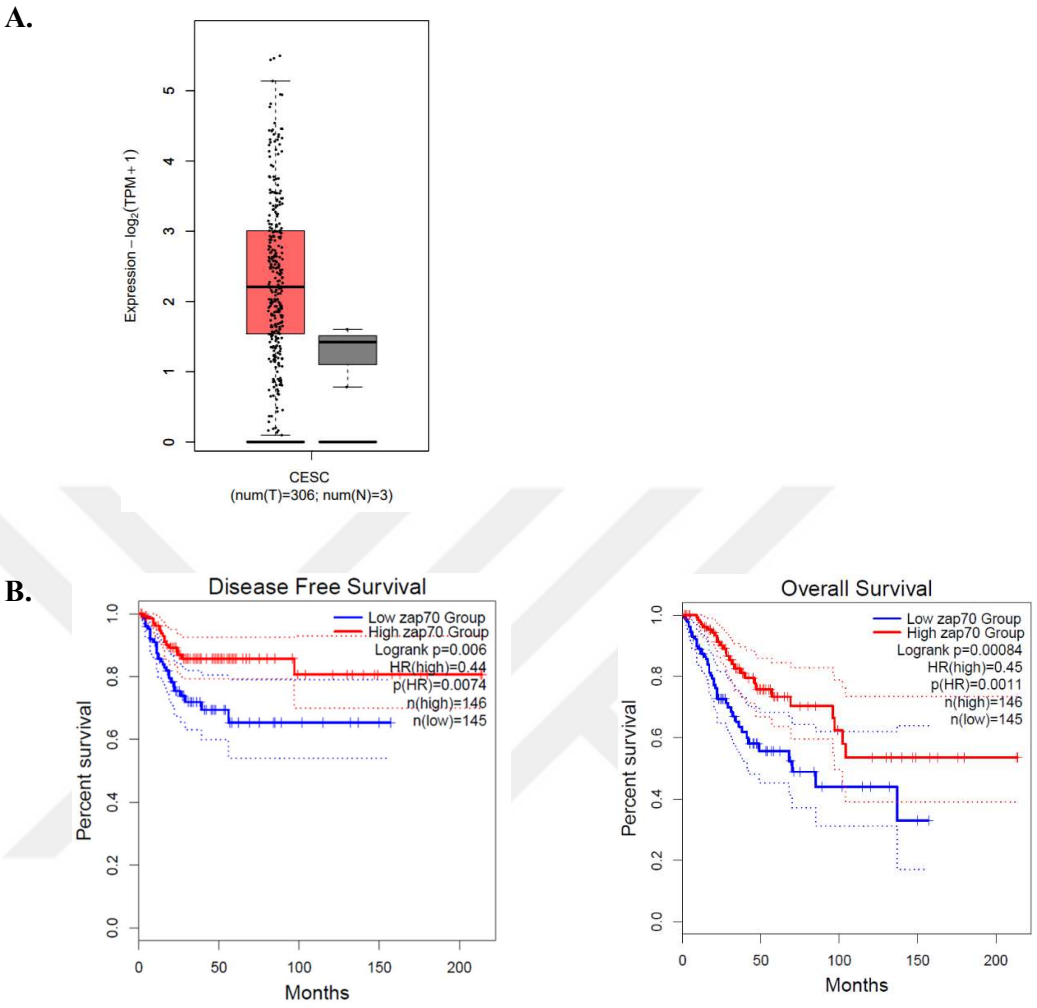


Figure 3.13. ZAP70 mRNA expression in CESC. A. The boxplots showing ZAP70 mRNA expression in CESC. The panel represent the analysis obtained from TCGA data from GEPIA2. **B.** Kaplan-Meier graphs showing overall survival and disease-free survival analysis.

Additionally, in order to elucidate the effect of ZAP70 expression in ovarian cancer, Kaplan-Meier survival curves were plotted by KM Plot [51] database. In paclitaxel/cisplatin chemotherapy treated grade1+2 and grade 2 patients, high ZAP70 expression significiantly decreased progression free survival (PFS) in comparison to low ZAP70 expression. In conclusion, ZAP70 might be a crucial prognostic marker for carcinogenesis and low ZAP70 expression correlated with better survival in chemotherapy treated ovarian cancer patients (Figure 3.14A- Figure 3.14B). Based on grade 2+3 and grade 3 ovarian cancer survival analysis, ZAP70 expression levels do not have any significant correlation of progression free survival upon paclitaxel+cisplatin treatment (Figure 3.14C- Figure 3.14D).

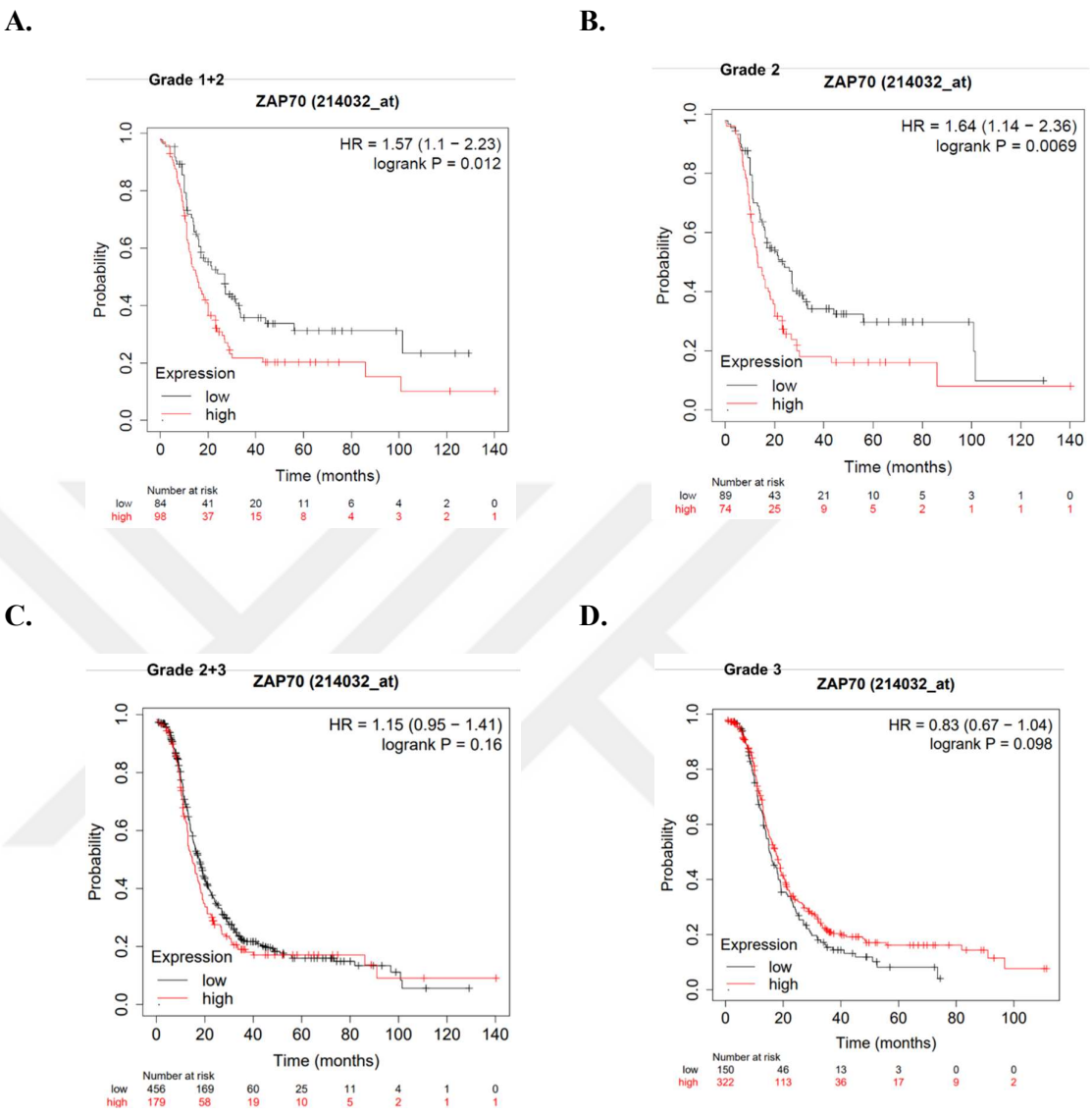


Figure 3.14. Kaplan-Meier survival graphs depicting grade specific correlation of ZAP70 expression level in ovarian cancer. The curves arranged by using mRNA gene chip data of KM Plot database, based on ZAP70 expression levels in ovarian cancer grades. **A.** Progression free survival of grade 1+2 ovarian cancer patient samples with paclitaxel/cisplatin treatment. **B.** Progression free survival of grade 2 ovarian cancer patient samples with paclitaxel/cisplatin treatment. **C.** Progression free survival of grade 2+3 and **D.** progression free survival of grade 3 ovarian cancer patient samples with paclitaxel/cisplatin treatment.

In literature, Huang et al. revealed that ZAP70 overexpression along with 5 other genes identifies as prognostic marker in colorectal cancer in response to radiation [45]. Accordingly, we analysed alterations of ZAP70 in colorectal cancer samples by using cBioPortal [48] Cancer Cell Line Encyclopedia (CCLE) (Broad, 2019). This analysis

indicate that colorectal cancer has one of the highest mutation rates in ZAP70 (Figure 3.15).

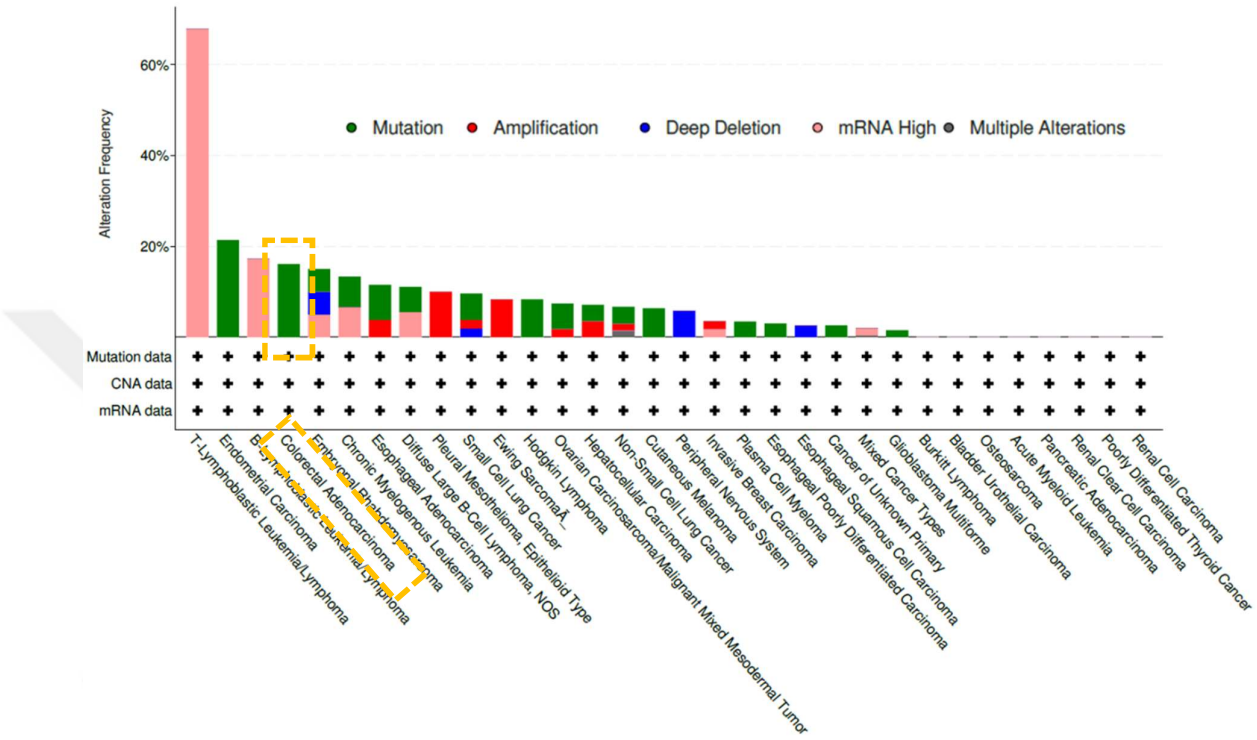


Figure 3.15. ZAP70 alterations in Colorectal Adenocarcinoma. According to cBioportal CCLE (Broad, 2019), colorectal adenocarcinoma has one of the highest alterations in ZAP70.

3.9 The role of ZAP70 overexpression in HEK293T cells

According to previous *in silico* analysis, renal cell carcinomas have the highest ZAP70 amplifications. We wanted to investigate the impact of ZAP70 overexpression in kidney cells to strengthen our research in epithelial cells. For this purpose, we have used HEK293T cells which are derived from human embryonic kidney and characterised as immortalized epithelial cells. HEK293T cells comprise SV40 T-antigen that enables high transfectable capability. We transiently expressed activated ZAP70 along with Myr-Akt1 as positive control and pBabe NEO negative control in HEK293T cells by conducting Lipofectamine 3000 protocol (Figure 3.15A). Also, tyrosine phosphorylation is elevated upon ZAP70 overexpression (Figure 3.15A).

ZAP70 overexpression in HEK293T cells enhanced phosphorylation of STAT3 in comparison to negative control which indicates STAT activation mediated by ZAP70 overexpression. Nevertheless, ERK1/2 phosphorylation levels do not change upon ZAP70 overexpression in HEK cells. To provide an insight of STAT3/myc axis, we have detected c-myc expression in HEK constructs. However, the c-myc signal is weak but overexpression of ZAP70 doesn't have any effect on myc protein expression in HEK293T cells (Figure 3.15B).

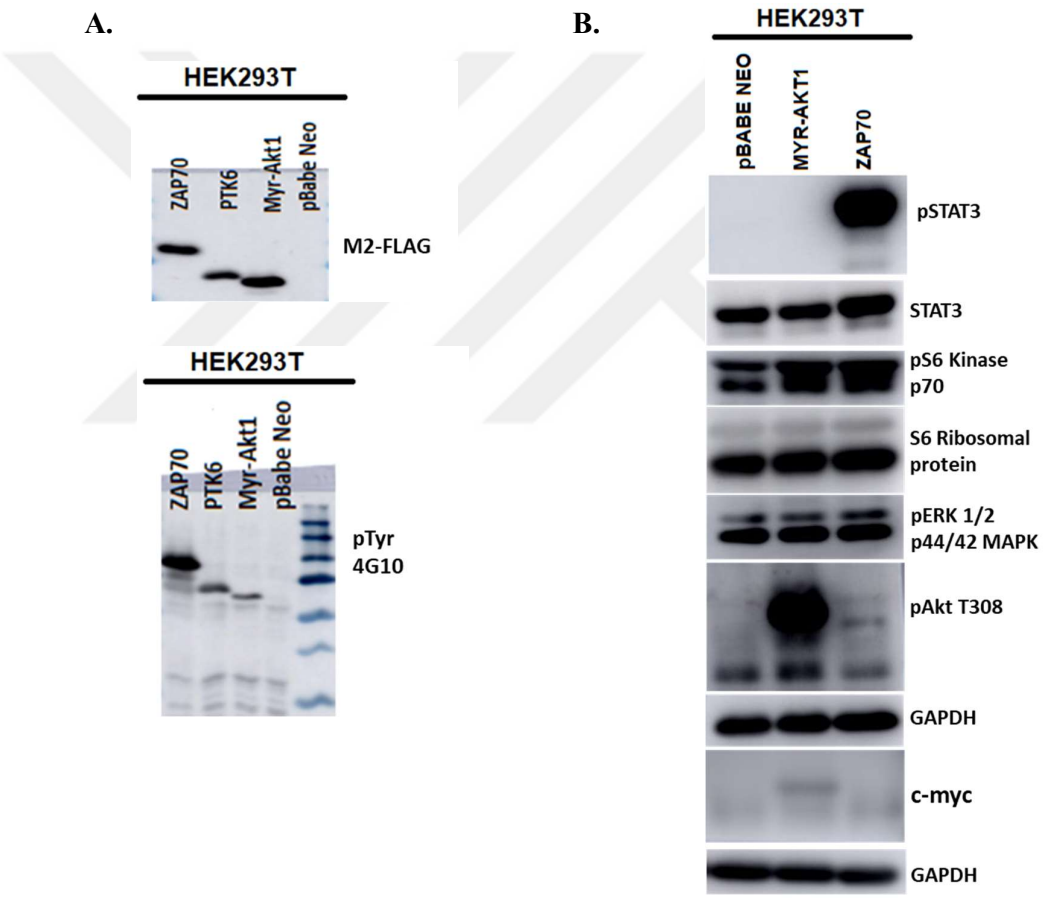


Figure 3.16. ZAP70 overexpression in HEK293T. A. Activated tyrosine expression detected by using M2-FLAG primary antibody. **B.** To elucidate the effect of ZAP70 in signaling pathway activation, downstream pathway components detected by western blot analysis.

CHAPTER 4

4. DISCUSSION

In this study, we first screened the activated TK library. Afterward, we found out that an activated tyrosine kinase pool has a role in PI3K functional compensation. We identified activated ZAP70, from a tyrosine kinase pool that plays a role in developing partial resistance to PI3K abrogation. Our findings on tyrosine kinase library screening demonstrated that C1-C4 pool expressing MEFs compensate growth upon p110α/p110β loss. Especially, activated ZAP70 expressing MEFs can compensate for growth as well as positive control upon loss of p110α / p110β (Figure 3.1).

Basically, ZAP70 plays a role in T-cell receptor signaling (TCR) pathway and involves in hematological malignancies [52]. In the first instance, we showed the function of ZAP70 in untransformed cell lines besides of its immunological function. Our analysis of cell viability assay justifies the TEL-ZAP70 expressing MEFs significantly enhance cellular proliferation in comparison to TEL-PTK6 MEFs and negative control. This analysis found evidence of active ZAP70 might have elevated proliferation ability via high levels of tyrosine phosphorylation (Figure 3.2). In Figure 3.2 we also showed TEL-ZAP70 protein expression in MEFs along with controls by using anti-M2-FLAG and anti-pTyr primary antibodies. According to immunoblots, TEL-ZAP70 expression in MEFs should be increased for getting significant results. However, such expression of ZAP70 can induce partial resistance to PI3K inhibition.

Based on literature, PI3K is the crucial regulator of cellular proliferation and PI3K abrogation has been linked to severe growth defects in cell system [19] [5]. In an attempt to abrogate PI3K signaling, we have used a molecular genetic system which enables to knock out of the endogenous p110α/p110β in MEFs. Our results suggest that, active ZAP70 could partially compensate growth upon p110α/p110β ablation. However, active PTK6 expressing MEFs line was not able to compensate growth in p110α/p110β knock out system Moreover, p110α and p110β are efficiently knocked out (Figure 3.3).

Furthermore, to inhibit the catalytic activity of PI3K, we have used a pharmacological approach that is clinically relevant to our research. Alpelisib (BYL719) is FDA approved p110α specific and AZD6482 (KIN193) is p110β specific small molecule

inhibitors. As a result of pharmacological inhibition of PI3K, in MEFs, a similar trend as the molecular genetic system was observed in cell viability. Although, the difference between TEL-ZAP70 MEFs and negative control MEFs in growth compensation upon PI3K abrogation was not significant. (Figure 3.4). Our results for this section were unsatisfactory, however the results from molecular genetic system for this approach are still promising. When these results are considered, ZAP70 might have a role in developing partial resistance to p110α/p110β inhibition.

As proposed by Siveen et al., STAT3 and MAPK signaling pathways are activated by activation of Src Family Kinases (SFK), which belong to the non-receptor tyrosine kinase family [8]. Furthermore, Warmuth et al. suggested that the STAT3 signaling cascade is crucial in carcinogenesis [53]. In Figure 3.5, we showed elevated STAT3 phosphorylation as well as dual-tyrosine phosphorylation of ERK in TEL-ZAP70 MEFs. In addition to this, a similar increase in p-STAT3 levels was obtained in RPE1 TEL-ZAP70 (Figure 3.9) and HEK293T TEL-ZAP70 (Figure 3.16) cells. Surprisingly, p-ERK1/2 and p-S6Kinase p70 expression levels did not change in TEL-ZAP70 RPE1-hTERT cells. But, p-S6Kinase p70 levels are elevated in HEK293T cells. These results highlights that, mTOR and MAPK signaling pathways might not be related to ZAP70 mediated resistance in our approach. Cha et al. noted that ZAP70 negatively regulates JAK/STAT3/c-MYC signaling axis to modulate differentiation capability in Mouse embryonic stem cells [44]. Even though, STAT3 activation positively correlated with ZAP70 expression in our research, we wanted focus on c-myc axis. We demonstrated that there is no difference in c-Myc levels in active -ZAP70 expressing cells. Our results confirm previous findings in the literature. Hence, the ZAP70 mediated compensation of growth upon PI3K abrogation might be explicated through STAT3 signaling pathway mechanistically. However, each cell response to tyrosine kinase mediated pathway activation may be different.

To further explore the function of ZAP70 in carcinogenesis, we suggested that ZAP70 has a role in anchorage independent growth on soft agar in untransformed cell lines. The MEFs lines are not able to grow in soft agar alone, yet H-Ras G12V expression provides the cells with an ability to form colonies in soft agar. In addition to this, we showed that ZAP70 has an additive effect in anchorage-independent growth together with H-Ras G12V in MEFs (Figure 3.6). We also observed similar findings in untransformed RPE1-hTERT cells that ZAP70 is capable of colony formation in soft

agar (Figure 3.10). Our results cast a new light on the function of ZAP70 in tumorigenesis as a tumor-initiating factor. ZAP70 has high oncogenic transforming potential while the disease is establishing. Even though our research was conducted in untransformed cell lines, it would be significant to elucidate the effects of ZAP70 in transformed cancer cells. Recently, a study investigated by Fu et al. identified ZAP70 as a novel target of tumorigenesis by regulation of invasion and migration in prostate cancer cells [43]. This study is relevant to our research in order to understand the role of ZAP70 in cancer cells. According to *in silico* analysis, we determined the role of ZAP70 in cancer patients data. We showed ZAP70 is mostly amplified in renal cell adenocarcinoma as well as ZAP70 mRNA expression is higher in tumor tissues in comparison to normal tissues in KIRC samples. Additionally, in lower grades of ovarian cancer patients which are treated with paclitaxel+cisplatin, low ZAP70 expression correlated with better survival. Moreover, Huang et al. demonstrated that ZAP70 overexpression has linked to response of radiation in colorectal cancer as a prognostic marker [45]. In conclusion our results indicate that ZAP70 might have a role in tumor initiation. Furthermore, in primary tumors, the effects of ZAP70 decrease as mutation acquired.

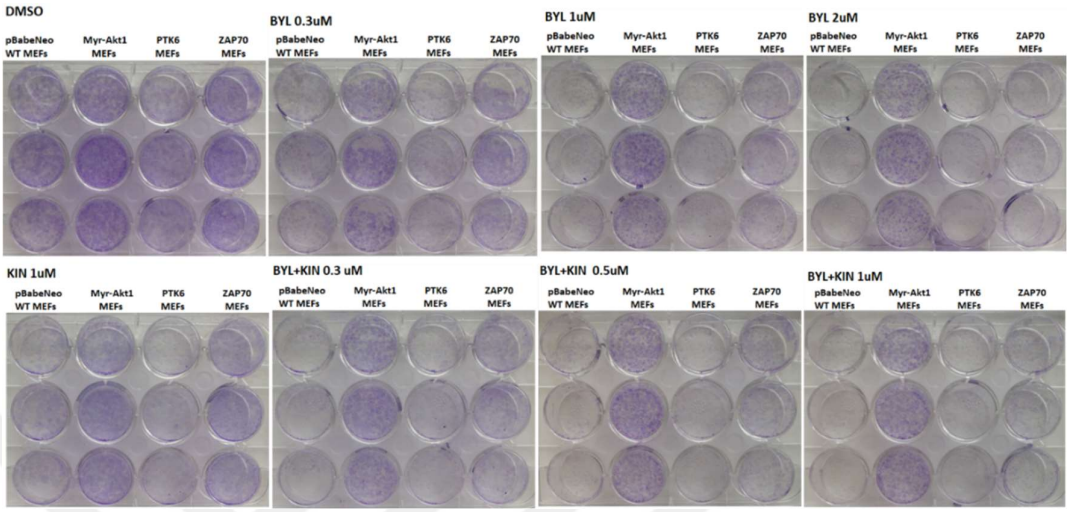
CHAPTER 5

5. CONCLUSIONS AND FUTURE PERSPECTIVES

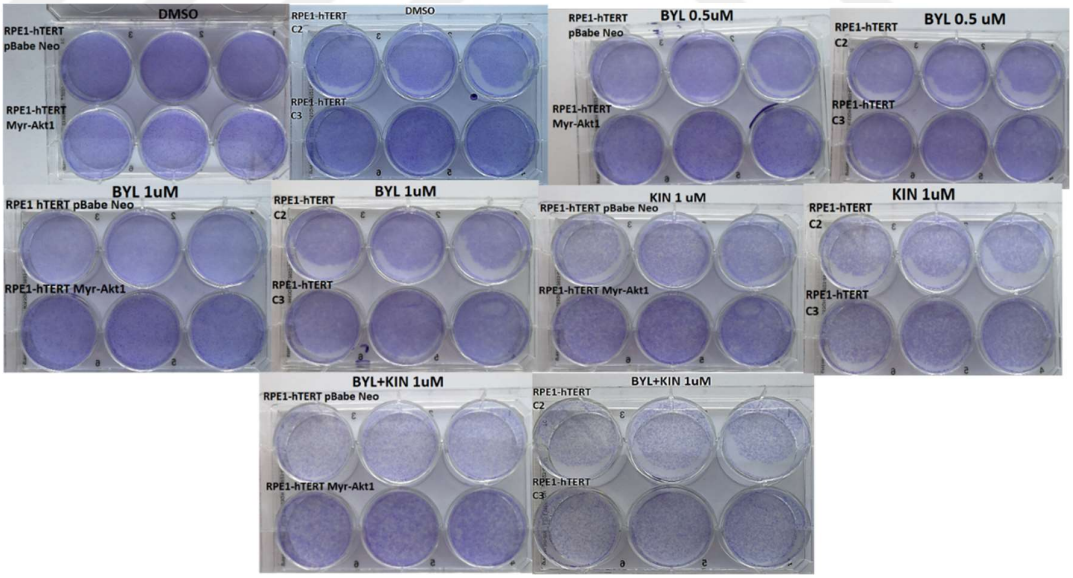
In this thesis, we first identified an individual tyrosine kinase, ZAP70, from a tyrosine kinase library that plays a role in developing partial resistance to PI3K abrogation. We started with the generation of TEL-ZAP70 expressing cell lines along with positive and negative controls. Further, we ablated PI3K pathway via the molecular genetic system and pharmacologic inhibition in MEFs. Activated ZAP70 could compensate growth upon PI3K abrogation in MEFs and RPE1-hTERT lines. Beside this, activated ZAP70 expression enhance cellular proliferation in untransformed breast epithelial MCF10A cells as well as MEFs. The activated ZAP70 induced signal transduction explored and found out that the STAT3/myc axis contributes to this pathway activation. In addition to that, potential role of activated ZAP70 in carcinogenesis was investigated. Our data indicated that activated ZAP70 has high transformation capability which could be crucial for initial stages of carcinogenesis. Furthermore, our *in silico* analysis supported the findings that ZAP70 might have a role to in transformed or cancerous cells. In conclusion, activated ZAP70 develops a partial resistance to PI3K abrogation in untransformed cells. Moreover, ZAP70 functions as a tumor-initiating factor in immortalized cells.

Future research on knocking-down of ZAP70 by using siRNA targeted constructs and P-pharmacological interventions might extend the explanations of the functions of ZAP70 in untransformed and cancer cells. Furthermore, in order to detect non-immunological substrates of ZAP70, phosphoprotein array or Mass spectrometry-based interactome analysis as well as phosphoproteomics approaches could be performed.

APPENDIX



Suplemented Figure 1: The crystal violet cell viability assay images of MEF lines. MEFs lines seeded into 12-well plates and treated with different concentrations of BYL719 and KIN193 inhibitors.



Suplemented Figure 2: The crystal violet cell viability assay images of RPE1-hTERT lines. RPE1-hTERT lines seeded into 6-well plates and treated with different concentrations of BYL719 and KIN193 inhibitors.

A	1	ERBB3
A	2	ERBB4
A	3	FES
A	4	FGR
A	5	FRK
A	6	HCK
A	7	IGF1R
A	8	ITK
A	9	MATK
A	10	NTRK2
A	11	PKMYT1
A	12	TEC
B	1	TXK
B	2	ZAP70
B	3	ERBB2
B	4	MET
B	5	LCK
B	6	NTRK1
B	7	PDGFRA
B	8	PDGFRB
B	9	TNK1
B	10	FLT3
B	11	EPHB4
B	12	DDR2
C	1	PTK2B
C	2	PTK6
C	3	ZAP70
C	4	NTRK3
C	5	PTK2
C	6	STYK1
C	7	FGFR2
C	8	LYN
C	9	RET
C	10	SYK
C	11	TYK2
C	12	ABL2
D	1	DDR1
D	2	EPHA3
D	3	FLT1
D	4	MUSK
D	5	LMTK2
D	6	JAK2
D	7	TYRO3
D	8	ALK
D	9	BTK
D	10	EPHA4
D	11	EPHA6
D	12	FER
E	1	FGFR1
E	2	FLT4
E	3	INSRR
E	4	KDR
E	5	EPHA2
E	6	ROR2
E	7	CSF1R
E	8	EPHA1
E	9	EPHB1
E	10	EPHB6
E	11	JAK1
E	12	FGFR3
F	1	SRC
F	2	EGFR
F	3	BMX
F	4	FYN
F	5	YES1
F	6	ABL1
F	7	BLK
F	8	CSK
F	9	MST1R (closed)
F	10	PTK7 (closed)
F	11	JAK3 (closed)
F	12	AXL (closed)
G	1	TIE1 (closed)
	A11	PKMYT1
	F1	SCR

Suplemented Figure 3: Activated tyrosine kinase library. The list of activated tyrosine kinases are depicted with letter code in this figure.

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