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Mechanisms of IKBKE Activation in Cancer

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Mechanisms of IKBKE Activation in Cancer

by

Sridevi Challa

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
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DEDICATION

This dissertation is dedicated to my kind and courageous mother.

I want to also acknowledge Smitha Pillai for her continuous support and encouragement during difficult times and for always being ready to listen to me, and Greg for giving me helpful suggestions.

Lastly, I want to thank my family for their constant support through my entire graduate school and beyond and for always trusting me with my decisions.

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ABSTRACT

Cancer is the second leading cause of death in the USA and it is expected to surpass heart diseases making it important to understand the underlying mechanisms of cancer. The efforts to target single signaling molecule showed little success in increasing the patient survival and it can be due to increased compensation for cell survival by alternative pathway activations. Hence comprehensive understanding of the alternative signaling pathways may help us treat cancer better. Chronic inflammation is attributed to increased risk of cancer and emerging studies show the growing importance of both canonical and non-canonical IκB kinases such as IKKα, IKKβ, IKBKE and TBK1 in human cancer pathogenesis. Initially identified as activator of NFκB pathway, IKBKE was shown to play an important oncogenic role by regulating multiple pathways downstream. Although IKBKE is implicated in tumorigenesis for over a decade, therapeutic targeting of this pathway has been a challenge. Recently, amlexanox and CYT387, which are in clinical trials for Type II diabetes and myeloproliferative disorders respectively, were identified as potential IKBKE inhibitors. In this study, we uncovered specific novel mechanisms of activation of IKBKE in tumor cells and the outcomes of targeting IKBKE pathway.

Oncogenic mutations are a cause of several human malignancies. Mutations in EGFR are observed in 15% of non-small cell lung cancer patients. While cells expressing these mutations respond better to the first generation TKIs, patients become resistant to these inhibitors due to secondary mutations in EGFR. These mutations were shown to make EGFR constitutively active even in the absence of ligands. Direct targeting of EGFR with secondary mutations has been

challenging as EGFR acquires novel mutations upon inhibitor treatment, which confer resistance to the EGFR-TKIs. Hence, it is important to improve our knowledge of the downstream signaling pathways of EGFR. Although PI3K, MEK signaling are well established, mutant EGFR was shown to activate several novel signaling pathways such as miRNA processing and autophagy that are implicated in resistance to EGFR-TKIs.

Here, we show that IKBKE acts downstream of mutant EGFR to activate the NFκB and AKT pathways. In addition, we show that mutant EGFR but not wildtype EGFR can directly phosphorylate IKBKE at Tyrosine 153 and Tyrosine 179 residues that are important for activation of IKBKE kinase. We also found that the IKBKE/TBK1 inhibitor Amlexanox exhibits increased efficacy in inhibiting cell viability in NSCLC cells with activating EGFR mutations. Furthermore, we also found that IKBKE inhibitors activate the MAPK pathway, and EGFR-TKI resistant NSCLCs exhibit enhanced response to co-treatment with IKBKE inhibitors and MEK inhibitors.

Similar to lung cancer, pancreatic cancer is a challenging disease due to lack of direct inhibitors of the KRas mutations that are observed in more than 95% of pancreatic cancer patients. IKBKE/TBK1 pathway is important for KRas signaling, but the efficacy of IKBKE inhibitors in pancreatic cancers is not well studied. Here, we show that IKBKE is an important target in pancreatic cancers that regulates pancreatic cell viability, cell migration and cancer stem cells. Importantly, we provide mechanistic insights into the effects of IKBKE inhibitors on specific signaling pathways. We found that IKBKE inhibition results in significantly increased expression of RTKs, such as ErbB3 and IGF1-R, which increases ERK1/2 activation. Our findings provide support for novel combination strategies for pancreatic cancer.

Metastasis is a poor prognostic factor for ovarian cancer. Although patients with early stage ovarian cancer with no distal metastasis exhibit a 70% 5-year survival rate, Stage IV patients with distal metastasis exhibit only 20% 5-year survival rate. Hence, ongoing efforts are aimed at targeting the pathways that regulate metastasis in ovarian cancers. IKBKE is upregulated in ovarian cancer patients, and IKBKE expression is known to regulate the expression of several genes important for cell motility in ovarian cancers. IKBKE is also implicated in chemo-resistance in ovarian cancer, and siRNA knockdown of IKBKE increases sensitivity towards chemotherapy. However, the mechanistic role of IKBKE in chemo-resistance in ovarian cancer is not known. EphA2 is another well studied oncogene in ovarian cancer as 70% of ovarian cancer patients exhibit elevated levels of EphA2. By activating Focal Adhesion Kinases (FAK), EphA2 can induce metastasis in ovarian cancers. In this study, we show that the clinical PARP inhibitor Olaparib (AZD2281) activates IKBKE by EphA2-mediated tyrosine phosphorylation. We also found that phosphorylation of EphA2 or IKBKE expression can be used as a biomarker for olaparib resistance.

Together, these studies have shed light on novel mechanisms of regulation of IKBKE and their importance in therapy resistance. These observations form a strong pre-clinical proof-of-concept to study the inhibitors further in the clinic.

CHAPTER 1
Background

Lung cancer

Lung cancer classification and its mutational landscape. Lung cancer is the 3rd leading cause of cancer-related death in both women and men. Approximately 220,000 people are estimated to die of lung cancer in 2016 in USA alone [1]. Lung cancers are divided into the following predominant subgroups: non-small cell lung cancer and small cell cancer which represent approximately 80% and 20% lung cancers, respectively [2]. The non-small cell lung cancer is further subdivided into lung adenocarcinoma (originating from the cells that produce mucous), squamous cell carcinoma (originating from lung squamous cells) and large-cell carcinoma (originating for several large cell types). The major risk factor for lung cancer is smoking [3]. Tobacco smoke leads to cytosine-adenine alteration, and patients who are smokers exhibit high levels of these mutations. Accordingly, lung cancer is one of the highly mutated cancer types [4]. As listed in Table 1, each histological subtype of lung cancers is marked by differential mutational status. For example, MAPK and PI3K pathways are frequently dysregulated in lung adenocarcinoma and squamous cell carcinomas, whereas NOTCH signaling and neuro endocrine differentiation genes are frequently deregulated in small cell carcinomas [5]. Such high levels of mutated pathways provide vast opportunities for selectively targeting cancer cells; it also imposes challenges due to high intra-tumoral heterogeneity. The unique treatment response exhibited by each subtype of lung cancer warrant a better understanding of these classifications. For example, EGFR mutations are observed exclusively in lung adenocarcinomas, and these patients respond well to EGFR-TKIs [6, 7]. Similarly, a high

1

response rate to ALK inhibitors is observed for lung adenocarcinoma patients as they exhibit high frequency of ALK rearrangements [8]. Hence, therapeutic regimens for lung cancers depend on the cell of origin and mutational landscape.

Table 1: Genetic alterations in various subtypes of lung cancer. Most frequent genetic alterations identified in different subtypes of lung cancer are shown.

Adenocarcinoma	Squamous cell carcinoma	Small cell carcinoma
TP53 (46%), KRAS (39%), EGFR (21%), NF1 (11%), PIK3CA (7%), BRAF (13%), MET (12%), ALK (8%), TERT (18%), TTF1 (14%), STK11 (17%), CDKN2A (24%),	TP53 (91%), TP63 (29%), PIK3CA (54%), HES1 (26%), CDKN2A (44%), PTEN (11%), SOX2 (43%), KEAP1 (12%), NFE2L2 (15%), RB1 (7%)	TP53 (92%), RB1 (88%), NOTCH1 (13%), EP300 (11%), CREBBP (10%), PTEN (9%), SOX2 (27%)

Therapeutic approaches for lung cancer. Chemotherapy and radiation therapy are primarily used as a frontline therapy for lung cancer patients depending on the stage and type of cancer. Stage IA, IB and IIA small cell cancer patients receive post-surgery chemotherapy [9] whereas Stage IIB and III patients exhibit good responses to combinations of etoposide/cisplatin with radiation therapy [10]. For late stage (Stage IV) patients, taxanes and Topoisomerase-I inhibitors are used as a second line therapy [11, 12]. Although targeted therapies such as Bcl2 inhibitors and HDAC inhibitors have been administered in combination with chemotherapy, they showed little benefit [9].

First line treatment for Stage I/II NSCLC patients includes surgery and adjuvant chemo- or radiotherapy depending on the size of the tumor [9]. Stage III patients receive a combination of surgery-chemotherapy and radiation to completely remove the tumor [13]. For stage IV patients, treatment is challenging as the tumor has spread to even distant sites. So a combination of chemotherapy, radiation and targeted therapies are included based on the mutational profile of

the patient's tumor. Investigational strategies such as combination of targeted therapies and immunotherapies have been evaluated with limited success in these patients [9]. For patients that do not respond well to these treatments, the treatment strategy includes combinations of anti-angiogenic agents such as Bevacuzimab with chemotherapy [14]. Given the observation that patients with mutations in EGFR are sensitive to EGFR inhibitors [7], the identification of driver pathways can assist in the development of novel treatment strategies. With an exception of KRas, we have been successful in directly targeting the driver mutation-activated proteins such as EGFR, ALK and c-Met in lung cancer [15].

KRas signaling in cancer. KRas is a 21KDa GTPase. KRas is activated upon binding to GTP, and conversion of GTP-bound KRas to GDP-bound KRas leads to its inactivation. KRas is present at the plasma membrane, and cues from upstream signals such as RTK activation leads to the exchange of GDP to GTP, thus activating the protein. This reaction is accelerated by a set of Guanine exchange factors (GEFs). In addition, the activation of KRas pathway is also regulated by GTPase-activating proteins (GAPs) such as RASA1 and NF1 that accelerate the conversion of GTP-bound Ras to GDP-bound Ras, thus shutting off the KRas activity. GTP-bound activated KRas signals downstream by activation of Raf-MEK-ERK pathway [16]. KRas signaling is frequently deregulated in cancer by the acquisition of mutations in KRas that lead to constitutive binding to GTP [17]. Since the K_d for this interaction is very high, targeting mutant KRas directly has been challenging [18]. In addition to mutations in KRas, this pathway can be deregulated by the aberrant activation of Ras-GEFs. Although mutations in GEFs are less frequent in human cancers, Ras-GEFs such as SOS are activated by the upstream RTKs that leads to their increased interaction with Grb2 with RasGEFs such as SOS proteins [19]. Whereas

gain-of-function mutations in GEFs are less frequent, loss-of-function mutations in RAS-GAPs are frequently observed in cancers and these mutations have implications in resistance to targeted therapies [20]. For example, RASA1 is frequently lost in metastatic melanomas [21] and NF1 is frequently lost in Desmoplastic melanoma (45%) and such NF1 loss is observed less commonly in other cancers (approximately 15%) [22, 23] and loss of NF1 was implicated in resistance to EGFR and BRaf inhibitors [24, 25].

In addition to frequent alteration in the pathway at Ras itself, the proteins downstream of KRas are also frequently deregulated in cancers. The most well studied downstream effector of KRas signaling is the MAP kinase cascade that is triggered upon activation of the serine threonine kinase Raf after interaction with KRas at the plasma membrane. This leads to downstream activation of MEK (MAP/ERK Kinase) and eventual activation of ERK that leads to the transcriptional activation of the Jun/AP-1 transcription factors [26]. BRaf mutations are frequent in melanoma, lung and colorectal cancers [27]. MEK mutations have also been identified and these mutations have been shown to be implicated in resistance to BRaf inhibitor resistance [28]. Even though targeting mutant KRas has not been successful in humans, the well-defined downstream signaling network of KRas provides a wealth of opportunities to inhibit this pathway. Hence several combination therapies that target various arms of KRas downstream signaling are being clinically evaluated [18].

EGFR signaling. Another important signaling pathway that is frequently altered in lung cancer is the receptor tyrosine kinase (RTK), Epidermal Growth Factor Receptor (EGFR). EGFR belongs to the family of erythroblastosis oncogene B (ErbB) receptor tyrosine kinase. The members of this family of proteins are membrane tethered proteins that are activated by

extracellular stimuli such as growth factor ligands. Although the ligands for ErbB2 are not identified, the other members of this protein family share most of the ligands [29], as shown in Figure 1. Upon ligand binding, these receptors form homodimers or heterodimers with other members of the family that lead to interaction of the intracellular kinase domains and activation by phosphorylation at tyrosine residues [30].

The phosphorylated intracellular domains of the RTKs act as docking sites for interaction with adaptor proteins such as Shc-Grb2 that leads to activation of MAPK pathway as described in previous section [31]. Similarly, EGFR activation also leads to docking of PI3K at the plasma membrane by Gab1 and subsequent activation of PI3Kinase [32]. Activated PI3Kinase is required for the production of Phosphatidylinositol (3,4,5)-trisphosphate (PIP3) from Phosphatidylinositol (4,5)-bisphosphate. PIP3 bound AKT/PKB is tethered to the plasma membrane through its PH domain, thus bringing it into close proximity with PDK1, which phosphorylates AKT on threonine 308 [33]. In addition, EGFR also induces the JAK-STAT signaling by activating JAK1 and JAK2 [34]. Together, MAPK, PI3K/AKT and JAK-STAT pathways are well known downstream effectors of EGFR signaling. In this study we identified IKBKE as a downstream effector of EGFR, highlighting another branch of signaling with therapeutic opportunities.
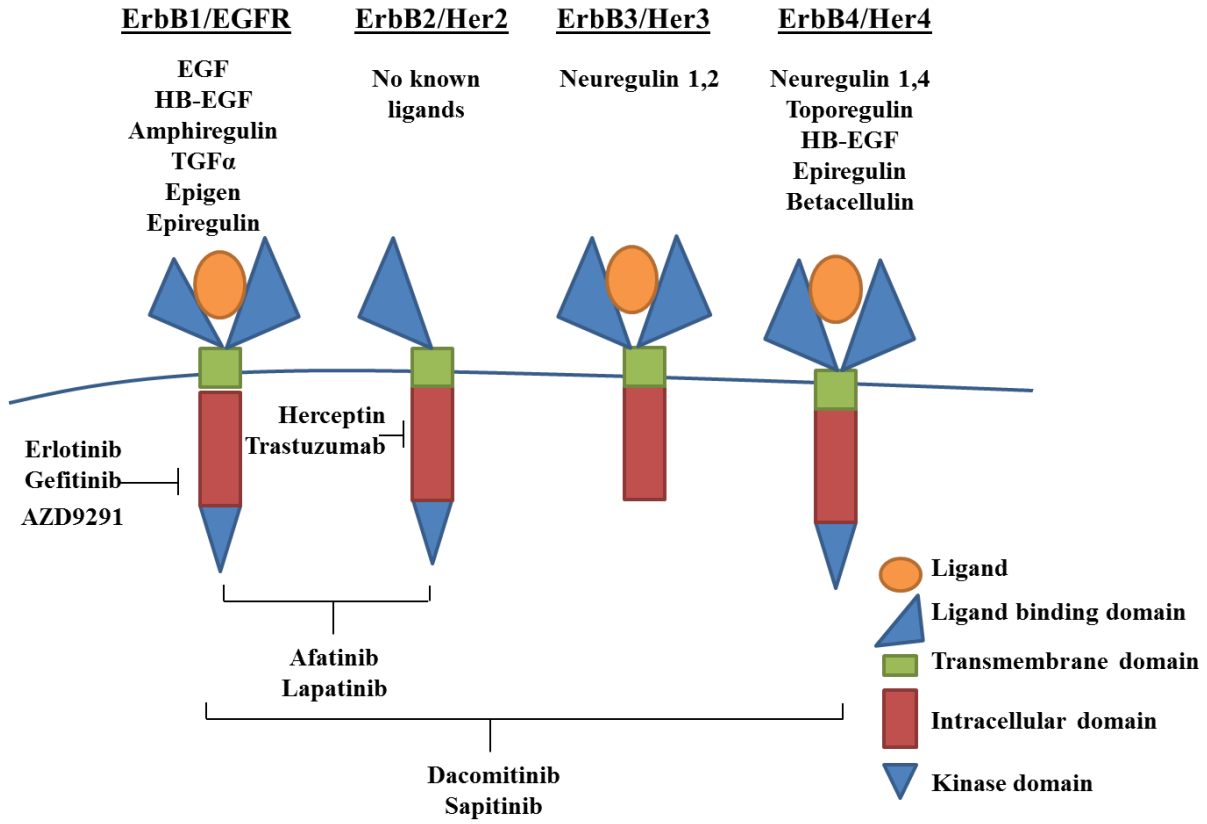
Figure 1: ErbB receptors: Ligands and inhibitors A schematic showing similarities and differences of ErbB receptor family of kinases. Binding of the ligand results in formation of homo/hetero-dimers of the RTKs leading to increseased autoactivation. Ligands for ErbB2/Her2 are not yet identified, but the ligands for each of the other ErbB receptor is shown on the top and the inhibitors developed to suppress these pathways are shown in the bottom.

Therapeutic challenges of Non-small cell lung cancer. KRas mutant lung cancers are a challenge as KRas itself is undruggable. However recent studies have identified small molecule or peptide based inhibitors of mutant KRas [35]. Unlike targeting KRas, we have been successful in targeting EGFR since antibody-based or small molecule inhibitors (tyrosine kinase inhibitors, TKIs) have been identified [36]. Although initial clinical trials did not show benefit from EGFR inhibitors, these studies helped to identify mutations in EGFR that sensitize patients to the EGFR inhibitors such as erlotinib and gefitinib [6, 7, 37]. Frequently identified EGFR mutations in patients include L858R, Deletions in exon 19 have been shown to induce conformational changes in the EGFR protein that enhance binding to the inhibitors (Figure 2) [7]. Although

patients showed more favorable initial response to EGFR TKIs, such as erlotinib and gefitinib, most of them exhibit resistance to these therapies [38]. The mechanisms involved in resistance to first line EGFR TKI therapy include loss of PTEN [39] acquired mutations in KRAS [40]. However, the most predominant mechanism of resistance to EGFR TKI is the acquisition of a second "gatekeeper" mutation in T790M in EGFR [41]. Mutation of the threonine residue at 790 to methionine inhibits the interaction of EGFR with the EGFR TKIs erlotinib and gefitinib, thereby promoting resistance.

To overcome this resistance, second generation TKIs such as afatinib were developed and FDA approved for use in lung cancer patients [42-46]. Afatinib shows activity against both EGFR containing mutations that are sensitive to first generation EGFR-TKIs and EGFR with the gatekeeper mutation [47]. Although this is a promising strategy to treat lung cancer patients with acquired EGFR TKI resistance, response to afatinib is limited due to increased toxicity due to inhibition of wild type EGFR in normal cells at effective dosage [48]. Third generation EGFR-TKIs such as AZD9291 showed better efficacy in lung cancer patients with EGFR mutation compared to Afatinib. AZD9291 is an irreversible inhibitor of EGFR that is selective for EGFR with mutations that sensitize to earlier TKIs and also the gatekeeper mutations. Interestingly, AZD9291 showed little to no activity against wild-type EGFR [49]. Furthermore, recent studies identify a tertiary mutation in EGFR that makes the cells resistant to even the second generation TKIs. Acquisition of a mutation in C797S in EGFR results in resistance to second generation TKIs [50, 51]. Mechanistically, since C797 residue in EGFR is essential for EGFR to bind to afatinib, mutation in this residue imparts resistance due to inability to bind to both afatinib and AZD9291 [51].
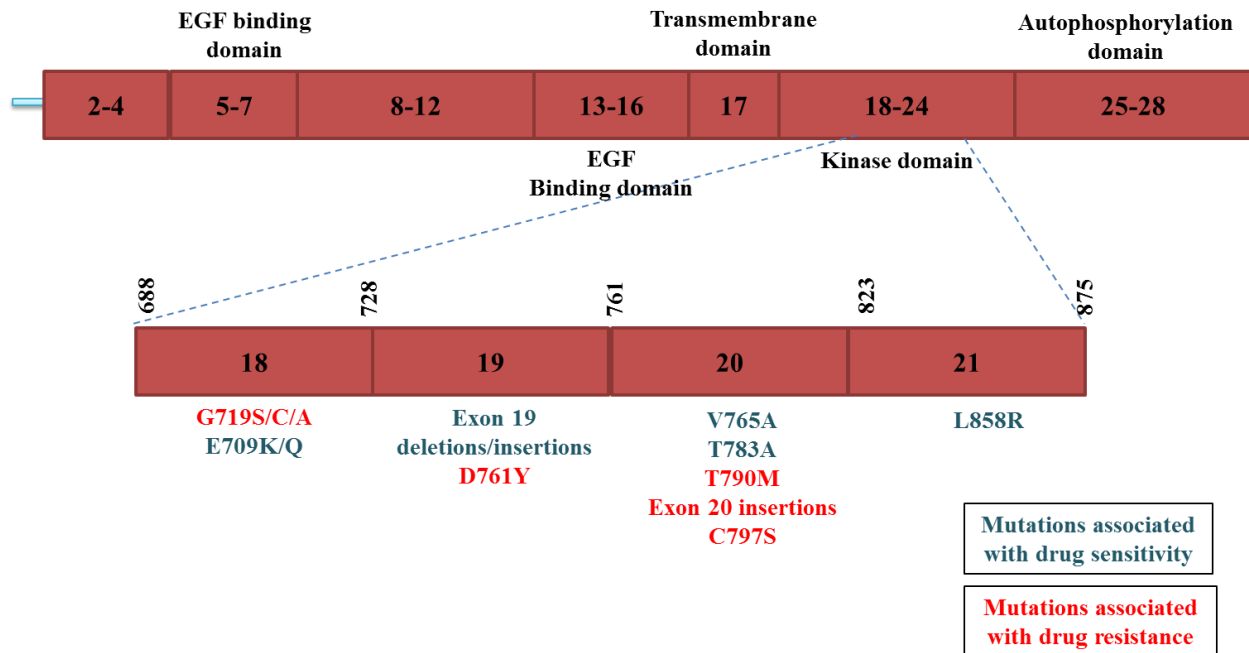
Figure 2: Gain of function mutations in EGFR. Deletions in exons coding the extracellular domain of EGFR (exons 2-15) are frequently observed in glioblastoma. Mutations in the kinase domain of EGFR (exons 18-24) are frequent in lung cancers. While several of these mutations make cells sensitive to EGFR TKIs (marked in blue), some mutations are implicated in EGFR-TKI resistance (marked in red).

Most of the RTK signaling pathways share similar downstream targets, hence there is a large proportion of overlap in signaling outcomes by various RTKs [52]. These alternative mechanisms help the cells to overcome phenotypic effect of EGFR-TKIs by atypical activation of other RTKs such as Met amplification, AXL activation or activation of other ErbB receptors [53-55]. Moreover, pre-existing clones with amplification in RTKs such as Met are shown to drive resistance to EGFR-TKIs [56]. Hence inhibitors that target multiple RTKs such as Lapatinib are now used for lung cancer.

Role of PI3K and MAPK pathways in EGFR-TKI resistance. Another well researched mechanism(s) of resistance to EGFR-TKIs is alternative mechanisms of activation of the downstream pathways such as MAPK, AKT and STAT signaling [52]. RTK activation and

downstream signaling homeostasis is achieved by several negative signals that counteract the positive signaling cues. For example, BRAF-V600E was shown to inhibit AKT activation by interaction with mTORC2 complex [57]. Similarly, under nutrient deprived conditions, inhibition of S6K activity was shown to relieve the suppression of PI3K activation by IRS-1 and activation of AKT [58]. Hence inhibition of the upstream RTKs relieves these feedback inhibitory pathways, leading to uncontrolled activation of the MAPK or PI3K pathways.

In addition, mutations in the MAPK and PI3K pathways are correlated with acquired resistance to EGFR inhibitors. While pre-existing KRAS mutation is a predictor of resistance to EGFR TKIs in lung cancers and Cetuximab in colorectal cancers [59, 60], acquired mutations in KRAS are shown to make colorectal cancers resistant to cetuximab [61]. Acquired BRAF and MEK1 mutations that lead to an EGFR-independent activation of MAPK signaling are associated with resistance to EGFR inhibitors [62-64]. Similarly, PI3KCA and PTEN mutations leading to constitutive AKT activation are also associated with acquired resistance to EGFR inhibitors [39, 65]. In addition, re-activation of these pathways results in phenotypic change in the tumors making them resistant to EGFR inhibitors. Epithelial-Mesenchymal transition was shown to play a role in acquired resistance to EGFR inhibitors and this could be inhibited by the inhibition of ERK1/2 or other RTKs such as AXL [62, 66]. Other phenotypic changes such as conversion of EGFR mutant NSCLC cells into SCLC cells are an interesting resistance mechanism to EGFR TKIs. These transformed cells were shown to have acquired Rb loss and neuroendocrine markers [67-69] and the role of MAPK or PI3K pathways in this cell transformation are yet to be studied.

In summary, these studies show the adaptability of cancers to targeted therapies and pathways that emerge due to such genetic and phenotypic changes will be good targets for overcoming resistance to targeted therapies.

Pancreatic cancer

Pathogenesis of Pancreatic cancer. Pancreatic cancer pathogenesis is a multi-step process involving accumulation of mutations. It includes, but not limited to the activation of oncogenic pathways such as KRas mutation and inactivation of tumor suppressors such as p53 or SMAD4 and accumulation of mutations in the genes involved in maintenance of genomic integrity [70-73]. One mode of transformation of pancreatic cells involve the progression of chronic pancreatitis to pancreatic ductal adenocarcinoma, and it was shown that patients with chronic pancreatitis showed increased risk for pancreatic cancer [74]. This progression could be more common in patients with familial conditions such as mutations in BRCA2 [75].

The different stages of pancreatic cancer transformation include progression of low grade PanINs (Pancreatic Intraepithelial Neoplasms) to high grade PDAC (Pancreatic Ductal Adenocarcinomas). This cellular transformation requires acquisition of mutations at various stages of disease [71]. For example, activation of ErbB receptors were observed at a lower frequency in the early PanIN stage (PanIN1a and PanIN1b) but this gradually becomes more frequent as the disease progresses [76]. Similarly, KRAS was found to be mutated in most of the pancreatic cancer patients, thus emphasizing the importance of targeting this pathway in pancreatic cancers [77]. Loss of tumor suppressive mechanisms is also important for progression of PanINs to PDACs. Firstly, CDKN2A loss due to mutation, promoter methylation or genetic loss of this region is observed in about 85% sporadic PDAC patients [77, 78]. Genetic loss of CDKN2A locus also leads to a loss of INK4A/ARF tumor suppressors that alter Rb and p53 signaling (Reviewed in [79]). Furthermore, loss of p53 is observed in later stages of pancreatic cancer pathogenesis (reviewed in [80]). But recent animal models showed that loss of p53 is observed at early stages of murine pancreatic cancer development [81]. In addition, loss of

SMAD4 is also observed at later stages of pancreatic cancer, further emphasizing the complex genetic events required for progression to an advanced PDAC [72].

Therapeutic opportunities and challenges of pancreatic cancer. Pancreatic cancer treatment has been challenging with only 8% of 5 year survival rate [1]. Early stage diagnosis of pancreatic cancer has been a challenge as patients remain asymptomatic until advanced stage PDAC is developed. Hence several efforts are being made to improve diagnostic modalities and biomarkers for early stages of pancreatic cancer [82, 83]. Adjuvant chemotherapy after surgery has shown improved survival in patients with resectable cancers [84, 85]. Chemo-radiation after surgery was evaluated in these patients with little benefit owing to the radiation induced c-MET activation resulting in more aggressive cancer [84, 86, 87]. For non-resectable patients, a regimen including an induction chemotherapy with a combination of gemcitabine and capecitabine, followed by capecitabine-radiation treatment showed better responses [88]. In the metastatic setting, although combined treatment with gemcitabine and EGFR TKI, Erlotinib was approved, only modest improvement in survival rate was observed [89]. Improved survival was observed with combined treatment with nab-paclitaxel and gemcitabine or with FOLFRINOX (a combinationation of folinic acid, 5-fluorouracil, irinotecan and oxaliplatin) [90, 91]. Although these studies showed improved survival in patients, the increase in survival is extremely modest (approximately 2months). Hence it is important to identify better treatment strategies for pancreatic cancer.
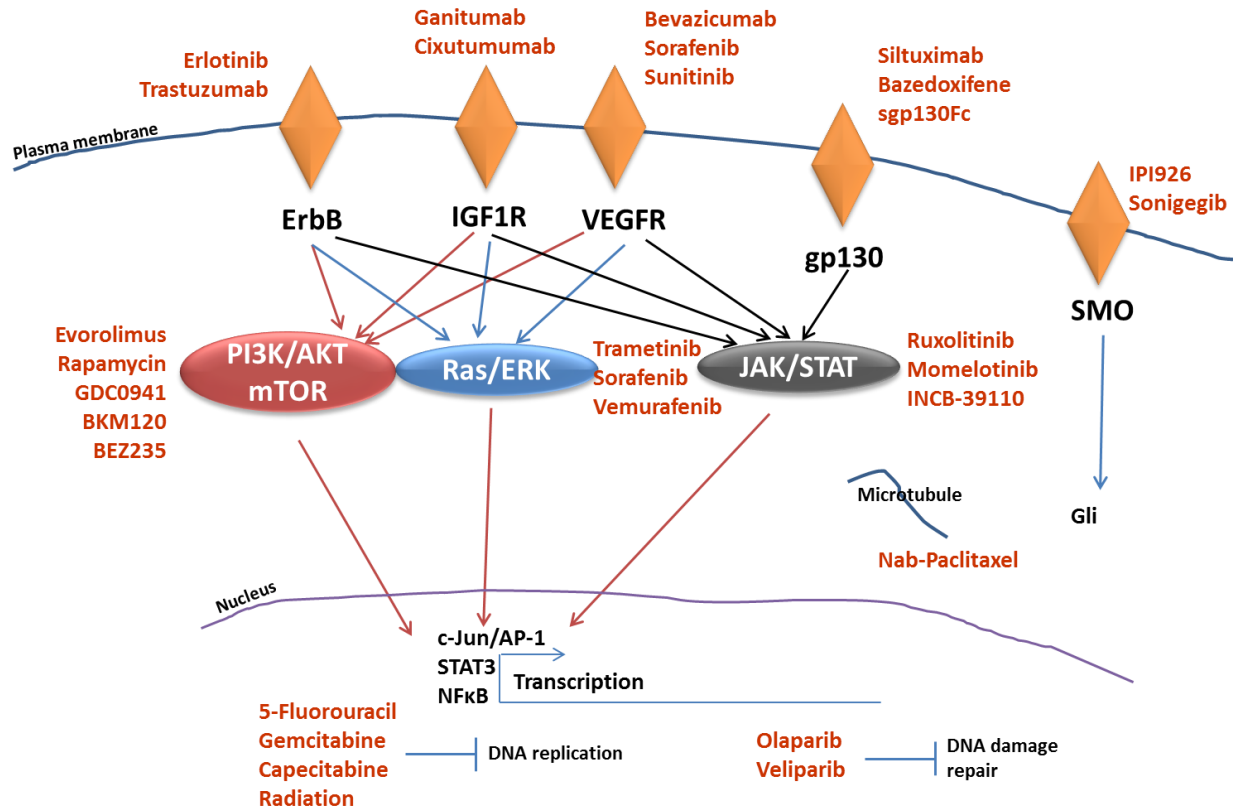
Figure 3: Pathways frequently altered in pancreatic cancers and opportunities for targeted therapies. The important signaling pathways for pancreatic cancer including RTKs and transcription factors are shown. Inhibitors to these pathways are highlighted in red.

One reason for such challenges in pancreatic cancer treatment can be attributed to the desmoplastic nature of PDACs [92]. Due to presence of large amounts of fibrous tissue, delivery of chemotherapeutic agents to the tumor site is extremely challenging and hence agents targeting tumor stroma such as Hedgehog inhibitors in combination with gemcitabine and nab-paclitaxel are clinically evaluated [93]. In addition to chemotherapeutic agents discussed above, several efforts aimed at evaluating the efficacy of targeted therapies in pancreatic cancer. Since the driver mutations in pancreatic cancer were identified in KRAS and targeting this pathway is a challenge, inhibitors of downstream signaling pathways were studied. Of note, clinical trials to evaluate the use of MEK inhibitors in combination with gemcitabine did not show significant

increase in progression free survival in chemotherapy-naïve PDAC patients [94]. As shown in figure 3, although there are several opportunities for targeted therapies in pancreatic cancer, none of these treatment strategies showed an impressive response in patients. This can be in part attributed to the resistance mechanisms that develop as discussed in the previous section and stromal response of tumor microenvironment on cancer cells [95, 96]. Another mechanism could be enrichment of cancer stem-like cells. Cancer stem-like cells are shown to have tumor initiating potential and are intrinsically resistant to chemotherapy [97]. Together these studies emphasize the importance of understanding the resistance mechanisms to targeted therapies and identify targetable pathways in cancer stem-like cells.

Ovarian cancer

  Signaling pathways and therapeutic opportunities for ovarian cancer. Ovarian cancer is the fifth leading cause of cancer related deaths in women. In 2016, it is predicted to have 22,280 new cases of ovarian cancer and around 15000 cases of cancer related deaths [1]. The major challenges of treating ovarian cancers are the lack of early diagnostics for these patients. Five year survival rate of the patients differ based on the stage of diagnosis; while stage 1 patients with cancer localized to ovaries show 90% survival, stage 2 patients with localized metastasis show 55-75% survival and Stage 3 and higher patients presented with distant metastasis have only 20-30% 5-year survival [1].

  Loss of tumor suppressors is very frequent in ovarian cancers. Loss of BRCA1 and BRCA2 is observed in 10-15% ovarian cancers [98-100]. Loss of BRCA genes result in impaired DNA damage response and hence patients with BRCA mutations showed better response to platin and taxol based therapies [101]. Genetic alteration in p53 is frequently observed (60-80%)

in ovarian cancer patients and unlike BRCA mutations, correlation to treatment responsiveness is not clear [102-104]. In addition to genetic loss of tumor suppressors, ovarian cancer cells have high levels of promoter methylation and other epigenetic loss of tumor suppressor function. For example, loss of ARHI and PEG3 in ovarian cancers occurs due to promoter methylation [105]. ARHI loss leads to inhibition of autophagic cell death, escape from dormancy and *in vivo* tumor growth [106]. Expression of PEG3 inhibits β-catenin protein levels and was shown to modulate p53 dependent apoptosis [107, 108].

Although alteration in the erbb family of RTKs is very rare, pathways downstream of RTK signaling are frequently mutated in ovarian cancers [104]. PI3K-AKT signaling is activated in over 70% ovarian cancers due to amplification of PI3KCA and AKT2 and these amplifications correlate with resistance to chemotherapy [109-111]. KRAS and BRAF mutations are frequently observed in borderline/low grade tumors showing its role in transformation of cells to carcinoma [112-114]. Another important downstream signaling of RTKs, the JAK-STAT pathway is also frequently upregulated due to increased production of IL6 and higher levels of IL6 in serum correlate with poor disease free survival [115-117]. Although inhibitors to the pathways discussed are available, curing ovarian cancer has been a challenge due to rapid development of resistance as described previously. Hence understanding the mechanisms of adaptive resistance might help in evaluating combinatorial treatments. Furthermore, since frequent epigenetic modulation is observed in ovarian cancers, inhibitors of epigenetic pathways such as HDAC and EZH2 may show some efficacy [118, 119]. In addition, combination treatment strategies that sensitize cells to chemotherapy are very important for ovarian cancers as these cells have high basal DNA damage [120, 121].

EphA2 signaling in ovarian cancer. EphA2 belongs to the Eph receptor family of RTKs, one of the largest classes of RTKs known till now. EphA2 can bind to the GPI-anchored or soluble EphrinA ligands [122]. This interaction initiates a bi-directional signaling that can be either tumor suppressive or oncogenic based on cell context: 1) The cells expressing Ephrin A ligands can inhibit EGFR signaling [123] or activate integrin-dependent adhesion by the activation of FYN and Integrin β1 signaling [124, 125] (Figure 4, A and B). 2) The cells in which EphA2 is activated by Ephrin A1 show tumor suppressive role by the activation of TSC2, inhibition of AKT and FAK pathways [126, 127] (Figure 4, A and B). Conversely, in the absence of Ephrin A1, activation of EphA2 leads to activation of RhoA and disruption of tight junctions, activation of FAK and AKT pathways that are important regulators of cell survival and cell motility [128]. Unlike the RTKs such as EGFR, ligand induced EphA2 tyrosine phosphorylation inhibits the downstream signaling pathways [128]. In the absence of ligand, EphA2 oncogenic activity is regulated by growth factors such as HGF and TNFα by increasing the EphA2 serine 897 phosphorylation [129, 130]. Moreover, EphA2 was shown to crosstalk with other RTKs such as ErbB2 to activate the downstream RhOA and ERK signaling [131] (Figure 4 C).

Oncogenic role of EphA2 can be further established due to its frequent amplification in cancers like glioma, breast cancer, prostate cancer and ovarian cancers [132-135]. More than 75% of ovarian cancer patients have amplified EphA2 expression and it is correlated with poor survival [132, 136]. EphA2 exerts it's oncogenic role in ovarian cancers by promoting vascular mimicry and angiogenesis and EphA2 knockdown using liposomal siRNAs showed 50% decrease in vascular density in an orthotropic ovarian cancer model [137-139]. Interestingly the siRNA mediated EphA2 knockdown sensitized chemo-resistant ovarian cancer cells to docetaxel in reducing tumor growth by synergistically reducing the micro-vesicle formation and increasing

apoptosis in *in vivo* murine models [140]. These studies together prove that EphA2 is an important therapeutic target in ovarian cancers and hence liposomal siRNA targeting EphA2 is in Phase I trial for advanced and recurrent cancers (NCT01591356). Although the use of siRNA mediated knockdown has shown promise as a therapeutic agent, complete knockdown of EphA2 is not achieved and hence there are efforts to increase the EphA2 knockdown levels by co-treatment with EphA2 siRNAs and miR-520d-3p that inhibits expression of both EphA2 and EphB2 [141].
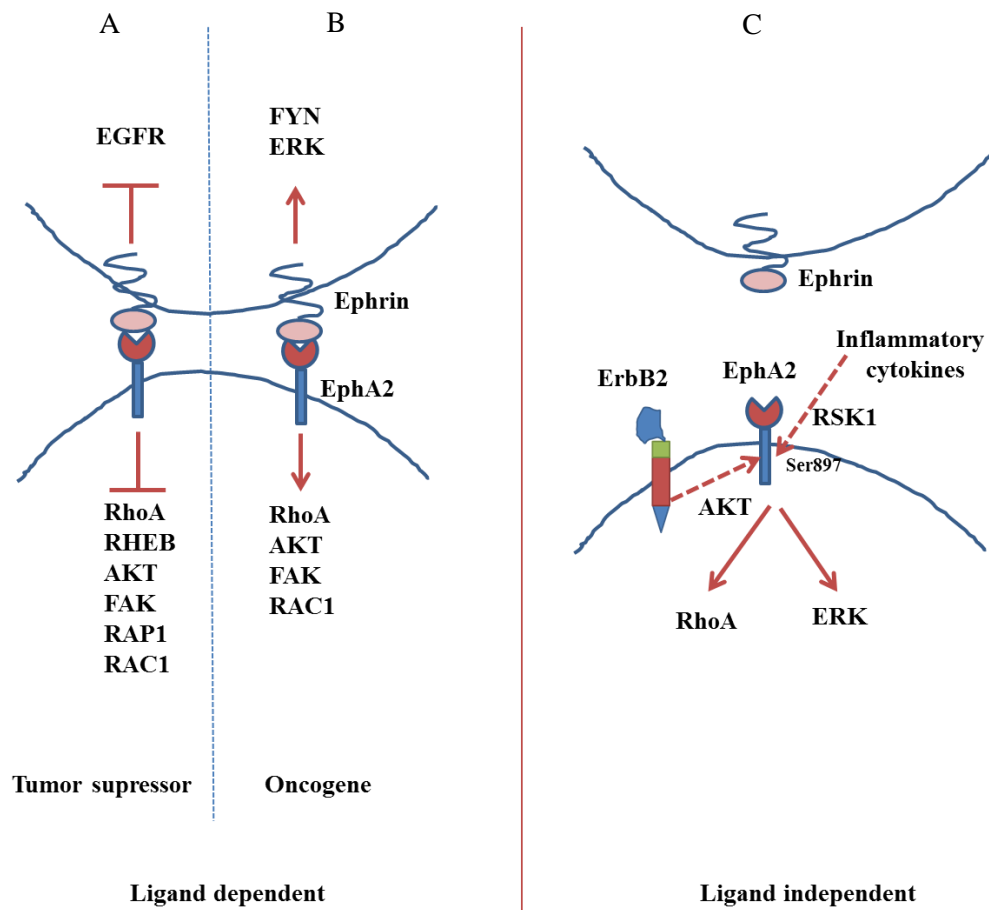
Figure 4: Schematic summarizing EphA2 signaling. Ligand dependent signaling can be either tumor-suppressive or oncogenic based on cell context (A and B respectively). Loss of EphrinA ligands or activation of alternate pathways such as ErbB2, inflammatory cytokines lead to ligand independent, oncogenic EphA2 signaling (C).

Success and limitations of PARP inhibitors for ovarian cancer. Poly(ADP-ribose) polymerase is a family of 17 members that contain ADP-ribosyltransferase (ART) domain. This family of enzymes is known to catalyze the addition of either a mono or poly ADP-ribosylation on target proteins by using NAD^+. The poly ADP-ribosylation is catalyzed by PARP1, PARP2, PARP5a and PARP5b while the remaining family members are known to exclusively catalyze a mono ADP-ribosylation. PARylation is thought to be an important posttranslational modification for DNA damage response [142]. PARP1 is required for both single strand and double strand break repairs and also for replication fork repairs [143, 144] . PARP1 is recruited to the DNA damage site and it mediates PARylation of several key regulators of DNA damage such as DNA topoisomerase II, histones and PARP itself [145]. After recruitment of the repair machinery to the damage site, PARP is dissociated from DNA. Another class of poly ADP-ribose glycohydrolases (PARG) removes the PAR chains from targets and PARP itself to recycle the PARP enzyme [144]. Initial studies using 3-aminobenzamide (an NAD^+ analog that can bind to PARP) showed that treating cells with this inhibitor led to trapping of PARP in the DNA bound state and inhibited PARylation [144]. The cells treated with 3-aminobenzamide showed strong sensitivity to chemotherapeutic agents and ionizing radiation that are known to cause single strand and double strand breaks respectively [144, 146].

The clinical use of PARP inhibitors became important with the observation that patients with either BRCA1 or BRCA2 mutations are sensitive to PARP inhibition, owing to the defects in homologous recombination mediated DNA damage repair [147, 148]. Since then several clinical trials were undertaken to evaluate the efficacy of combining PARP inhibitors with either chemotherapy or radiation (NCT01264432, NCT02470585, NCT01033123, NCT01033292, NCT01445418). Combined treatment with VEGF inhibitor bevacuzimab and chemotherapy was

FDA approved for treating platin-resistant, recurrent ovarian cancers and efficacy of addition of PARP inhibitors to this combination regimen is currently evaluated (NCT00989651, NCT01459380). Since it was previously shown that mutations in PTEN lead to PARP inhibitor sensitivity, combined inhibition of PI3K-AKT and PARP inhibitors is currently studied in high grade serous ovarian cancers (NCT01623349) [149].

The good clinical outcome of PARP inhibitors in the past decade has garnered interest in understanding the underlying mechanisms of action of these inhibitors and pathways that might lead to resistance to these inhibitors. The well understood mechanism of resistance could be attributed to the ability of cancer cells to restore homologous recombination by acquisition of secondary mutations to restore BRCA function or loss of TP53BP1 [150, 151]. Even though clinical studies showed additional biomarkers that can be used to evaluate patient response to PARP inhibition [152], we lack comprehensive understanding of functions of PARP inhibitors and mechanisms that can shed light on developing better combinations to increase their efficacy. Part of this study focused on identifying receptor tyrosine kinases that may play a role in PARP inhibitor resistance.

Essential role of IKBKE signaling in cancer and immunity

IKBKE (Inhibitor Of Kappa Light Polypeptide Gene Enhancer In B-Cells, Kinase Epsilon) is a non-canonical IKK (IκB kinase) that shows only 33% homology to the canonical IKKs, IKKα and IKKβ but has 65% sequence homology to the other non-canonical IKK, TBK1. Together with TBK1, the non-canonical IKKs are frequently shown to be important for innate immunity and cell transformation [153]. Although TBK1 expression is not yet shown to be dysregulated, IKBKE was shown to be over expressed in several cancers [154-158]. Hence,

although IKBKE and TBK1 share most of the substrates, few signaling pathways were shown to be unique to these kinases [153]. In this section we review various downstream signaling pathways of IKBKE that are important for both tumor immunity and cancer cell transformation.

IKBKE signaling in inflammation. Chronic inflammation is a well-known risk factor for cancer. Patients with inflammatory disorders such as pancreatitis and Crohn's disease were shown to have increased risk for cancer [74, 159]. In addition, chronic inflammation can lead to re-modeling of the tumor microenvironment thus accelerating cancer progression. Hence it is important to understand the molecules regulating inflammatory pathways in cancer and to identify inhibitors of this pathway. Macrophages are key cells that get activated upon tissue damage. Tissue injury leads to recruitment of macrophage progenitor cells to the damage site and the cytokines and chemokines in the microenvironment promote their differentiation to mature macrophages. Mature macrophages produce a plethora of growth factors that re-wire the microenvironment to repair tissue damage [160]. Similarly, obesity is associated with low levels of chronic inflammation and it is shown that obesity is an important risk factor for cancer [161].

IKBKE was first identified as an activator of the NFκB pathway by inhibition of IκB kinase in response to IL1 and LPS in RAW264.7 macrophages [162] (Figure 5). It was previously shown that high fat diet increased the expression of IKBKE in adipose tissue macrophages as a result of increased inflammation. Moreover, knockout of IKBKE was shown to protect mice from high fat induced obesity [163]. Furthermore, IKBKE was also shown to be important for the chronic inflammation in adipose tissues by limiting inflammasome priming in macrophages [164]. Taken together, these studies show the importance of IKBKE in

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inflammation and suggest the utility of IKBKE inhibitors for obesity and cancer. Indeed, an IKBKE inhibitor, Amlexanox is in Phase 2 clinical trial (NCT01975935) for obesity and Type II diabetes [165] and a JAK/IKBKE inhibitor CYT387 is in clinical trials for lung cancer (NCT02258607) [166].
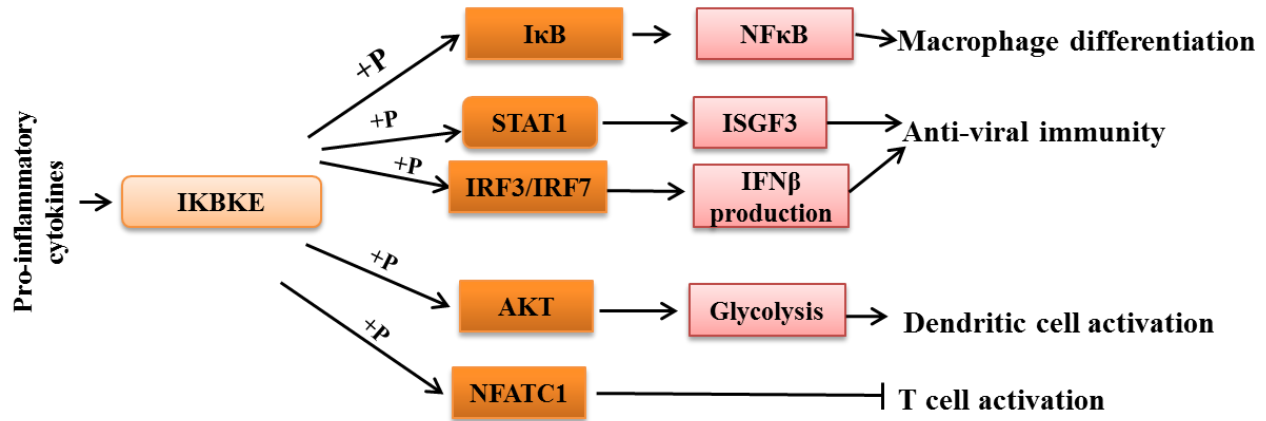
Figure 5: Multifaceted roles of IKBKE in regulation of immune cells. IKBKE regulates various immune-related processes by phosphorylating a plethora of substrates. As shown here, IKBKE regulated macrophage differentiation by activating NFκB, anti-viral immunity by regulating STAT and IRF pathways, dendritic cell activation by the modulation of glycolysis and T-cell immunity by suppressing the function of NFATc1.

IKBKE in NFκB pathway activation. NFκB is an important oncogene in cancers. This pathway modulates tumorigenesis by multifaceted regulation of the tumor microenvironment. NFκB can regulate tumor cell growth by regulation of STAT3 activity. In addition NFκB pathway can also assist in tumorigenesis by the expression of inflammatory cytokines that modulate the tumor microenvironment to induce tumorigenesis. Classically, NFκB activation is under tight regulation by several proteins such as IκB that binds to NFκB and sequesters it to cytoplasm, thus inhibiting its function. Inflammatory stimuli lead to phosphorylation mediated degradation of IκB and activation of NFκB pathway. The canonical IκB kinases IKKα and IKKβ are well studied oncogenes [167].

Since identification of IKBKE as an NFκB modulator, several key downstream effectors were identified that relay the effects of IKBKE activation. NFκB pathway itself is regulated by IKBKE in several different ways. While IKBKE can directly phosphorylate IκB preferentially at Serine 36 [162], IKBKE was also shown to phosphorylate CYLD, a deubiquitinase and a negative regulator of NFκB at serine 418 and inhibits its activity [168]. IKBKE was also shown to enhance NFκB activity by direct phosphorylation of p65-RelA subunit at Serine536 [169]. Although the canonical IKKs are able to phosphorylate p65 at Ser536, IKBKE was also shown to phosphorylate RelA at a specific site of Serine 468 in response to T-cell co-stimulation [170]. All of these downstream targets of IKBKE together regulate NFκB pathway and induce the production of inflammatory cytokines such as IL6.

IKBKE in anti-viral immunity. Pathogen recognition and clearance is an important function of the immune cells. Immune cells express receptors such as Toll-like Receptors (TLRs) that are activated by the polysaccharides in pathogens. Activated TLRs activate downstream transcription factors such as Interferon response factors (IRF) and NFκB pathways to induce the production of Type I interferons or inflammatory cytokines respectively. IKBKE is a key regulator of innate immunity by regulation of both IRF and NFκB pathways. IKBKE is essential for TLR mediated activation of IRF3 and IRF7, IKBKE directly phosphorylates IRF3 at Serine386 and induce nuclear localization and DNA binding of IRF3 that results in an increased production of IFNβ in response to sendai viral infection [171]. IKBKE-mediated activation of both the NFκB and IRF3 pathways lead to complete response to the dsRNA mediated viral immunity [172]. Although TBK1 was shown to be more potent activator of IFNβ in response to LPS and poly(I:C) than IKBKE, loss of TBK1 could be rescued by the expression of IKBKE

[173, 174]. In addition to the activation of IRF3 and NFκB pathways, IKBKE was shown to directly phosphorylate STAT1 inducing the formation of IFN-stimulated gene factor 3 (ISGF3) while suppressing formation of gamma activated factor (GAF) (Figure 5). Thus IKBKE plays an important role in balancing the levels of Type I and Type II interferon pathways [175].

IKBKE in tumor immunity. Cancers evade being scavenged by the immune system by activating several key inhibitory pathways that either inactivate the immune system or lead to T-cell or B-cell anergy. Recent efforts have focused on inhibiting these inhibitory molecules such as PD-L1, CTLA4 to activate immunity against tumor cells. The PD-L1 and CTLA4 inhibitors showed promising results in various cancers. In addition, inflammation is a major contributor to tumorigenesis. As we already know, inflammatory cytokines such as IL6 are pro-tumorigenic by the activation of JAK-STAT pathway, inflammation can also have pro-tumorigenic roles by modulation of innate immunity and macrophage activation (reviewed in [176]).

IKBKE is predominantly expressed in T cells and macrophages and was shown to be activated by the stimulation of TCR signaling and by LPS treatment respectively in these cells [162, 177]. Hence it is reasonable to believe that IKBKE might play a role in activation of these immune cells. Indeed, it was recently shown that by phosphorylation and inhibition of NFATc1, IKBKE is able to inhibit T cell activation (Figure 5). In addition, IKBKE is also involved in the maintenance of Th17 cells by altering the IL17 signaling by phosphorylation of Act1 and also by the phosphorylation and inactivation of GSK3α [178, 179]. Together, these studies suggest the importance of IKBKE in inflammation and immune exhaustion that might contribute to tumor progression. Hence IKBKE inhibitors will show anti-tumorigenic activity by suppression of inflammation.

IKBKE in cancer cells. In addition to extensive studies focusing on the role of IKBKE expression in immune cells, several studies have reported the oncogenic functions of IKBKE in tumor cells. Initially, IKBKE was identified as an important oncogene in breast cancers where it was shown to be amplified in approximately 30% of breast cancer patients. It was also shown that activated IKBKE in collaboration with activated MEK is sufficient to transform cells and overcome the loss of PI3K pathway activation [158]. Since then several groups identified IKBKE as an important protein for cell transformation in several cancer models [157, 180]. This tumorigenic potential of IKBKE can be attributed to the important downstream signaling pathways of IKBKE. As shown in Figure 6, a major downstream pathway of IKBKE is the NFκB signaling, where IKBKE was shown to also activate this pathway by direct phosphorylation of p65-RelA at Ser 536 [169]. Oncogenicity of IKBKE in breast cancers can also be attributed to the phosphorylation and inhibition of a key breast cancer tumor suppressor, CYLD that is a known NFκB pathway inhibitor [168]. IKBKE also phosphorylates TRAF2, leading to increased ubiquitination and activation of TRAF2 and it was shown that this pathway synergizes with CYLD suppression to increase breast cancer cell survival [180].

In addition to modulation of NFκB pathway, IKBKE was also shown to directly phosphorylate and activate the AKT pathway independent of PI3Kinase by several groups [157, 181]. By direct phosphorylation at threonine 308 and serine 473 residues in AKT, IKBKE was shown to activate AKT. AKT/PKB signaling is an important oncogenic pathway as activation of AKT leads to suppression of the tumor suppressive transcription factor, FOXO3a that leads to enhanced cell survival and evasion of apoptosis [182]. In addition, AKT signaling is critical for cell metabolism and translation by modulation of mTOR activation and pS6Kinase activity [183]. Another oncogenic function of IKBKE could be attributed to its ability to directly inhibit

FOXO3a function. Phosphorylation at serine 664 residue in FOXO3a by IKBKE was shown to inhibit the nuclear localization of FOXO3a that results in decreased protein stability [184].
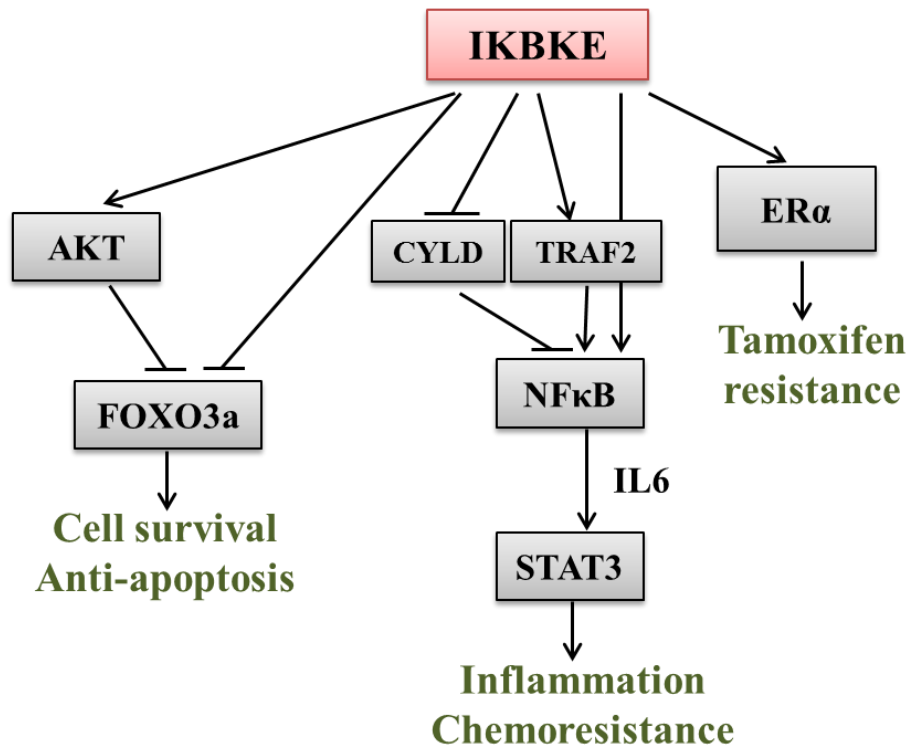
Figure 6: Downstream pathways of IKBKE that play a role in tumorigenesis. IKBKE shows oncogenic activity in cancer cells by phosphorylating important signaling targets such as AKT, ERα. IKBKE was shown to activate STAT3 by increased production of IL6 due to NFκB activation.

In addition to promoting cell survival, IKBKE pathway was implicated in resistance to chemotherapy and targeted therapies. IKBKE was shown to phosphorylate ERα at Ser 167 leading to an increased transcriptional activity of ERα and resistance to Tamoxifen [185]. Another study showed that similar phosphorylation of ERα36, a splice variant of ERα with no transcriptional activity, leads to stabilization of the ERα36 protein [186]. Moreover, overexpression of IKBKE correlates with poor patient survival and chemoresistance in ovarian cancers and another study identified IKBKE as a critical regulator of cell invasion and metastasis by modulation of expression of genes related to tumor metastasis [156, 187]. Although it is not

yet known about the mechanisms by which IKBKE over-expression leads to chemotherapeutic resistance, one study showed that upregulation of BCL2 expression could be a reason for IKBKE-induced chemoresistance in gliomas [155].

These data together showed that IKBKE plays an important role in tumor immunity and in cell autonomous regulation of cancer cell survival and metastasis. By regulating several important downstream signaling pathways, IKBKE can be considered as a gene at the nexus of cell transformation and hence targeting this pathway will have a multi-pronged effect on cancer growth.

Mechanisms of regulation of IKBKE expression and function. Previous studies on signaling regulated by IKBKE have demonstrated its important roles in tumorigenesis and therapy-resistance [154-156, 184]. Hence, it is important to understand the pathways that lead to dysregulation of IKBKE expression or function. IKBKE is located in the 1q32 amplicon and it is frequently amplified in cancers, and particularly in breast cancers as shown in Figure 7. Aberrant expression of IKBKE can also be attributed to the activity of several important oncogenic transcription factors. It was previously shown that IKBKE expression was previously shown to be induced by CK2α overexpression and inhibition of CK2α resulted in reduced IKBKE expression leading to decreased NFκB activation [188]. Overexpression of IKBKE by Gli transcription factors was previously shown to be an important mechanism in KRas mediated pancreatic tumorigenesis [189]. Furthermore, our group has previously shown that IKBKE expression is upregulated by STAT3 in response to tobacco carcinogen and this leads to resistance to chemotherapy in Non-small cell lung cancers [154]. In this study it was identified

that NSCLC patients showed a correlation of STAT3 activation with IKBKE expression further emphasizing the clinical relevance of STAT3-mediated IKBKE expression.
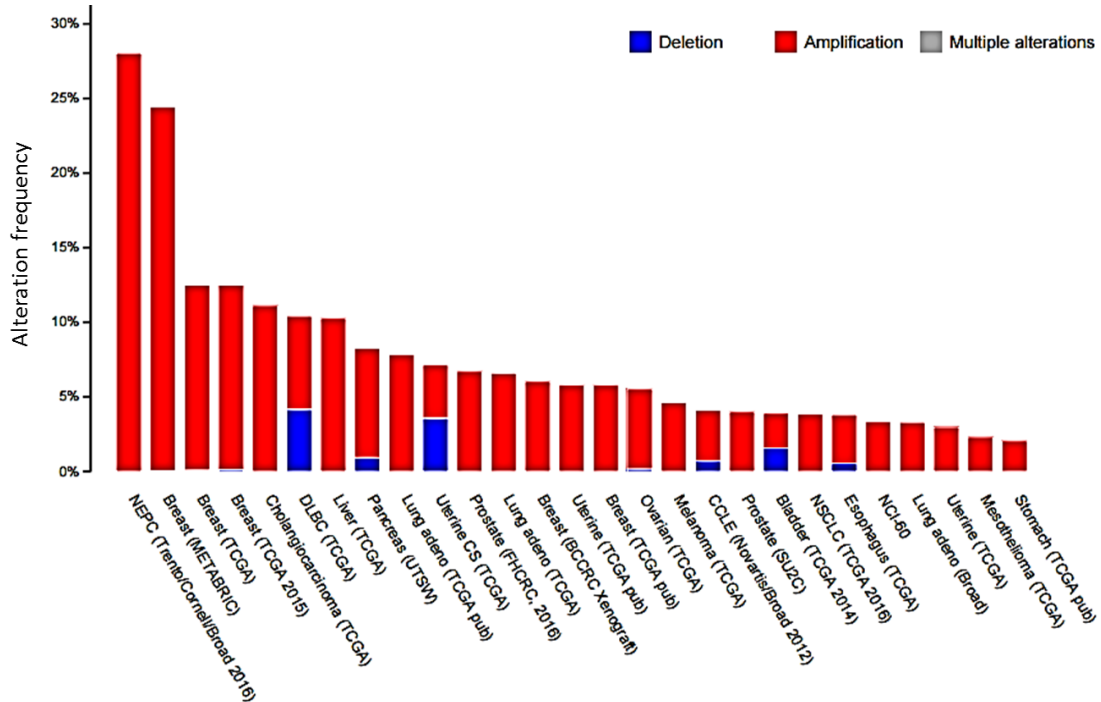
Figure 7: Copy number analysis of IKBKE in various cancers. Copy number analysis of IKBKE locus in various cancers were obtained from cBioPortal [22]. IKBKE is amplified in several cancers with highest observed in breast cancer patients.

Tumor microenvironment plays a critical role in cell transformation and drug resistance. IKBKE could be activated by the pro-inflammatory cytokines such as IL1β and IL6 produced by T cells and macrophages at least in part due to STAT3 activation [166]. Indeed it was shown that interaction with activated macrophages increase IKBKE expression in adipocytes that is inhibited by Vitamin B6 treatment [190]. Interestingly, high fat diet was also shown to enhance IKBKE expression in adipocytes due to chronic inflammation and activation of the NFκB pathway [163]. These data together strongly suggests that IKBKE activation could be a

consequence of chronic inflammation and that this pathway will be a good target for therapy in inflammatory disorders such as obesity and cancer.

Post-translational modifications of IKBKE. Post-translational modifications represent an important regulatory mechanism of protein function. Cancer cells often gain growth phenotype by aberrant modifications of proteins. Other than genetic amplification, mutations in IKBKE gene are not significantly associated with cancers. Hence it is possible that IKBKE could be hyper-activated by post-translational regulation. To this end several modifications of IKBKE were identified (Figure 8). Firstly, IKBKE gets auto-phosphorylated at Serine 172 and Threonine 501 residue that are augmented by IFNβ treatment [162, 191]. In addition IKBKE was also shown to be ubiquitinated at Lysine 30 and Lysine 401 residues in response to TNFα treatment and this K63-linked ubiquitination was shown to regulate IKBKE kinase activity. These ubiquitination events were shown to increase IKBKE activity by affecting the substrate binding ability of IKBKE and inhibition of the canonical NFκB signaling [192]. Furthermore, IKBKE was also shown to be SUMOylated in response to DNA damage that results in an increased localization of IKBKE in PML bodies and subsequent activation of downstream signaling [193]. Together, these data show the importance of post-translational modifications of IKBKE in regulating its function in response to various stimuli such as inflammation and DNA damage. Even more, tyrosine phosphorylation, an important post-translational modification of IKBKE, and their effect on IKBKE kinase activity are yet to be identified.
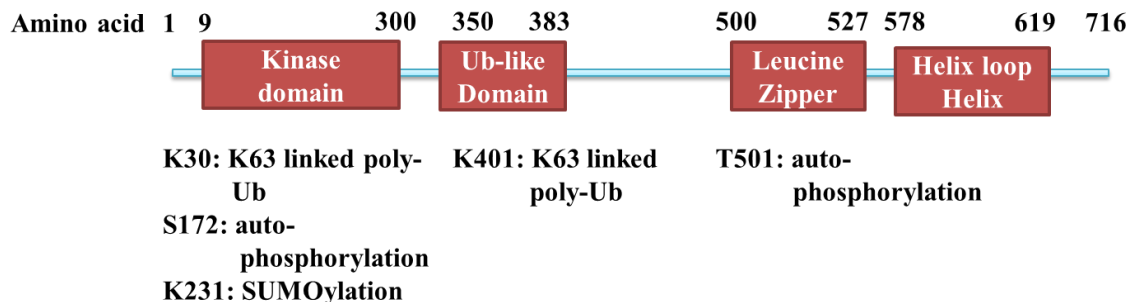
Figure 8: Summary of known post-translational modifications in IKBKE protein. Various post-translational modifications in IKBKE are shown.

Although the crystal structure of IKBKE is not completely solved, structure of TBK1, a close homolog of IKBKE was solved and based on the homology of TBK1 and IKBKE protein sequences, we could get structural insights into function of IKBKE. Essentially, it was noted that the ubiquitin-like domain (ULD) in IKBKE folds back onto the Kinase domain and that this interaction is important for activation of IRF3 pathway by IKBKE. Furthermore, deletion of ULD or the residues important for the ULD-kinase domain interaction failed to activate IRF3 in response to LPS or Poly(I:C) [194]. Furthermore, another study showed that the poly-ubiquitination of TBK1 at the Lys 30 and Lys 401 (that were also conserved in IKBKE as discussed above) is required for phosphorylation at Serine 172, kinase activation and substrate phosphorylation [12]. Together, these data show that post-translational modifications of IKBKE may affect the intra-molecular and inter-molecular interactions thus altering the substrate activation.

In summary, although there was success in identifying targeted therapeutic opportunities for cancer, development of resistance to these therapies remains as a major challenge. In this study we 1) identified a novel signaling pathway mediated by EGFR with activating mutations and 2) studied the outcome of IKBKE inhibition on cancer cell signaling. We further demonstrated that EphA2-IKBKE axis is important for PARP inhibitor resistance in ovarian

cancers. Together, these results provide a rationale for clinical evaluation of combinational therapies using IKBKE inhibitors for the treatment of cancers.

CHAPTER 2

IKBKE is a substrate of EGFR and a therapeutic target in non-small cell lung cancer with activating mutations of EGFR

Introduction

EGF receptor (EGFR) is the major driver pathway of non-small cell lung cancer (NSCLC), especially lung adenocarcinoma [37]. Activating mutations of EGFR have been detected in 15% and 30% of NSCLC in Western and Asian populations, respectively [36]. EGFR-mutated NSCLCs depend on EGFR signaling for their survival and growth. While MAPK, PI3K/AKT and the STAT pathways have been shown to be the major signaling cascades activated by EGFR and mediate EGFR oncogenic signaling [36], the molecular mechanism of active mutant EGFR driving NSCLC growth remains elusive.

Activating EGFR mutations dictate responsiveness of NSCLC to reversible EGFR tyrosine kinase inhibitors (TKIs) and despite promising initial responses, virtually all patients progress because of the acquisition of resistance [36]. Secondary EGFR-T790M mutation that prevents binding of TKIs to EGFR, is the most common resistance mechanism and accounts for ~60% of patients after disease progression [41, 195]. Second generation irreversible, EGFR-TKIs have been developed and extensively studied. Despite promising preclinical evidences of activity against EGFR-mutated cell lines harboring the T790M mutation [42, 47, 196], these inhibitors as monotherapy have failed to overcome T790M-mediated resistance in patients [44-46], In addition to efforts of developing and characterizing "third generation" EGFR-TKIs that

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are designed to target T790M, inhibition of EGFR downstream signaling, such as MEK inhibitor and PI3K/Akt/mTOR inhibitors, have been used to overcome resistance [64, 197, 198].

IKBKE is a non-canonical IKK kinase family member frequently upregulated/activated in NSCLC [154] and its overexpression leads to malignant transformation in various cell types [158]. In addition to phosphorylation of IRF3, IRF7 and STAT1 [172, 175, 177, 191], IKBKE can activate the NF-κB pathway by inhibiting several key regulators of NF-κB pathway [168, 169, 180, 199], which lead to induction of CCL5 and IL-6 and subsequent activation of JAK/STAT3 pathway [166, 200]. In addition, we and others have demonstrated that IKBKE activates AKT by direct phosphorylation of AKT-Thr308 and Ser473, independent of PI3K and mTORC2 [157, 181]. IKBKE can also exhibit its oncogenic function by phosphorylation and degradation of FOXO3a [184]. In this study, we showed that wild-type and mutant EGFR interact with IKBKE and IKBKE is directly activated by mutant but not wild-type EGFR. And we report that the IKBKE inhibitor amlexanox, a small-molecule therapeutic presently used in the clinic to treat aphthous ulcers, asthma and obesity [165, 201, 202], synergized with MEK inhibitor AZD6244 leading to significant reduction of tumor growth in NSCLC cells bearing EGFR mutation.

Materials and methods

Cell culture, plasmids and antibodies. NSCLC cells were obtained from the Moffitt Cancer Center Lung Cancer SPORE Cell Core and maintained in RPMI 1640 containing 10% fetal bovine serum and 1% Penicillin/streptavidin [203]. Knockdown of IKBKE was performed using shRNAs described previously [154]. siRNAs specific for EGFR were obtained from IDT

Technologies. The sequences for EGFR siRNAs are: siEGFR1: Sense 5'-GCAACAUCUCCGAAAGCCATT-3', antisense 5'- UGGCUUUCGGAGAUGUUGCTT-3' and siEGFR2: sense 5'-GGAGCACAAGCCACAAGUCTT-3', antisense 5'-GACUUGUGGCUUGUGCUCCTT-3'. Afatinib was purchased from LC Biotech. Amlexanox and AZD6244 were from Sigma.

The pCMV-Tag3b Myc-IKBKE plasmid was obtained as described previously [156]. Tyrosine -153 and -179 residues were mutated to phenylalanine using the Stratagene Quick Change kit according to the manufacturer protocol. GST-fusion peptides (20 amino acids) spanning the phosphorylation tyrosines and their mutant forms were obtained by cloning oligonucleotide into pGEX-4T1 vector. Truncations of IKBKE were obtained by PCR amplification and ligation into pCMV-Tag3b vector.

Antibodies against pIKBKE-Ser172, pAKT-Thr308, pAKT-Ser473, AKT, pEGFR-Y1068 and GAPDH were purchased from Cell Signaling Technologies. EGFR, Myc, pan-phospho-tyrosine (pTyr) and GST antibodies were purchased from Santa Cruz Biotechonology. IKBKE antibody was from Sigma. For the pIKBKE-Y153 antibody, 2 rabbits were immunized with peptides containing the sequence Ac-EGQSI(pY)KLTDFC-amide and the sera was affinity purified using non-phosphorylated peptide (New England Peptide).

Western blot, immunoprecipitation and *in vitro* kinase assays. Western blot analysis was performed as described previously [157]. For detection of phosphorylated IKBKE-tyrosine, cell lysates were immunoprecipitated with anti-Myc (for transfected myc-IKBKE) or anti-IKBKE antibodies, followed by Western blot with pTyr antibody. For co-immunoprecipitation, cells expressing myc-IKBKE and EGFR were lysed in a lysis buffer containing 50 mM Tris-

HCl, 300 mM NaCl, 4 mM EDTA, 1 mM DTT, 0.5% CHAPS. IKBKE and EGFR kinase assays were performed as previously described [157, 204]. Briefly, Recombinant EGFR and recombinant IKBKE were incubated in EGFR kinase assay buffer for 30 minutes at 30°C. The reaction was stopped by addition of SDS PAGE loading buffer. IKBKE kinase assays were performed with myc-IKBKE purified from cells using c-myc antibody. Purified IKBKE was incubated with GST-IκB in IKBKE kinase buffer at 30°C for 30 minutes and the reaction was stopped by addition of SDS PAGE loading buffer followed by boiling at 103°C for 5minutes. Recombinant IKBKE and EGFR were purchased from Life Technologies and Sigma, respectively.

MTT and clonogenic assays. Cells indicated in the figure legends were plated in 96-well plates at a density of 4,000 cells/well. After culture for 12 hours (hrs), the growth media was replaced with media containing indicated amount of Amlexanox (IKBKE inhibitor) or AZD6244 (MEK inhibitor) and the combination of these two inhibitors. After 48 hrs, MTT assay (Sigma) was performed according to manufacturer's protocol. Drug synergy was calculated using CompuSyn software [205]. For clonogenic assay, cells were plated at a density of 500 cells/well in a 12 well plate and treated with the IKBKE inhibitor or MEK inhibitor and their combination. After culture for 9 days, the cells were fixed with crystal violet stain.

Mass spectrometry. IKBKE from *in vitro* EGFR kinase assay reactions were separated on SDS-PAGE. The band corresponding to IKBKE was in-gel digested with trypsin and the phosphorylated peptides were identified by MS/MS.

Confocal microscopy and immunohistochemistry. After transfection of GFP-IKBKE and EGFR, the cells were fixed with 10% Formalin containing methanol and the cells were stained with EGFR antibody. Co-localization of IKBKE and EGFR in H1975 cells were

analyzed using confocal microscopy [184]. Immunohistochemistry was performed as previously described [154]. High pH antigen un-masking solution (Vector laboratories) was used for antigen retrieval.

Cell migration/invasion assay, cell cycle analysis and xenograft study. Cell migration and invasion assays were performed as described previously [206]. Cell cycle analysis was performed using PI staining and followed by flow cytometry.

Three cell lines H1975, HCC827 and H1650 were injected subcutaneously into 7-week old female nu/nu mice (5×10^6/mouse, Charles River). After the tumors have reached a volume of 100 mm^3, mice were randomized into four groups i.e., vehicle, Amlexanox (50mg/kg), AZD6244 (25mg/kg) and combination of Amlexanox and AZD6244. The mice were treated five days a week by oral gavage. Tumor volumes were measured using calipers. The animal experiments were performed according to the IACUC protocol.

Statistical analysis. Student's *t* tests were used to analyze statistical significance. P≤0.05 was considered as statistically significant. The error bars indicate standard deviation.

Results

NSCLC cell lines with activating EGFR mutation are more sensitive to IKBKE inhibition: activating mutations of EGFR activate IKBKE. We have previously shown frequent overexpression of IKBKE in NSCLC [154]. To investigate its functional significance in NSCLC, we knocked down IKBKE in a panel of NSCLC cell lines. Interestingly, we observed that except PC9, cells harboring the activating mutations of EGFR, including EGFR-T790M mutant H1975 and H820, are more sensitive to IKBKE knockdown than NSCLCs with wild-type EGFR (Figure 9A). Further analysis of IKBKE activation, by measuring pIKBKE-Ser172,

revealed that IKBKE was highly activated in the EGFR mutant cell lines (Figure 9B). We further examined if activating mutations of EGFR induces IKBKE kinase activity. Following transfection of various EGFR mutants and myc-IKBKE, we immunorecipitated IKBKE and performed *in vitro* IKBKE kinase assay. Notably, we found that IKBKE kinase activity was significantly induced by active EGFR mutants but not wild-type EGFR and that EGF stimulation failed to activate IKBKE (Figure 9C). Collectively, these findings suggest that IKBKE is
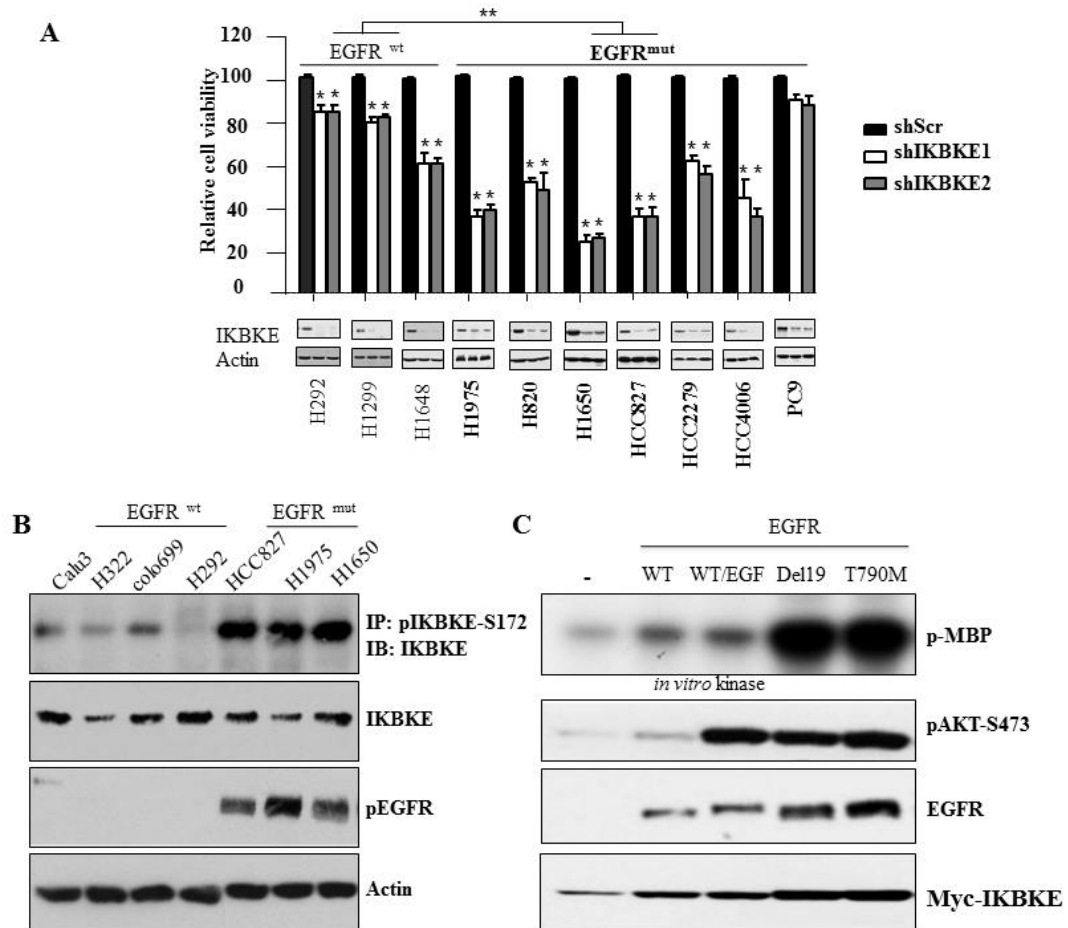
Figure 9: Activation of IKBKE by activating mutations of EGFR. (A) A panel of NSCLC cell lines were transfected with two different shRNAs targeting IKBKE and viable cells were measured using MTT assay. (B) Lysates from NSCLC cell lines were subjected to immunoprecipitation (IP) with pIKBKE-Ser172 antibody followed by Western blot with IKBKE antibody. (C) H1299 cells were transfected with myc-IKBKE and wild type and mutant EGFR. Following treatment with and without EGF, myc-IKBKE was immunoprecipitated with myc antibody and then subjected to *in vitro* kinase assay using MBP as substrate (top). Western blot was carried out with indicated antibodies (panel 2-4). * $p<0.05$ and ** $p<0.005$

regulated by activating mutations of EGFR and could serve as a therapeutic target and a prognostic marker in NSCLC, especially in the tumors with EGFR mutations.

IKBKE directly interacts with EGFR. It has been shown that Kaposi sarcoma-associated herpesvirus GPCR (kGPCR) directly interacts with IKBKE, resulting in activation of IKBKE [207]. To understand the mechanism by which IKBKE is activated by mutant EGFR, we first investigated if activating mutation of EGFR is able to form a complex with IKBKE. Following expression of EGFR$^{L858R/T790M}$ and myc-IKBKE in H1299 cells, co-immunoprecipitation revealed that the mutant EGFR interacted with IKBKE (Figure 10A). Furthermore, inhibition of EGFR kinase activity has no effect on this interaction in H1975 (Figure 10B). These data imply that EGFR kinase activity is dispensable for its interaction with IKBKE.

To examine if this interaction is direct, we performed *in vitro* binding assay using recombinant GST-tagged intracellular domain of EGFR$^{L858R/T790M}$ and IKBKE proteins. Wild-type EGFR or EGFR$^{L858R/T790M}$ was immunoprecipitated with IKBKE antibody following incubation of recombinant GST-IKBKE and EGFR^{WT} or EGFR$^{L858R/T790M}$ proteins *in vitro* (Figure 10C). In addition, an *in vitro* GST-pulldown assay by incubating GST-EGFR or GST-vector with lysates from HEK293T cells expressing myc-IKBKE revealed that myc-IKBKE is specifically pulled down by GST-EGFR$^{L858R/T790M}$ or GST-EGFR^{WT} (Figure 10D). Interestingly, IKBKE also bound to wild-type EGFR and addition of EGF ligand led to dissociation of EGFR-IKBKE complex (Figure 10E). To identify the domains in IKBKE that are critical for its interaction with EGFR, we generated constructs expressing the myc-tagged N-terminal/kinase and C-terminal domains of IKBKE. Immunoprecipitation revealed that N-terminal region of

IKBKE bound to EGFR (Figure 10F). Collectively, these results suggest that intracellular

domain of EGFR directly interacts with N-terminal region of IKBKE.
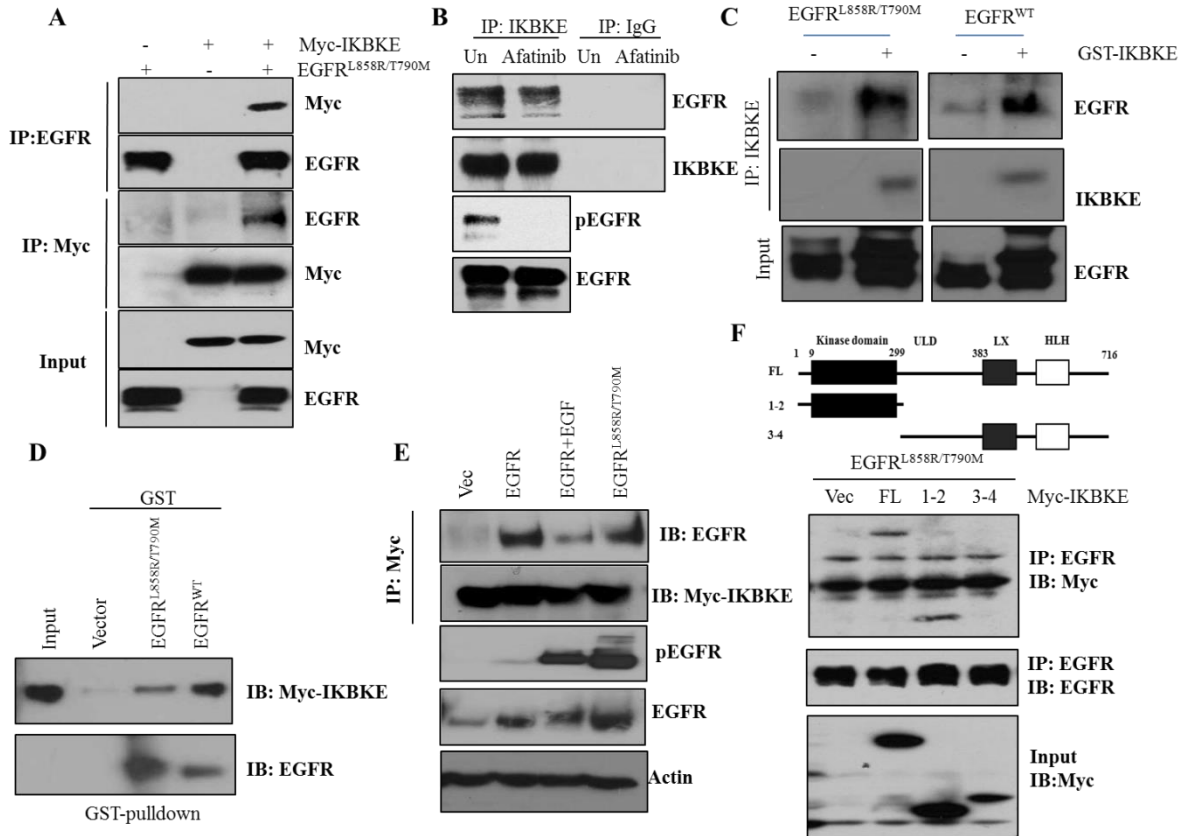
Figure 10: IKBKE is a binding partner of mutant EGFR. (A) Lysates from H1299 cells co-transfected with myc-IKBKE and EGFR$^{L858R/T790M}$ were subjected to co-IP with antibodies against EGFR (Panels 1 and 2) and Myc (panels 3 and 4). (B) H1975 cells which harbor EGFRL$^{858R/T790M}$ mutation were treated with and without EGFR inhibitor Afatinib (200nM) for 16 hours and were subjected to IP with IKBKE antibody and immunoblot with EGFR antibody. (C and D) *in vitro* binding assay was performed using recombinant GST-EGFR and GST-IKBKE. IKBKE was immunoprecipitated with IKBKE antibody and followed by Western blot with EGFR antibody (C). Recombinant GST-EGFR was incubated with lysates from HEK293T cells overexpressing myc-IKBKE (lanes 3 and 4) or vector (lanes 2) in *in vitro* binding buffer followed by GST pulldown and detection with myc antibody (D). (E) H1299 cells were transfected with the indicated plasmids and upon treatment with EGF for 15 minutes, IKBKE was immunoprecipitated using Myc antibody. EGFR bound to IKBKE was detected using EGFR antibody. pEGFR and EGFR expressions were shown in panels 3 and 4. (F) N-terminal region of IKBKE binds to EGFR. HEK293 cells were transfected with truncation mutants of myc-IKBKE (left) and EGFR$^{L858R/T790M}$ and then were subjected to IP with EGFR antibody and immunoblot with myc antibody.

EGFR phosphorylates IKBKE at tyrosine-153 and -179 residues. Having demonstrated EGFR direct interaction with IKBKE, we sought to identify if IKBKE is phosphorylated by EGFR at tyrosine residue(s). Following co-expression of myc-IKBKE with EGFR$^{L858R/T790M}$ or EGFR^{WT} in H1299 cells, we immunoprecipitated IKBKE with myc antibody and then immunoblotted the immunoprecipitates with pan-phosphotyrosine (pTyr) antibody. Figure 11A showed that tyrosine phosphorylation of IKBKE was induced by EGFR$^{L858R/T790M}$ but not by EGFR^{WT} even when treated with EGF ligand. We further investigated if inhibition of EGFR will inhibit the tyrosine phosphorylation of IKBKE. Two activating mutation of EGFR cell lines H1975 and HCC827 were treated with Afatinib, a second generation irreversible inhibitor of EGFR [43], and then were subjected to immunoprecipitation with IKBKE antibody and immunoblotting with pan-pTyr antibody. The phosphotyrosine level of IKBKE was significantly reduced by Afatinib in both cell lines (Figure 11B and 11C). However, Afatinib had no effect on IKBKE-tyrosine phosphorylation in either H292, in which EGFR is amplified, or H322, in which EGFR^{WT} was ectopically overexpressed (Figure 11C). In addition, decrease in phosphotyrosine of IKBKE was observed after knockdown of EGFR in EGFR-mutant H1975 cells but not in wild-type EGFR H292 and H322 cells (Figure 11D). We next examined if EGFR directly phosphorylates IKBKE. *In vitro* kinase assay with recombinant EGFR$^{L858R/T790M}$ or EGFR^{WT} and IKBKE showed that IKBKE was phosphorylated by EGFR$^{L858R/T790M}$ but not by EGFR^{WT} (Figure 11E). These data strongly suggest that constitutively active mutant EGFR is a *bona fide* kinase leading to IKBKE tyrosine phosphorylation.
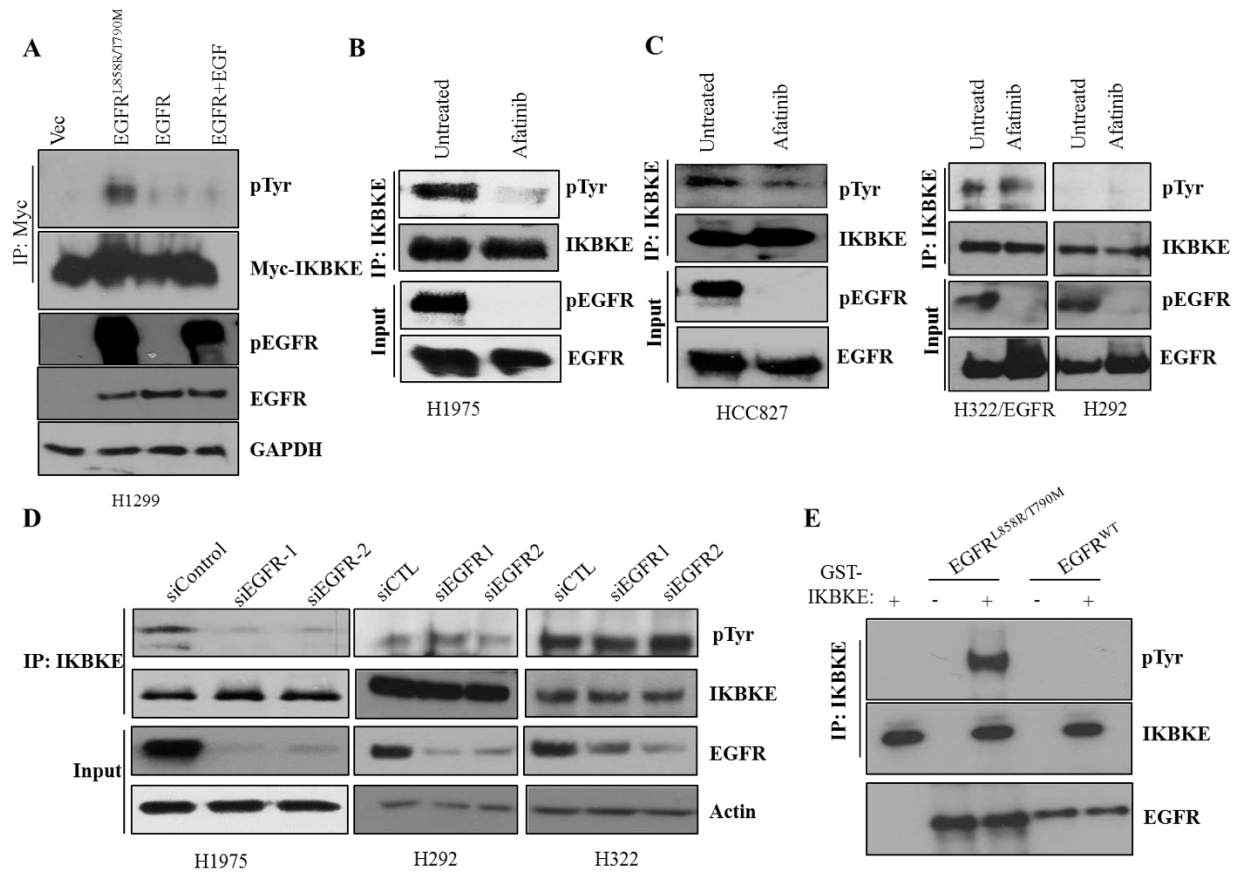
Figure 11: EGFR$^{L858R/T790M}$ phosphorylates IKBKE at tyrosine residues. (A) H1299 cells were cotransfected with indicated plasmids. Following IP with myc antibody, immunoprecipitated IKBKE was blotted with pan-phospho-tyrosine antibody (pTyr, top). Panels 2 and 4 show the immunoprecipitated IKBKE and expression of transfected EGFR, respectively. (B-C) Indicated cells were treated with 200nM afatinib for 16 hours and the endogenous IKBKE was immunoprecipitated followed by Western blot with indicated antibodies. Panels 3 and 4 are immunoblots with phospho- and total-EGFR antibodies. (D). Indicated cells were transfected with two siRNAs targeting EGFR and a control siRNA. After incubation for 48 hrs, the cells were lysed and endogenous IKBKE was immunoprecipitated followed by Western blot with indicated antibodies. Knockdown of EGFR was shown in panel 3. (E) GST-IKBKE was incubated with GST-EGFR$^{L858R/T790M}$ or GST-EGFR^{WT} *in vitro*. After IP with IKBKE antibody, the immunoprecipitates were blotted with indicated antibodies (panels 1 and 2). Bottom panel shows GST-EGFR used for the assay.

To identify the residues phosphorylated by EGFR, recombinant IKBKE from *in vitro* EGFR kinase assay was subjected to mass spectrometric analysis. Five tyrosine residues were identified as potential phosphorylation sites by EGFR. To further define the EGFR phosphorylation residue(s) in IKBKE, *in vitro* EGFR kinase assay was performed using GST-fused peptides for each of potential phosphorylation tyrosine sites and their mutant (i.e., 20 amino acids of IKBKE with the candidate tyrosine and its phenylalanine substitution in the middle). We noted that EGFR phosphorylated IKBKE-Y153 and -Y179 more significantly than the other tyrosine residues while their tyrosine to phenylalanine (Y-F) mutants failed to be phosphorylated by EGFR *in vitro* (Figure 12A). In support of this finding, we found that the N-terminal region of IKBKE, in which Y45, Y-153 and Y-179 reside, was predominantly phosphorylated by EGFR (Figure 12B). We further created the Y-F mutation for each candidate phosphotyrosine residue in full length myc-tagged IKBKE. Following co-expression of these mutants with constitutively active EGFR, immunoblot analysis of the myc-IKBKE immunoprecipitates showed that IKBKE-Y153F and IKBKE-Y179F present lower phosphotyrosine level than wild-type and other mutants of IKBKE (Figure 12C). Notably, IKBKE with both Y153 and Y179 mutation largely lost the EGFR-induced tyrosine phosphorylation (Figure 12D).
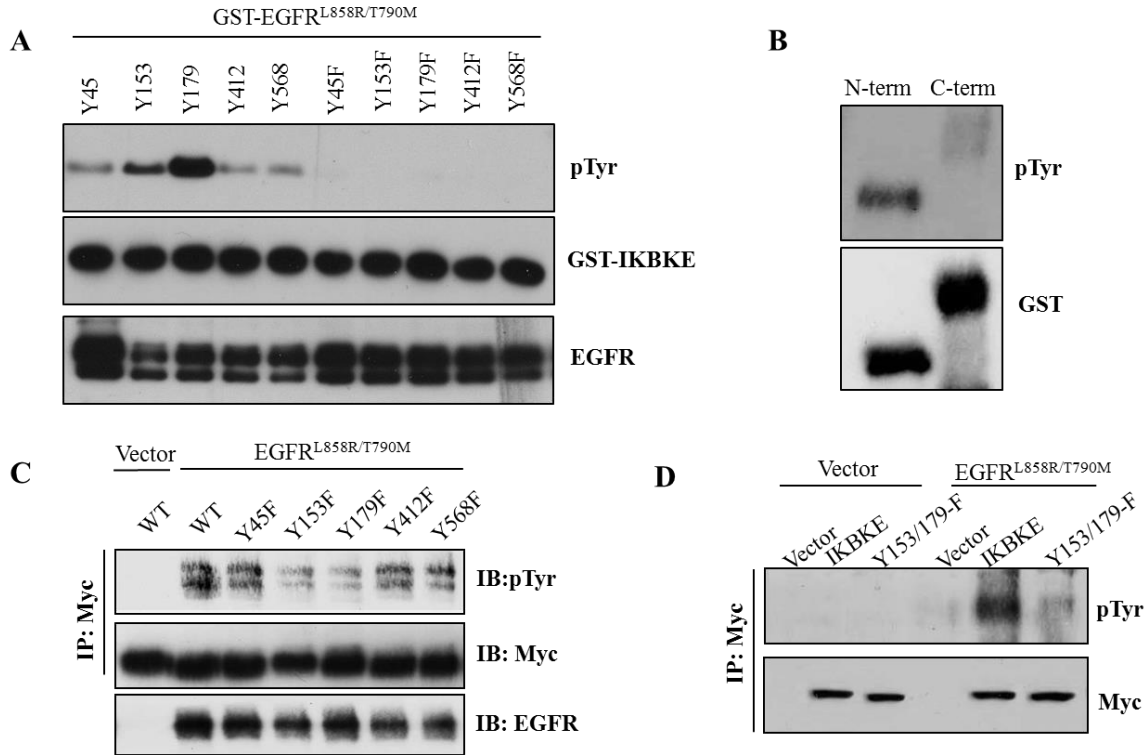
Figure 12: Identification of IKBKE tyrosine residue(s) phosphorylated by EGFR. (A) Following *in vitro* incubation of recombinant EGFR$^{L858R/T790M}$ with indicated GST-fusion IKBKE peptides spanning each of the phosphorylation tyrosine residues, the GST-fused IKBKE peptides were immunoblotted with pTyr antibody. (B) In vitro EGFR kinase assay was carried out by incubation of recombinant EGFR$^{L858R/T790M}$ with GST-fusion N-terminal and C-terminal domains of IKBKE. The GST-IKBKE-NT and GST-IKBKE-CT were then blotted with pTyr antibody. (C) Indicated myc-IKBKE plasmids were co-expressed with EGFR$^{L858R/T790M}$. After IP with myc antibody, myc-IKBKEs were immunoblotted with indicated antibodies. (D) H1299 cells were transfected with indicated plasmids and the IKBKE was immunoprecipitated with myc antibody and then immunoblotted with pTyr (upper) and myc (bottom) antibodies.

We further generated specific pIKBKE-Y153 antibody. To determine the antibody specificity, we initially incubated GST-fused IKBKE-Y153 peptides with and without recombinant constitutively active EGFR and then immunoblotted with pIKBKE-Y153 antibody. We observed that EGFR phosphorylated IKBKE-Y153 is recognized by pIKBKE-Y153 antibody (Figure 13A). We also found that the pIKBKE-Y153 antibody could detect endogenous level of

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pIKBKE-Y153 in H1975 cells, which was reduced by either pre-treatment of the cell lysate with alkaline phosphatase or IKBKE knockdown (Figure 13B). Moreover, pIKBKE-Y153 antibody could not recognize the IKBKE-Y153F but reacted with IKBKE-Y179F and wild-type IKBKE in the presence of constitutively active EGFR (Figure 13C). Depletion of EGFR by either siRNA or small molecule inhibitor Afatinib resulted in significant reduction of pIKBKE-Y153 (Figure 13D and 13E). NSCLC cells with EGFR mutation showed higher levels of pIKBKE-Y153 levels (Figure 13F). Even though we could not reach statistical significance due to limited sample size, NSCLC patients with EGFR mutations showed higher levels of pIKBKE-Y153 than patients with EGFR wildtype (Figure 13G). These data show that our pIKBKE-Y153 antibody specifically recognizes endogenous p-IKBKE-Y153 and IKBKE is a *bona fide* substrate of EGFR.
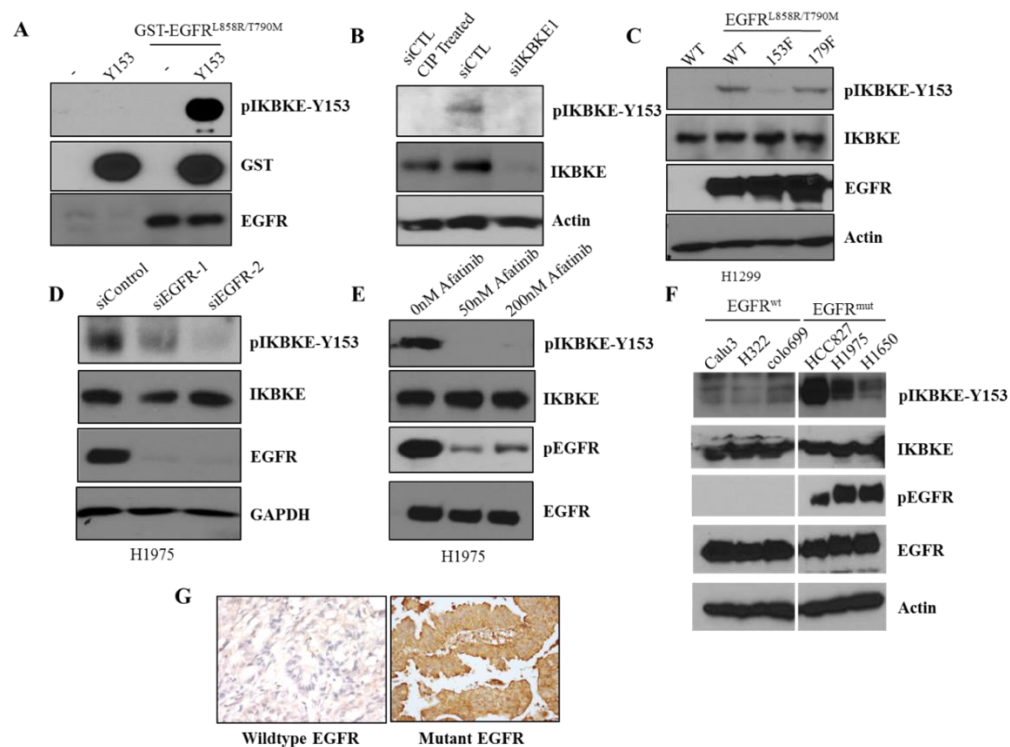
Figure 13: Characterization of pIKBKE-Y153 antibody. (A) Following *in vitro* EGFR kinase assay by incubation of recombinant EGFR$^{L858R/T790M}$ with GST-fusion peptides spanning IKBKE tyrosine 153 (as shown in Figure 10A), the reaction was immnoblotted with indicated antibodies.

Figure 13 (continued)

(B) Western blot analysis was performed with pY153-IKBKE antibody in H1975 cells that were transfected with IKBKE siRNA or control siRNA and the lysates from control siRNA were further treated with calf intestinal phosphatase (lane 1) prior to SDS-PAGE. (C) H1299 cells co-transfected with EGFR[L858R/T790M] and wild type, Y153F and Y179F mutants of IKBKE and then were subjected to western blot with indicated antibodies (D-E) After H1975 cells were treated with control siRNA (siCTL) and 2 siRNAs against EGFR for 48 hours (D) or with indicated amount of afatinib for 16 hours (E), western blot analysis was performed with indicated antibodies. (F) Western blot analysis of 3 wild type and 3 mutant EGFR NSCLC cell lines with indicated antibodies. (G) Representative images of immunohistochemical staining of NSCLC TMAs using pY153-IKBKE antibody. EGFR mutated NSCLC specimen exhibited higher staining (right) when compared to wild type EGFR tumor (left).

Phosphorylation of IKBKE by EGFR increases IKBKE kinase activity and IKBKE mediates EGFR signaling and cellular function. We further investigated whether EGFR phosphorylation of IKBKE regulates its kinase activity. Fifure 14A showed that overexpression of wildtype IKBKE with EGFR[L858R/T790M] led to a significant increase in pIKBKE-Ser172 and IKBKE kinase activity. While mutation of the tyrosine 153 and 179 residues led to a significant decrease in EGFR-mediated IKBKE activation and IKBKE with both tyrosine residues mutated showed a complete loss in auto-activation and kinase activity when co-expressed with EGFR[L858R/T790M] (Figure 14A). To confirm this observation in lung cancer cells, H1299 cells were transfected with myc-IKBKE, myc-IKBKE-Y153F/Y179F, which was no longer phosphorylated by EGFR, together with and without EGFR[L858R/T790M]. IKBKE was immunoprecipitated with myc antibody and then subjected to *in vitro* kinase assay using IκB as a substrate. We found that expression of EGFR[L858R/T790M] significantly induced IKBKE kinase activity and that IKBKE-Y153F/Y179F mutant not only lost basal kinase activity but also could not be activated by EGFR[L858R/T790M] (Figure 14B).

We examined whether EGFR phosphorylation of IKBKE affects downstream signaling of IKBKE. We and others have previously showed that IKBKE is an AKT kinase kinase, i.e., IKBKE activates AKT by direct phosphorylation of AKT-T308 and -S473 [157]. Thus, we co-expressed myc-IKBKE or myc-IKBKE-Y153F/Y179F with and without EGFR$^{L858R/T790M}$ in H1299 cells. Immunoblot analysis showed that pAKT-T308 and -S473 were induced by expression of IKBKE which was further enhanced by co-expression of IKBKE and EGFR$^{L858R/T790M}$. However, expression of IKBKE-Y153F/Y179F failed to activate AKT. Also expression of constitutively active EGFR had no effect on IKBKE-Y153F/Y179F toward AKT activation (Figure 14C). In addition, we found that knockdown of endogenous IKBKE in EGFR$^{L858R/T790M}$ transfected H322 and in EGFR mutation bearing H1975 cells significantly decreased phosphorylation of AKT and RelA/p65 (Figure 14D and 14E).
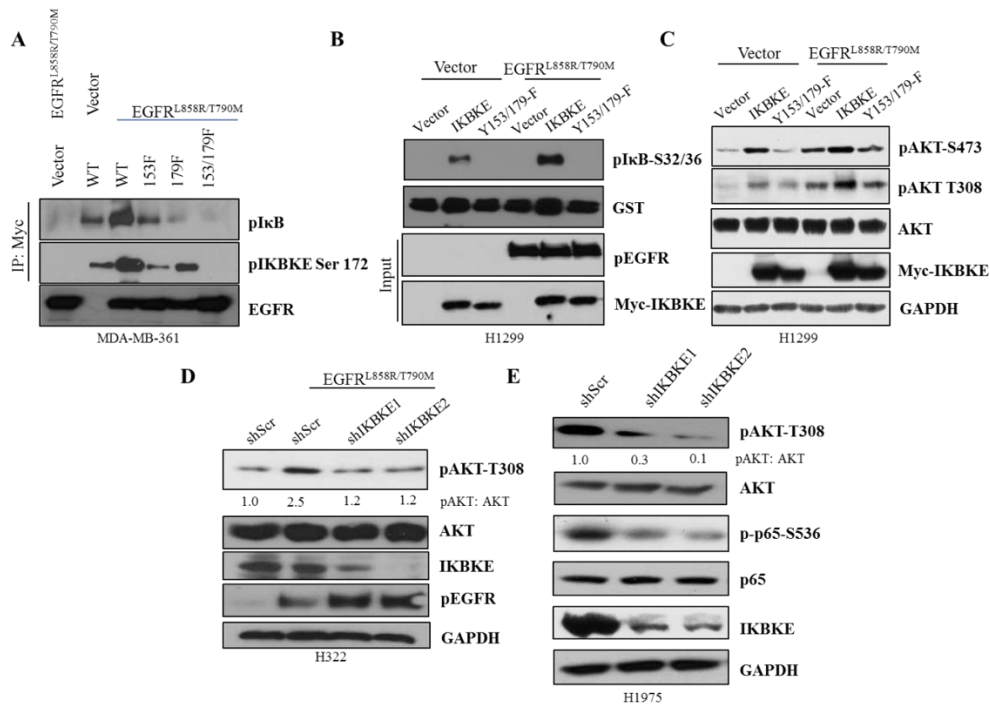
Figure 14: IKBKE-Y153/Y179 phosphorylation by activating mutant EGFR regulates its signaling. (A and B) *in vitro* IKBKE kinase assay was performed using myc-IKBKE

Figure 14 (Continued)

immunoprecipitated from MDA-MB-361 (A) and H1299 (B) cells transfected with indicated plasmids and GST-IκB as a substrate (C) H1299 cells were transfected with indicated plasmids and then immunoblotted with indicated antibodies. (D and E) Indicated plasmids were introduced to H322 (D) and H1975 (E) cells. After incubation for 48 hours, immunoblot was performed with indicated antibodies.

Moreover, depletion of IKBKE in H1975 cells with activating mutations of EGFR inhibited cell survival, growth and invasion (Figure 15A-C). We further investigated the significance of EGFR phosphorylation of IKBKE-Y153/Y179 in EGFR cellular function. We expressed wild type IKBKE or nonphosphorylatable IKBKE-Y153F/Y179F and EGFR$^{L858R/T790M}$ individually and in combination in H1299 cells.   Boyden chamber assays showed that while expression of IKBKE alone has no significant effects on cell migration and invasion, co-expression of IKBKE and constitutively active EGFR significantly induces these phenotypes. Notably, constitutively active EGFR-promoted cell migration and invasion were inhibited by expression of IKBKE-Y153F/Y179F mutant (Figure 15D and 15E).   Collectively, these data indicate that constitutively active EGFR induces IKBKE kinase activity through phosphorylation of Y153 and Y179 and that IKBKE is an important downstream target of EGFR to mediate EGFR signaling and function.
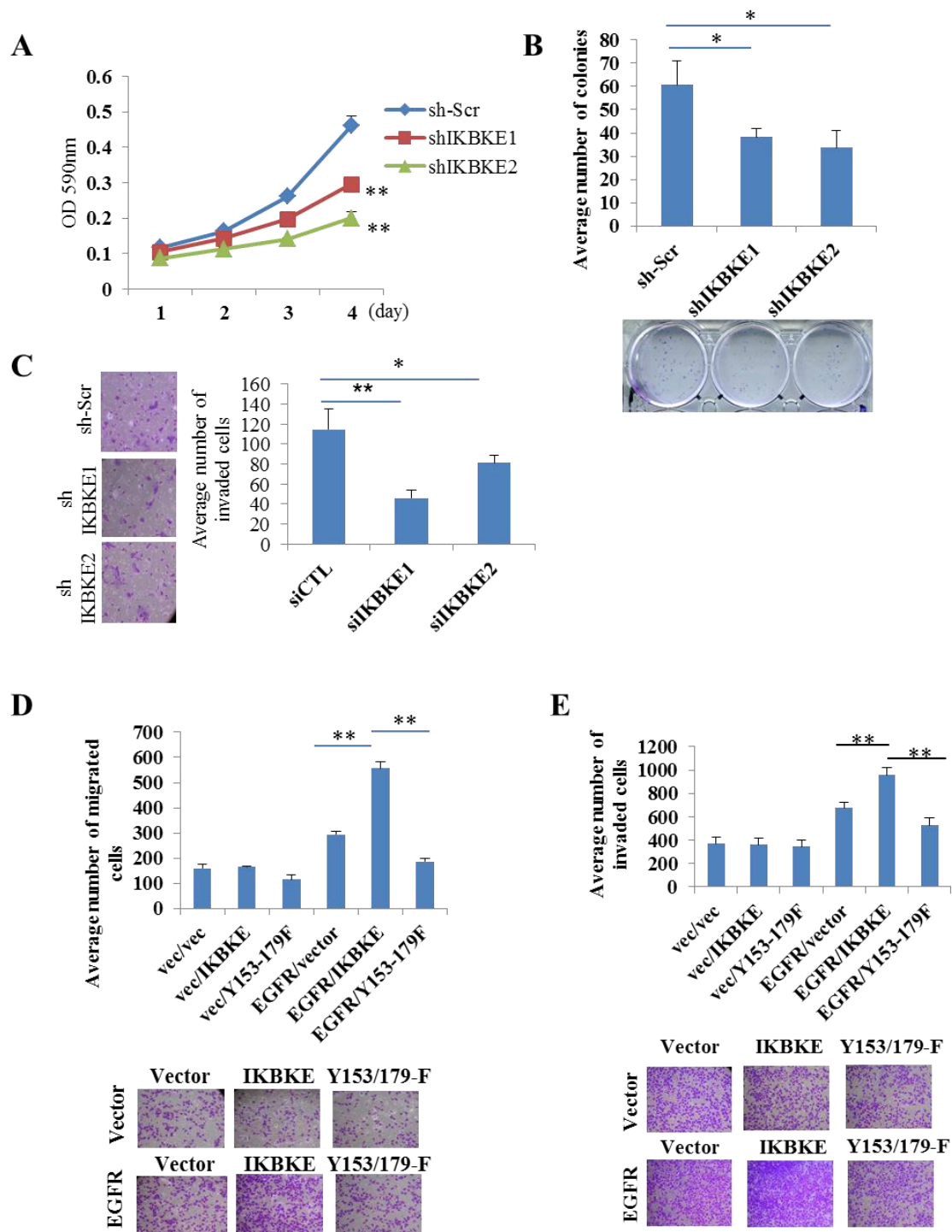
Figure 15: Depletion of IKBKE reduces active EGFR cellular function. Following knockdown of IKBKE in H1975, cell viability (A), colony growth (B) and cell invasion (C) were measured. (D and E) H1299 cells were transfected with indicated IKBKE plasmids with vector or EGFR$^{L858R/T790M}$ and then assayed for migration (D) and invasion (E). * $p<0.05$ and ** $p<0.005$

Therapeutic targeting of IKBKE for NSCLC cells with EGFR mutation and TKI resistance. A recent study identified an anti-inflammatory drug, amlexanox, as a selective inhibitor of IKBKE/TBK1 [165]. Because IKBKE is directly activated by activating mutation of EGFR and amlexanox is an approved small-molecule therapeutic presently used in the clinic to treat aphthous ulcers [201], and in clinical trial for obesity [202], we assumed that amlexanox could preferentially inhibit cell survival in NSCLCs with activating EGFR mutation including acquired EGFR^{T790M}. We treated a panel of NSCLC cell lines with increasing concentrations of amlexanox and found that the EGFR mutant cell lines, including acquired-resistant lines H1975 and H1650, are more sensitive to amlexanox (GI$_{50}$ 20 and 30μM) compared to the NSCLCs with wild type EGFR (GI$_{50}$ >80μM) (Figure 16A). Interestingly, amlexanox treatment led to a moderate increase in pERK1/2, while decrease in pAKT, in EGFR mutant H1975 and H1650 cells (Figure 16B, and 16C), suggesting the feedback activation of MAPK pathway upon IKBKE inhibition.

In addition to IKBKE and AKT, EGFR mutations also significantly activate MAPK in NSCLC [36]. Moreover, our data show that inhibition of IKBKE further induces pERK1/2 in EGFR mutant NSCLC cells. These findings prompted us to examine the synergistic anti-tumor activity of combination of IKBKE inhibitor amlexanox with MEK inhibitor AZD6244, a potent ATP-noncompetitive inhibitor of MEK1/2 and currently in phase II/III clinical trial [208]. We treated H1975 (Figure 16D-F) with amlexanox and AZD6244 individually and their combination for 48 hours. Cell viability analysis showed that whereas inhibition of either IKBKE or MEK reduced cell survival, inhibition of both pathways exhibited significant synergy with combination index values lower than 1.0 (Figure 16E). We also observed that amlexanox and AZD6244 synergistically reduced colony growth in both cell lines (Figure 16F).

Figure 16: IKBKE inhibitor Amlexanox preferentially inhibits cell viability in NSCLC cells with activating mutations in EGFR and synergizes with MEK inhibitor. (A) A panel of NSCLC cell lines (grey lines indicate EGFR wildtype and black lines indicate EGFR mutant cells) were treated with increasing concentrations of Amlexanox for 72 hours and then subjected
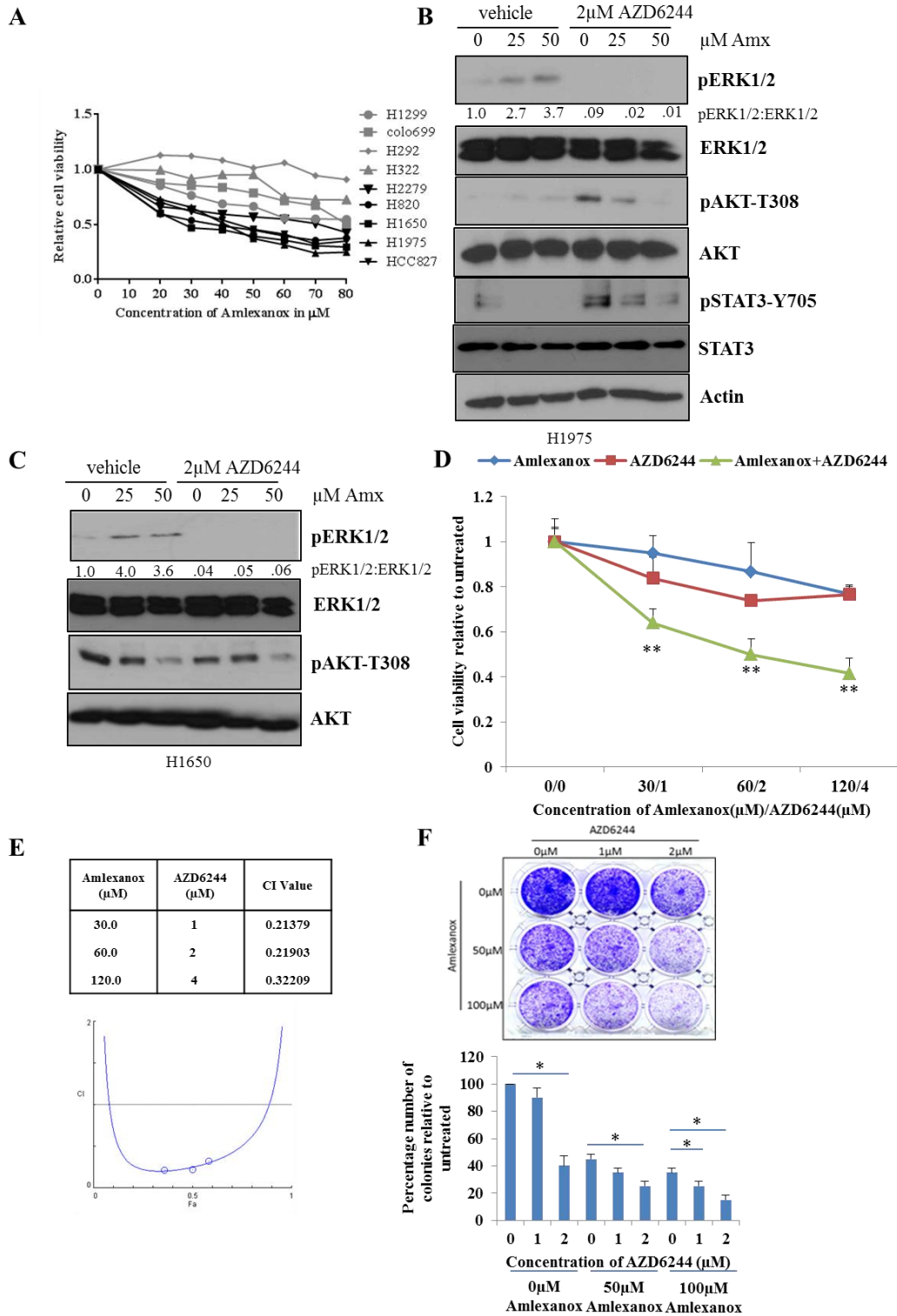
Figure 16 (Continued)
to MTT assay. (B and C) H1975 (B) and H1650 (C) cells were treated with indicated compounds for 24 hours and then were immunoblotted with indicated antibodies. (D-F) H1975 cells were treated with Amlexanox and AZD6244 individually and the combination. Cell viability (D) and colony growth (F) were assayed. One-way ANOVA was used to calculate statistical significance for cell viability and student's t test was used for the colony growth assay. Combination indices for the combination treatment was computed using CompuSyn (E). * $p<0.05$ and ** $p<0.005$

Next, we asked whether the combination of amlexanox with AZD6244 could inhibit EGFR mutant-driven tumor growth and surmount EGFR-TKI resistance *in vivo*. Xenograft experiments were carried out with 2 EGFR mutant, TKI-resistant cell lines (H1975, H1650) and one EGFR mutant and TKI-sensitive cells (HCC827). We noted that amlexanox alone only moderately repressed the tumor growth in these cells (no statistical significance). However, the combination of amlexanox and AZD6244 resulted in significant reduction in tumor growth and tumor weight (Figures 17A-B and 17D-E). Immunoblot analysis of the xenograft lysates revealed that the tumors treated with amlexanox alone expressed elevated pERK1/2 which is consistent with our *in vitro* findings (Figures 17G, 17H). Significantly, the tumors treated with both amlexanox and AZD6244 displayed low levels of pAKT and pERK1/2 (Figures 17G, 17H). Interestingly HCC827 xenografts that are known to be sensitive to EGFR-TKIs did not respond well to the combined treatment (Figure 17C and F) and upon examination of xenograft tissues, we found that amlexanox did not induce ERK1/2 activation (Figure 17I). Immunohistochemical staining of the H1975 and H1650 xenograft sections with Ki67 showed that combination treatment with amlexanox and AZD6244 more significantly inhibited cell proliferation compared with the tumors treated with either one alone (Figure 17J and 17K). Together, these data suggest that IKBKE is an important therapeutic target in NSCLC with activating EGFR mutations and

that combination of inhibitors of IKBKE and MEK could be a promising therapeutics for EGFR-

TKI resistance.
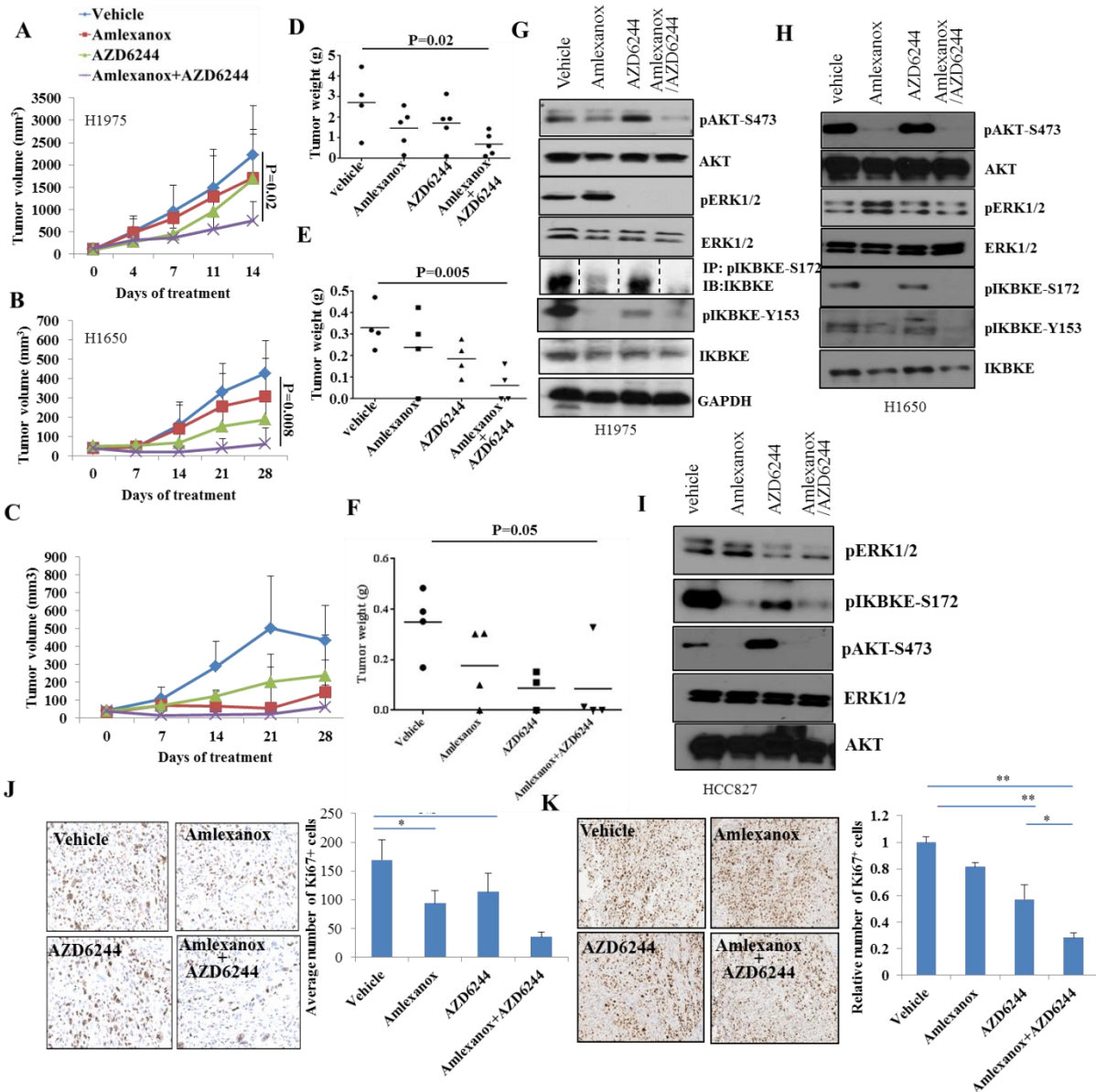
Figure 17: Amlexanox and AZD6244 synergistically inhibit EGFR inhibitor-resistant NSCLC tumor growth. (A-C) *in vivo* tumor growth of H1975 (A), H1650 (B) and HCC827 (C) xenografts. (D-F) Tumor weight of H1975 (D), H1650 (E) and HCC827 (F) xenografts. (G-I) Western blot analyses of H1975 (G), H1650 (H) and HCC827 (I) tumor lysates was performed with indicated antibodies. (J and K) Proliferation in H1975 (J) and H1650 (K) xenografts was determined by immunohistochemical staining with Ki67 (left) and was quantified (right). The images were acquired at 20X magnification. * p<0.05 and ** p<0.005

Discussion

Accumulating evidence indicates that EGFR mutations are key drivers for NSCLC by initiating several signal transduction cascades, principally the MAPK, AKT and JNK pathways. Here, we demonstrated that IKBKE is a direct downstream target of activating mutations of EGFR. This is supported by three key findings: First, IKBKE directly binds to EGFR. Second, constitutively active EGFR phosphorylates IKBKE-Y153/Y179 *in vitro* and *in vivo*. Third, IKBKE, but not IKBKE-Y153F/Y179F, is activated by activating mutation of EGFR.

EGFR mutations are frequently detected in NSCLC patients, [209]. Transgenic murine models have shown that expression of mutant EGFR in the lung induces lung adenocarcinoma [210]. More importantly, NSCLCs with EGFR mutations exhibit sensitivity to EGFR-TKIs such as erlotinib and gefitinib; however, acquired resistance develops after a median of 9-14 months [38]. The most common mechanism of TKI resistance is a second-site mutation (T790M) in the EGFR kinase domain. EGFR^{T790M} allele also has been detected in a minority of tumors with primary resistance to these drugs (10). Our study showed that IKBKE is directly activated by activating mutations of EGFR including L858R, del19 and T790M. Furthermore, we demonstrated that knockdown of IKBKE largely inhibits EGFR-driven NSCLC cell survival. In addition, amlexanox, an IKBKE inhibitor currently in clinical trial for obesity and diabetes [202], preferentially inhibits cell viability in EGFR-mutant NSCLC cell lines. Although third-generation EGFR inhibitors, such as AZD9291, CO-1686 and WZ4002, are capable of inhibiting mutant EGFR with T790M, recent studies have shown that patients develop acquired resistance to these inhibitors due to new activating EGFR-C797S mutation [50, 51]. The role of IKBKE activation and therapeutic potential of IKBKE inhibitors in the cells bearing this mutation are yet to be studied. However, our data showed that IKBKE was activated by various mutants of

EGFR, and thus inhibition of IKBKE should reduce EGFR-C797S mutation driven cell growth and survival. Interestingly, inhibition of IKBKE moderately induces ERK1/2 activation even though the MAPK pathway is already activated in EGFR mutant NSCLC (Figures 16B, 16C, 17G and 17H). In agreement with these findings, combination of amlexanox with MEK inhibitor AZD6244 synergistically inhibited cell survival and xenograft tumor growth in EGFR-mutant NSCLC cells including EGFR^{T790M} mutation. Of note, it was previously shown that AZD6244 feedback activation of AKT leads to resistance to MEK inhibition [211]. Indeed, analysis of xenograft lysates from AZD6244-treated mice showed elevated level of AKT activation whereas the combination of amlexanox and AZD6244 abrogated both feedback pathways (Figure 17G and 17H). Taken together, these findings suggest that IKBKE is a critical mediator of cellular function of activating mutations of EGFR and that combinational inhibition of IKBKE and MAPK could be an effective therapeutic strategy for NSCLC with EGFR mutation and EGFR-TKI resistance, especially those driven by secondary EGFR mutation.
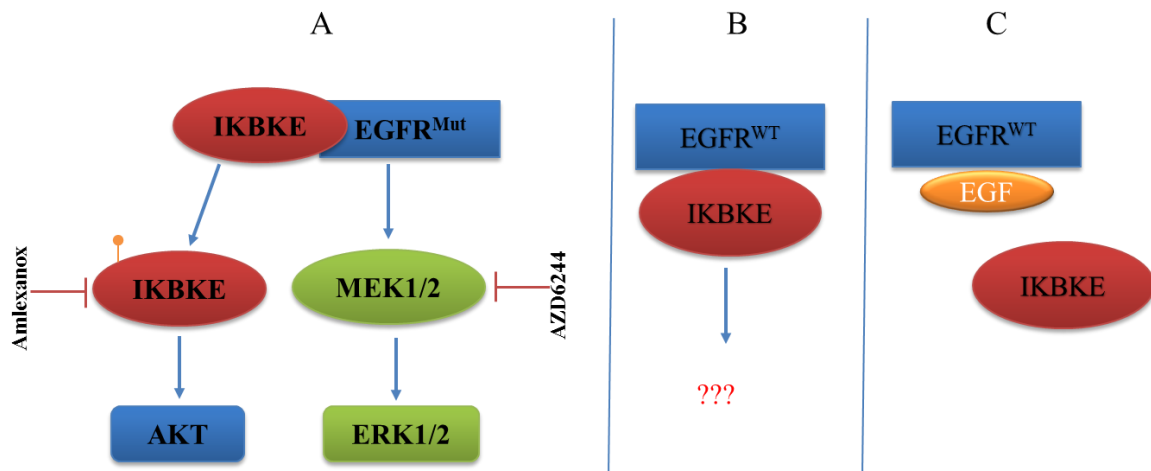
Figure 18: Schematic of IKBKE activation by EGFR. Mutant EGFR binds to and phosphorylates IKBKE (A). Although wildtype EGFR interacts with IKBKE (B), addition of EGF ligand led to disruption of this interaction (C). Since MEK/ERK pathway is another important signaling pathway downstream of mutant EGFR, co-targeting IKBKE and MEK1/2 shows better effect.

Extensive studies have focused on downstream targets of IKBKE, however, the upstream kinases responsible for activation of IKBKE are not yet known. In this study, we showed a novel mechanism of regulation of IKBKE, i.e., activating mutations of EGFR, but not EGF-stimulated wild type EGFR directly phosphorylate and activate IKBKE (Figure 18). This is the first study to identify tyrosine phosphorylation of IKBKE, thus underlining the importance of IKBKE in tumor cells. Interestingly, the EGFR phosphorylation residue, IKBKE-Y153 is conserved cross several species and also in its close homology kinase TBK1 but not in IKKα and IKKβ. Hence, active EGFR mutant may also phosphorylate TBK1. Indeed, a recent study showed that in the absence of ligand, wild-type EGFR constitutively interacts with TBK1 and IRF3 leading to activation of TBK1, while underlying mechanism is currently unknown, subsequently TBK1 phosphorylates IRF3 that results in transcription of target genes. When EGF is added, EGFR/TBK1/IRF3 complex is disrupted and EGFR now activates canonical downstream signaling pathways such as ERK and AKT [212]. In agreement with these findings, our data also show the interaction between wild-type EGFR and IKBKE which is reduced by EGF stimulation. Further investigation is needed to understand the mechanism and physiopathological significance of IKBKE interaction with wild-type EGFR.

Classically, activated EGFR triggers downstream signaling through recruiting various cytoplasmic proteins that transduce and regulate the EGFR function. The proteins recruited to active EGFR include many Src homology 2 (SH2) and phosphotyrosine binding (PTB) domain containing proteins which binds to the tyrosine phosphorylated residues in EGFR. However, recent studies showed that activated EGFR could form a complex with the proteins that do not have SH2 or/and PTB domain, such as RNA helicase A, TBK1 and IRF3 [212, 213]. In this study, we showed that N-terminal region of IKBKE, in which there is no sequence homology to

SH2 and PTB motifs, interacts with EGFR. Thus, we predict IKBKE/EGFR interaction via an unconventional manner which needs further investigation. Nevertheless, our study suggest that IKBKE is a direct downstream effector of activating mutant EGFR, including T790M mutation responsible for half of acquired TKI resistance in NSCLC and that inhibition of IKBKE and its feedback activation of MAPK could be an effective therapeutic strategy for secondary EGFR-mutation associated TKI-resistant NSCLC.

CHAPTER 3

Targeting IKBKE and its feedback circuit in pancreatic cancer

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer-related deaths in United States with an estimated 41,780 deaths in 2016. Prognosis for these patients is extremely poor with an estimated 5-year survival of 8% [1]. KRAS is the driver mutation in 95% of PDAC patients and directly targeting mutant KRas has been challenging. Therefore strategies aimed at targeting downstream signals of KRas are highly desired and under intense investigation. Current treatment strategies for pancreatic cancer include surgery, chemotherapy, and chemo-radiation therapies. Patients who become resistant to these treatments present with metastatic tumors that are highly lethal. Cancer stem cells with self-renewing capacity were previously identified in PDAC and in addition to tumor initiation, pancreatic cancer stem cells were implicated in resistance and cancer metastasis [97]. Hence identification of molecule(s) that are downstream of KRas and that can control pancreatic cancer stem cells will lead to new treatment strategies for this malignancy.

Inhibitor of I-KappaB kinase enhancer (IKBKE) has been identified as an important oncogene in several cancers [156, 158, 184, 187]. Overexpression of IKBKE was reported to be resulted from genetic changes such as copy number gain and transcriptional activation in breast cancer [158]. We have previously shown that STAT3 induced IKBKE transcription and resulted in chemotherapy resistance in lung cancers [154]. It was also shown that KRas induced IL1-β could upregulate IKBKE levels in NSCLC cells and these cells were sensitive to inhibition of

IKBKE/TBK1 [166]. Another study identified IKBKE as an important transcriptional target of Gli1 and a mediator of oncogenic activity of Gli1 in PDACs.[189] Moreover, upregulation of IKBKE induces cell transformation, cell survival, proliferation, migration/invasion and chemoresistance whereas depletion of IKBKE exerts opposite effects [154, 156-158, 180, 184, 187, 189, 214]. While it belongs to IKK kinase family, IKBKE only shares ~27% homology with IKKα and IKKβ and 64% with TBK1. IKBKE activates NFκB through phosphorylation of IκB-Ser32, CYLD-Ser418, TRAF2-Ser11 and p65-Ser536 [162, 168, 169, 180], which leads to induction of CCL5 and IL-6 and subsequent activation of JAK/STAT3 pathway [166, 215]. We have previously shown that IKBKE directly phosphorylates AKT at Ser473 and Thr 308 leading to its activation independent of PI3K and mTORC2 complex [157]. IKBKE was shown to be upregulated in ~30% breast cancers [158]. We have also shown that IKBKE is upregulated in and ~50% pancreatic cancers and increased expression of IKBKE is associated with poor patient survival [214]. Together, these studies show that IKBKE plays a pivotal role in human malignancy and could be a critical therapeutic target for PDAC.

Recent studies identified that two small molecule compounds, Amlexanox and CYT387, directly inhibited IKBKE/TBK1 kinase activity [165, 166, 215]. Amlexanox is FDA approved for the treatment of aphthous ulcers and it was shown to reduce obesity in mice on high-fat diet by sensitizing the cells to insulin [165]. CYT387 is a known JAK1/JAK2 inhibitor that is in clinical trials for myeloproliferative disorders [216-218]. CYT387 was also shown to inhibit IKBKE/TBK1 by direct inhibition of its kinase activity [166, 215]. In this study we show that depletion of IKBKE reduces cell viability and cancer stem cell property in PDAC. While IKBKE inhibitors significantly inhibit *in vivo* tumor growth and metastasis of PDAC cells, they also

activate the RTK/MAPK pathway, and thus they synergize with MEK inhibitor, trametinib, to further hinder PDAC growth and metastasis.

Materials and methods

Cell lines and reagents. The PDAC cells were purchased from ATCC and used within 6 months of thawing. L3.6pl, MiaPaca2, Panc1 were maintained in DMEM (Gibco) media with 10% FBS and 1% Penicillin-Streptamycin. ASPC1, T3M4, BXPC3 were cultured in RPMI 1640 containing 10% FBS and 1% Penicillin-Streptamycin. HPNE-vec and HPNE-Kras were cultured in media containing 75% DMEM with reduced glucose, 25% Medium M3 Base (Incell Corp Catalog # M300F-500), 5% FBS, 10ng/ml recombinant human EGF, 750ng/ml puromycin. Amlexanox was purchased from Abcam and CYT387, trametinib were from Selleckchem. CYT387 and trametinib used for *in vivo* studies were purchased from APExBIO. The shRNAs and expression plasmids for IKBKE were described previously [156, 184]. siRNAs for ErbB3 (Mission siRNA: SASI_Hs01_00196190) and IGF-1R (Mission siRNA: SASI_Hs01_00126194) were purchased from Sigma. Cell transfection was performed using Lipofectamine 2000 (Life Technologies) according to manufacturer's protocol. All of the antibodies except IKBKE (Sigma), IGF-1R (Santa Cruz), Ki67 (Abcam) and CD31 (Abcam) were purchased from Cell Signaling Technologies.

Cell viability and drug synergy studies. For cell viability, cells were plated at a density of 4000 cells/well in a 96-well plate with quadruple wells for each treatment. siRNA transfections were performed 24 hours after plating the cells. Inhibitor treatments were performed as indicated in figure legends. Cell viability was measured 72 hours after treatment with Thiazolyl Blue Tetrazolium Bromide (MTT) (Sigma) according to manufacturer's protocol.

For drug synergy studies, cells were treated with increasing concentrations (at equal ratios) of the inhibitors and cell viability was measured as described previously [219]. Combination indices for each treatment were calculated using Chou-Talalay method in CompuSyn [205].

Migration and invasion assays. Cell migration and invasion assays were performed as described before [220]. Indicated amount of inhibitors were added to the top chamber and the cells were allowed to migrate or invade at 37°C for 16 hrs.

3-D matrigel spheroid culture. Cells were cultured in "3D on top format" as described previously [221] with slight modifications. Reduced growth factor matrigel (2.5%, Corning) was used as the matrix and cells were cultured for 4 days. 3-D spheroids were counted using an inverted microscope at 10x magnification.

Cancer stem cells assays. Cancer stem cell populations were determined using Aldefluor kit from Stemcell Technologies according to manufacturer's protocol. Sphere assay was performed by growing cells at a density of 5000 cells/well in ultra-low attachment 6-well plates as described before [222]. For self-renewal assays, the spheroids were trypsinized and single cells were obtained by passing through a 40μm filter. 2000cells/well were re-plated and grown in non-adherent conditions as described before.

RNA isolation and RT-qPCR. RNA was isolated using Trizol reagent from Life technologies according to the manufacturer's instruction. RT-qPCR was performed as described previously [220]. Primer sequences are as follows: ErbB3: Forward 5'-ACAGTCTGCTGACTCCTGTT-3', Reverse 5'- GAACTGAGACCCACTGAAGA-3' IGF-1R: Forward 5'- TGTGTGGACCGTGACTTCTG -3', Reverse 5'-GGACCTTCACAAGGGATGCA -3'

Phospho-RTK arrays. Phospho-RTK arrays (R&D Biosystems) were used as described previously [223] with slight modifications. The RTK membranes were incubated with 50µg of lysates from L3.6pl cells treated with 2µM CYT387 or vehicle for 24 hours or 250µg of lysates from L3.6pl cells treated with 2µM CYT387 or vehicle for 36 hours. Detection of activated RTKs was performed according to the manufacturer's protocol.

Animals and Treatments. Female athymic nude mice (6 weeks old, 20-23 g) were purchased from Charles River (Wilmington, MA, USA). Mice (n = 20) were orthotopically implanted with luciferase expressing L3.6pl cells (one million in 50 µl) to the pancreas. After one week mice were randomly divided into treatment and control groups. The mice were treated with vehicle or Amlexanox at 50mg/kg for 5 days a week. For CYT387 treatments, the mice were divided into four groups as follows: Vehicle, CYT387, trametinib and CYT387 plus trametinib. The mice were treated as described before [215] at 10mg/kg or 2.5mg/kg of CYT387 and trametinib respectively for 5 days a week. Drug treatments were performed by oral gavage. The tumor volume was measured once a week using IVIS 200 (Xenogen). After treatment for 4 weeks, the animals were sacrificed and tumor weight and liver metastasis were recorded. Half of the tumor tissues were immediately immersed in liquid nitrogen for biochemical analyses and another half were fixed in buffered formalin for histological analysis. The care and use of the animals reported in this study were approved by Institutional Laboratory Animal Care and Use Committee (IACUC) and as per the guidelines of the National Institute of Health (NIH).

Statistical analysis. One way ANOVA was used for analyzing the animal experiments and two-tail unpaired *t*-test was used for analyzing statistics for all the experiments performed.

Results

IKBKE knockdown decreases PDAC cell viability, cancer stem cells and invasion.
We have previously identified that IKBKE expression is elevated in approximate 50% of PDAC patients by immunohistochemical staining of tissue microarray [214] and the higher levels of IKBKE correlate with poor patient survival. Moreover, we found that IKBKE expression is higher in early stages of pancreatic cancer suggesting its role in tumor initiation [214]. Here we further showed that IKBKE expression is elevated in several PDAC cell lines (Figure 19A). To study the dependence of PDAC growth on IKBKE expression, we depleted IKBKE in a panel of PDAC cells using two different siRNAs and found that viability of these cells were lower when IKBKE is depleted (Figure 19B). We noted that cells with wildtype KRas and low IKBKE were resistant to IKBKE knockdown. Since pancreatic cancer stem cells (CSCs) are intrinsically resistant to many therapeutic agents and highly tumorigenic and are responsible for PDAC metastasis and treatment failure [97], we examined the effects of IKBKE on pancreatic CSCs. The population of pancreatic CSCs evaluated by ALDH staining was significantly lower in IKBKE siRNA transfected cells when compared with control siRNA (Figure 19C). Moreover, stem cell sphere growth was also significantly reduced in IKBKE knockdown cells (Figure 19D). In addition, depletion of IKBKE led to decreased migration and invasion (Figure 19E and 19F) in L3.6pl cells. Collectively, these results demonstrate that IKBKE plays a critical role in PDAC cell survival, CSC growth and invasion.
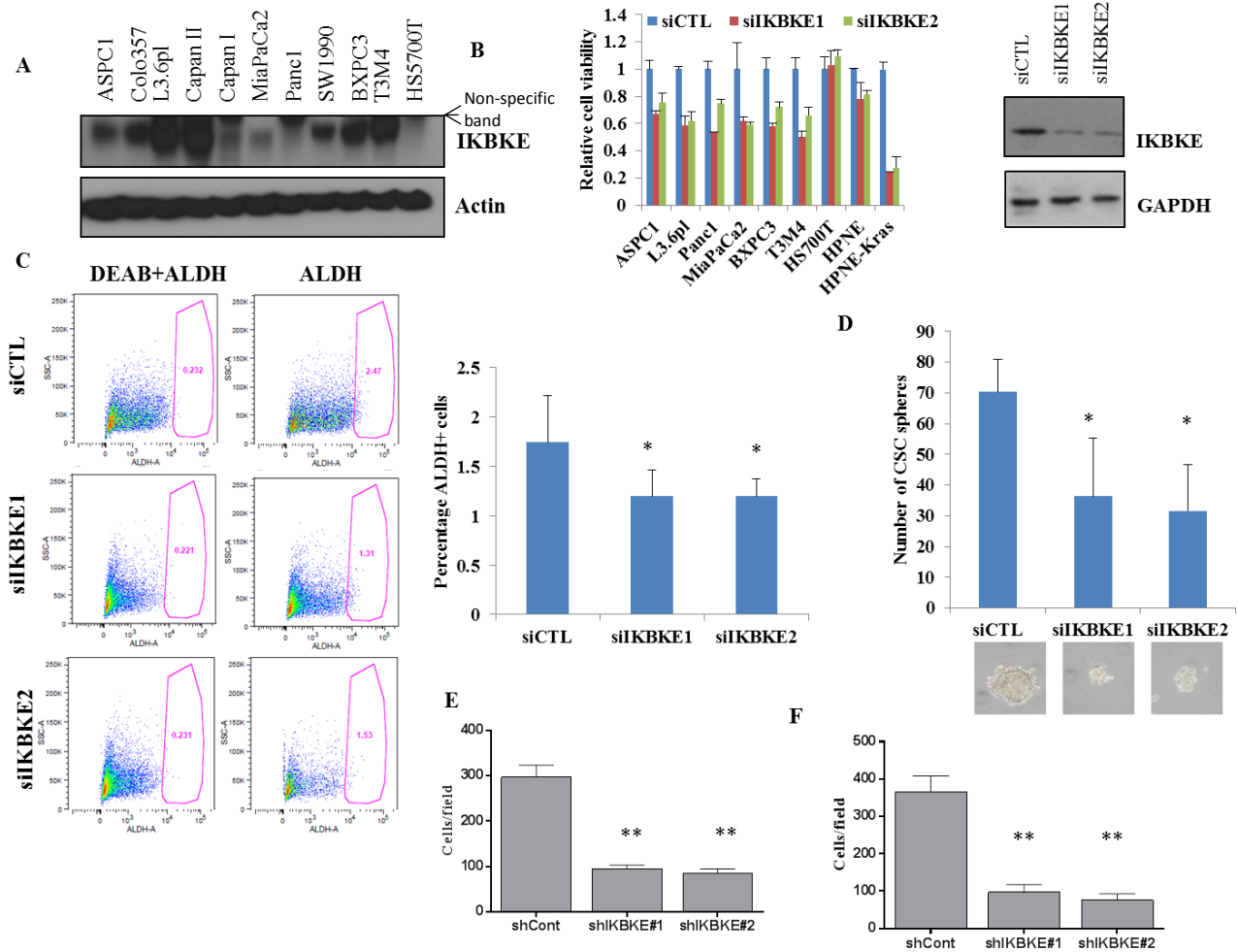
Figure 19: IKBKE is an important oncogene in Pancreatic Ductal Adenocarcinoma (A) Lysates from KRas^{mut} or KRas^{wt} PDAC cells were subjected to immunoblot with IKBKE and actin. (B) A panel of PDAC cells were transfected with two siRNAs for IKBKE. MTT assay was performed 72 hours after transfection. Knockdown efficiency of the siRNAs is shown. (C) Aldefluor assays were performed on L3.6pl cells with IKBKE knockdown or control cells as described under "Methods" section. DEAB was used as a control. (D) CSC spheres from control or IKBKE siRNA transfected l3.6pl cells. (E and F) L3.6pl cells transfected with control siRNA or IKBKE siRNAs were plated in boyden chambers for migration (E) or invasion (F) assays. *p<0.05 and **p<0.002

The effect of pharmacological inhibition of IKBKE on PDAC phenotype. CYT387 was recently identified as an inhibitor of IKBKE [165, 166]. To study the efficacy of CYT387 in PDACs, we treated 5 PDAC cells with increasing doses of CYT387 (Figure 20A) and measured the cell viability using MTT assay. In agreement with the IKBKE-knockdown data (Figure 19),

inhibition of IKBKE with CYT387 reduced cell invasion (Figure 20B) and migration (Figure 20C), the percentage of ALDH$^+$ cells (Figure 20D) in L3.6pl cells. Taken together, these data show that inhibitors of IKBKE could have an antitumor effect in highly metastatic PDAC cells.
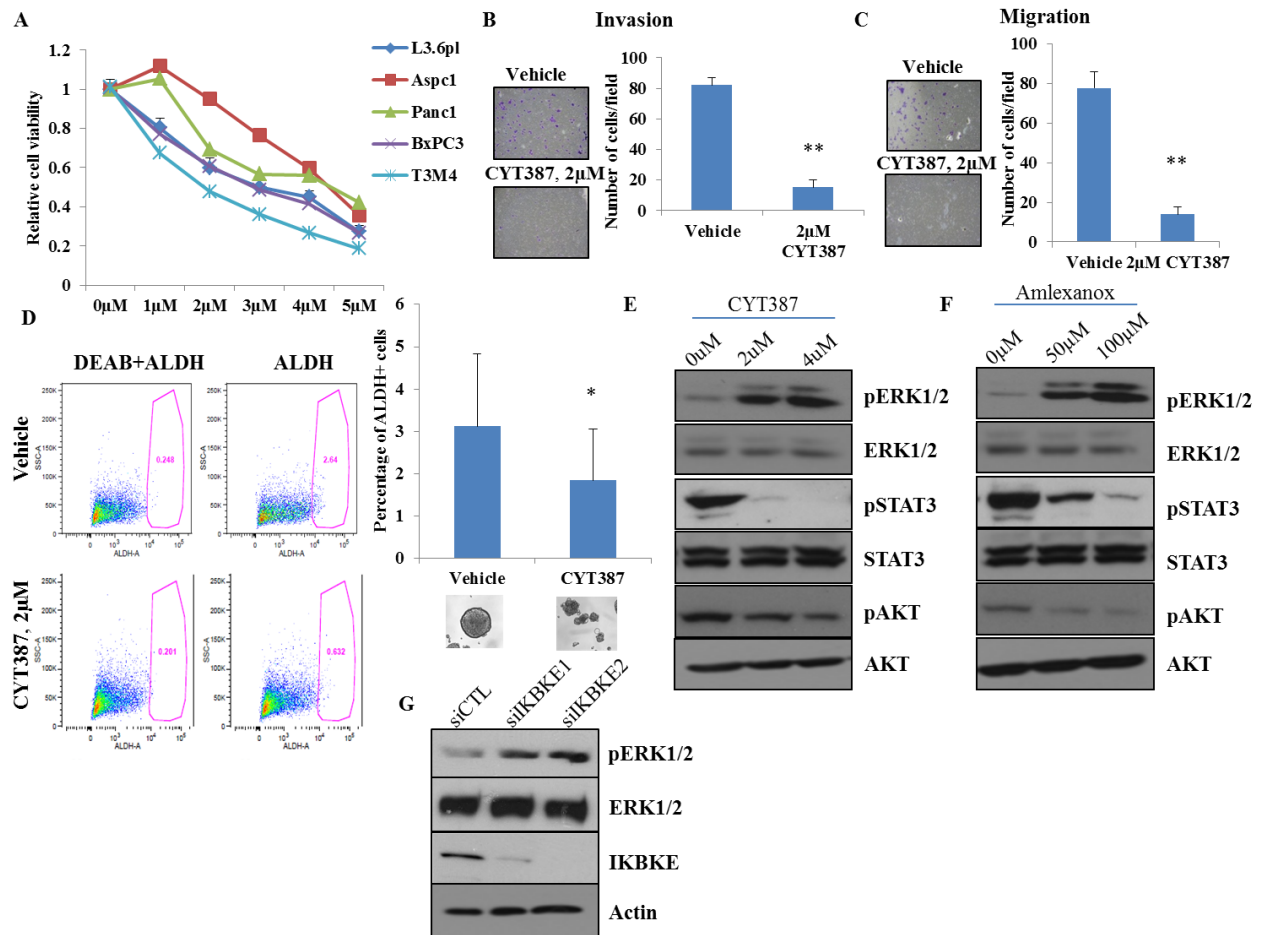
Figure 20: IKBKE/TBK1 inhibitor CYT387 reduced cell viability, cell motility and cancer stem cell populations in PDAC cells (A) Panel of PDAC cells were treated with increasing doses of CYT387 and MTT assay was performed after 72 hours. (B and C) L3.6pl cells were plated with indicated amount of CYT387 or vehicle for cell invasion (B) or migration (C) assays. (D) L3.6pl cells were treated with 2μM CYT387 for 96 hours and ALDH$^+$ cancer stem cell population was assessed using Aldefluor kit. Quantification and size of the spheroids are shown. (E and F) Western blot analysis of l3.6pl cells treated with different doses of CYT387 (E) or amlexanox (F) for 24 hours show decrease in phosphorylation of STAT3 and AKT and increase in phosphorylation of ERK1/2. G, Western blot analysis of L3.6pl cells transfected with control or IKBKE specific siRNAs for 72 hours *p<0.05 and **p<0.002

Inhibition of IKBKE leads to feedback activation of RTKs/MAPK pathway by reactivation of FOXO3. To understand the effect of IKBKE inhibitors on downstream signaling, immunoblot was performed in L3.6pl cells treated with increasing doses of CYT387 (Figure 20E) or amlexanox (Figure 20F) for 24 hrs. We found that both inhibitors abrogate pAKT and pSTAT3, two major downstream targets of IKBKE [157, 166].
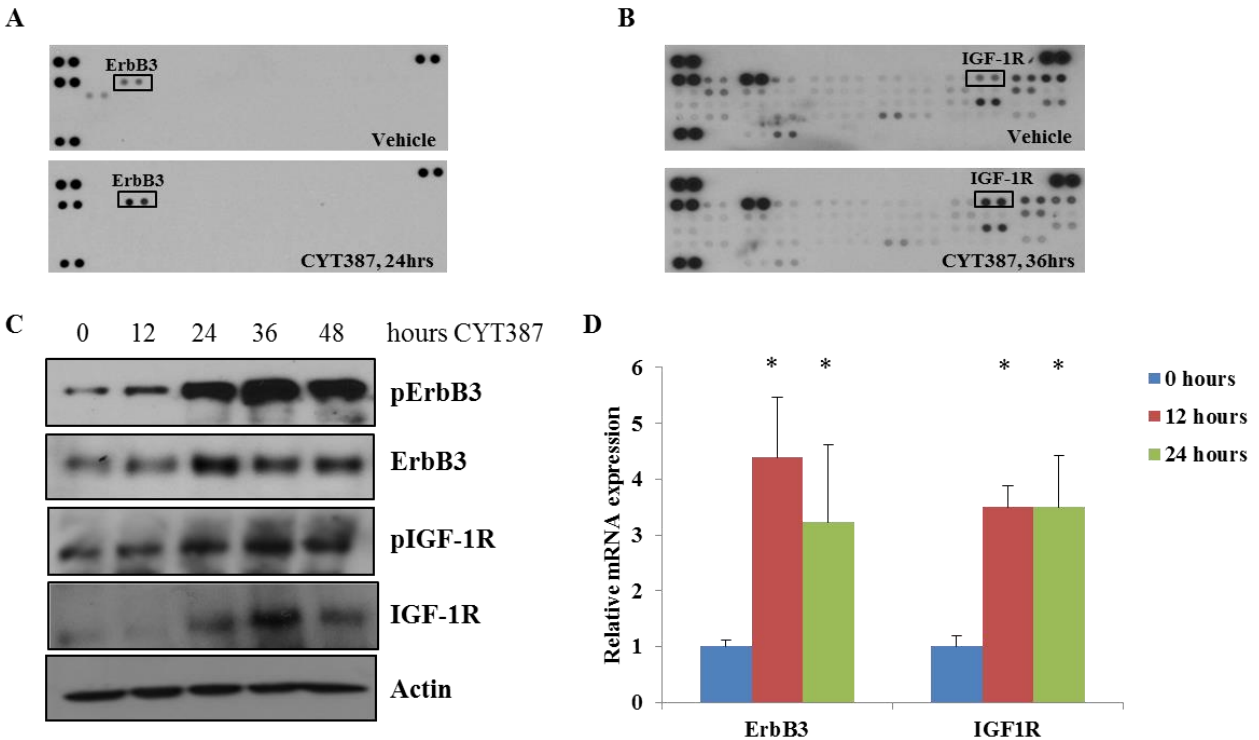
Figure 21: Inhibition of IKBKE leads to transcriptional upregulation of several RTKs (A) 50µg of lysates from L3.6pl cell treated with 2µM CYT387 for 24 hours were incubated with phospho-RTK arrays. Western blot with pan Tyrosine antibody revealed activation of ErbB3. (B) Phospho-RTK array analysis with 250µg of lysates from L3.6pl cells treated with 2µM CYT387 for 36 hours showed significant activation of IGF-1R. (C) Western blot analysis of lysates from L3.6pl cells treated with 2µM CYT387 for indicated times showed increase in phosphorylation and protein expression of IGF-1R and ErbB3. (D) Quantitative PCR with ErbB3 and IGF-1R primers using RNA from L3.6pl cells treated with 2µM CYT387 for indicated amount of time. Actin was used as a control. *$p < 0.05$

We previously showed that IKBKE inhibition led to activation of ERK1/2 pathway [219] in Non-small cell lung cancer cells. Here we found that IKBKE inhibition led to significant increase in pERK1/2 in PDAC cells (Figure 20E, 20F). To confirm that this feedback activation

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of ERK1/2 is due to inhibition of IKBKE, we performed Western blot analysis with lysates from IKBKE-knockdown or control siRNA-treated L3.6pl cells. IKBKE knockdown led to similar elevation of pERK1/2 (Figure 20G).

It was previously shown that inhibition of AKT leads to feedback activation of RTK/MAPK by reactivation of FOXO3 which induces RTK transcription [223]. Since IKBKE can inhibit FOXO3a by direct phosphorylation [184] or by activation of AKT, we hypothesized that inhibition of IKBKE-induced pERK1/2 could be resulted from RTK activation. By hybridization of the phospho-RTK array with CYT387 treated and untreated cell lysates, we found that CYT387 treatment led to marked increase in phosho-ErbB3 and phospho-IGF-1R (Figures 21A and 21B). Time course studies with CYT387 showed that ErbB3 and IGF-1R activation was observed by 24 hours. Furthermore, CYT387 treatment resulted in an increase in expression of ErbB3 and IGF-1R at protein (Figure 21C) and mRNA levels (Figure 21D).

We next examined if ErbB3 and IGF1R mediate IKBKE inhibition mediated feedback ERK1/2 activation. We transfected L3.6pl cells with siRNAs of ErbB3 and IGF1R followed by CYT387 treatment and found that knockdown of ErbB3 or IGF1R significantly decreased CYT387-activated ERK1/2 (Figure 22A). Similarly, inhibition of ErbB3 activity with a pan-ErbB inhibitor AZD8931 led to significant reduction in ERK1/2 activation when L3.6pl cells were treated with CYT387 (Figure 22B) or by IKBKE knockdown (Figure 22C). We also observed that amlexanox-mediated ERK1/2 activation could be inhibited by AZD8931. Together, we confirmed that ErbB3 activation is required for IKBKE inhibition mediated ERK1/2 activation.
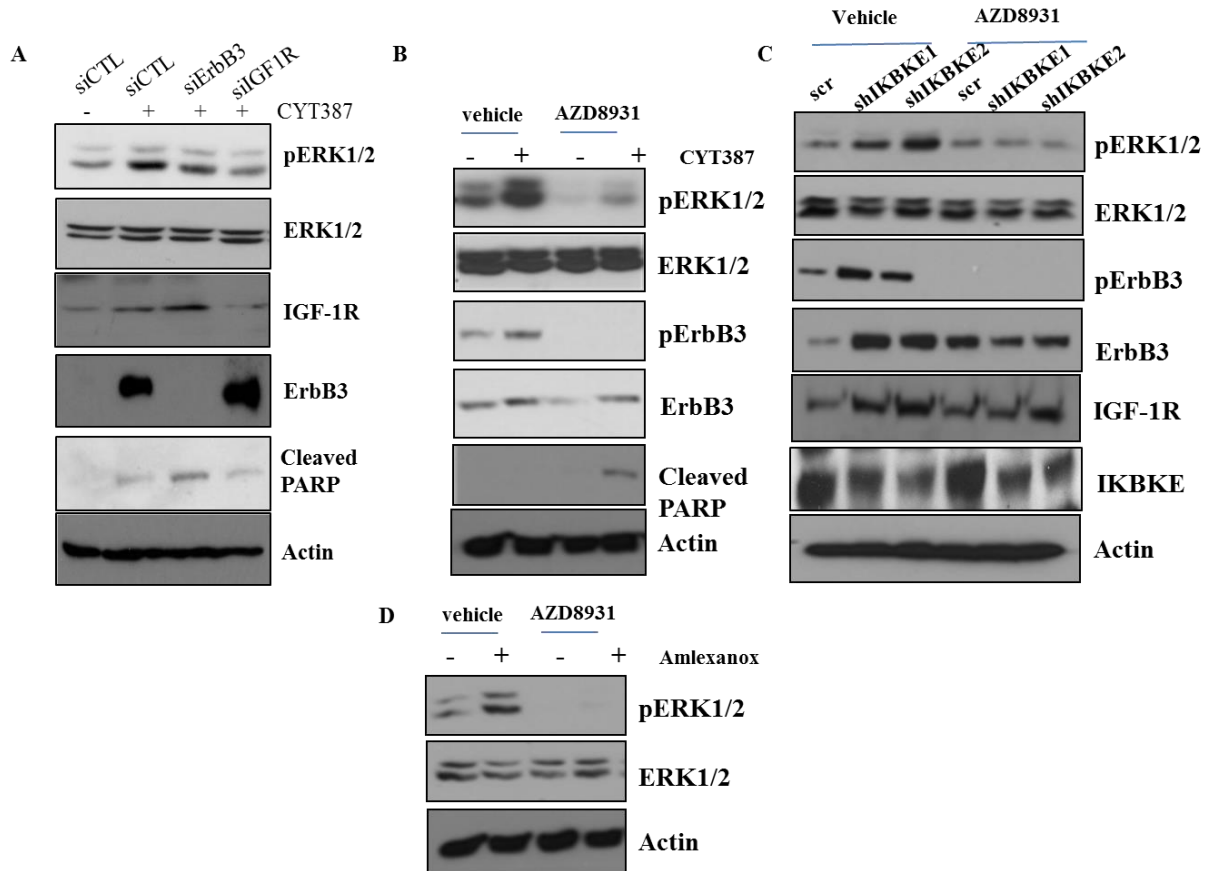
Figure 22: Inhibition of ErbB3 or IGF-1R inhibits CYT387 mediated ERK1/2 activation.
(A) L3.6pl cells were transfected with control siRNA or siRNAs targeting ErBB3 or IGF-1R. 24 hours later the cells were treated with 2uM CYT387 for additional 24 hours and western blot was performed using indicated antibodies. (B) L3.6pl cells were treated with CYT387 for 24 hours followed by treatment withpan-ErbB inhibitor AZD8931 for 5 hours and western blot was performed. (C) IKBKE knockdown cells or control cells were treated with AZD8931 for 5 hours and lysates were subjected to western blot. (D) L3.6pl cells were treated with amlexanox for 20 hours prior to AZD8933 treatment for 4 hours and western blot analysis was performed.

Having shown that IKBKE inhibition led to feedback activation of ErbB3 transcription, we wanted to study the mechanism involved in this. FOXO3a was shown to act as a transcription factor to upregulate ErbB3 and IGF-1R expression upon AKT inhibition [223]. Since we previously showed that IKBKE can inhibit FOXO3a activity through activation of AKT or by direct phosphorylation, we hypothesized that FOXO3a is activated with IKBKE inhibition using CYT387. Indeed, we found that CYT387 inhibited phosphorylation of FOXO3a but induced the expression of total FOXO3a, ErbB3 and IGF-1R (Figure 23A). Knockdown of FOXO3

significantly reduced CYT387-induced expression of ErbB3 and IGF-1R at protein and mRNA levels as well as pERK1/2 (Figure 23A and 23B). These results indicate that pharmacological and genetic inhibition of IKBKE feedback activates the MAPK pathway through induction of RTKs by reactivation of FOXO3.
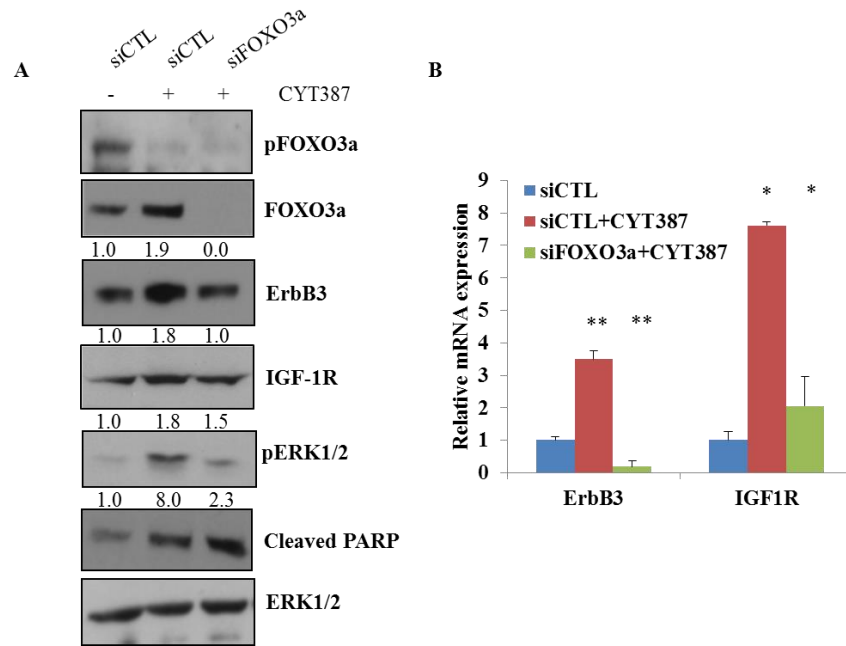
Figure 23: FOXO3a is required for activation of ErbB3/IGF1-R with CYT387. (A) l3.6pl cells were transfected with control or FOXO3a specific siRNAs. After 36 hours, the cells were treated with CYT387 for additional 24 hours and western blot was performed using indicated antibodies. The band intensities were quantified using ImageJ software. (B) l3.6pl cells were treated as described in (A) and RNA was isolated. Quantitative PCR with ErbB3 and IGF-1R primers were performed. *p<0.05 and **p<0.002

Combination of IKBKE and MEK inhibitors inhibited PDAC cell survival and cancer stem cell renewal. Since inhibition of IKBKE feedback activates the MAPK pathway, we next investigated the effect of combinational IKBKE and MEK inhibitor on PDAC cell viability, proliferation and cancer stem cell growth. CYT387 synergized strongly with trametinib, a FDA-approved MEK inhibitor, in l3.6pl cells (Figure 24A). Furthermore, the combination treatment resulted in less colony growth than either alone (Figure 24B) and a

dramatic decrease in cancer stem cell self-renewal (Figure 24C). Western blot analysis revealed that trametinib completely abrogates CYT387-induced ERK activation (Figure 24D). In agreement with a previous report [224], we found that trametinib treatment alone induced pSTAT3 that was inhibited by CYT387 treatment (Figure 24D). Notably, combination treatment led to significant increase in PARP cleavage (Figure 24D).
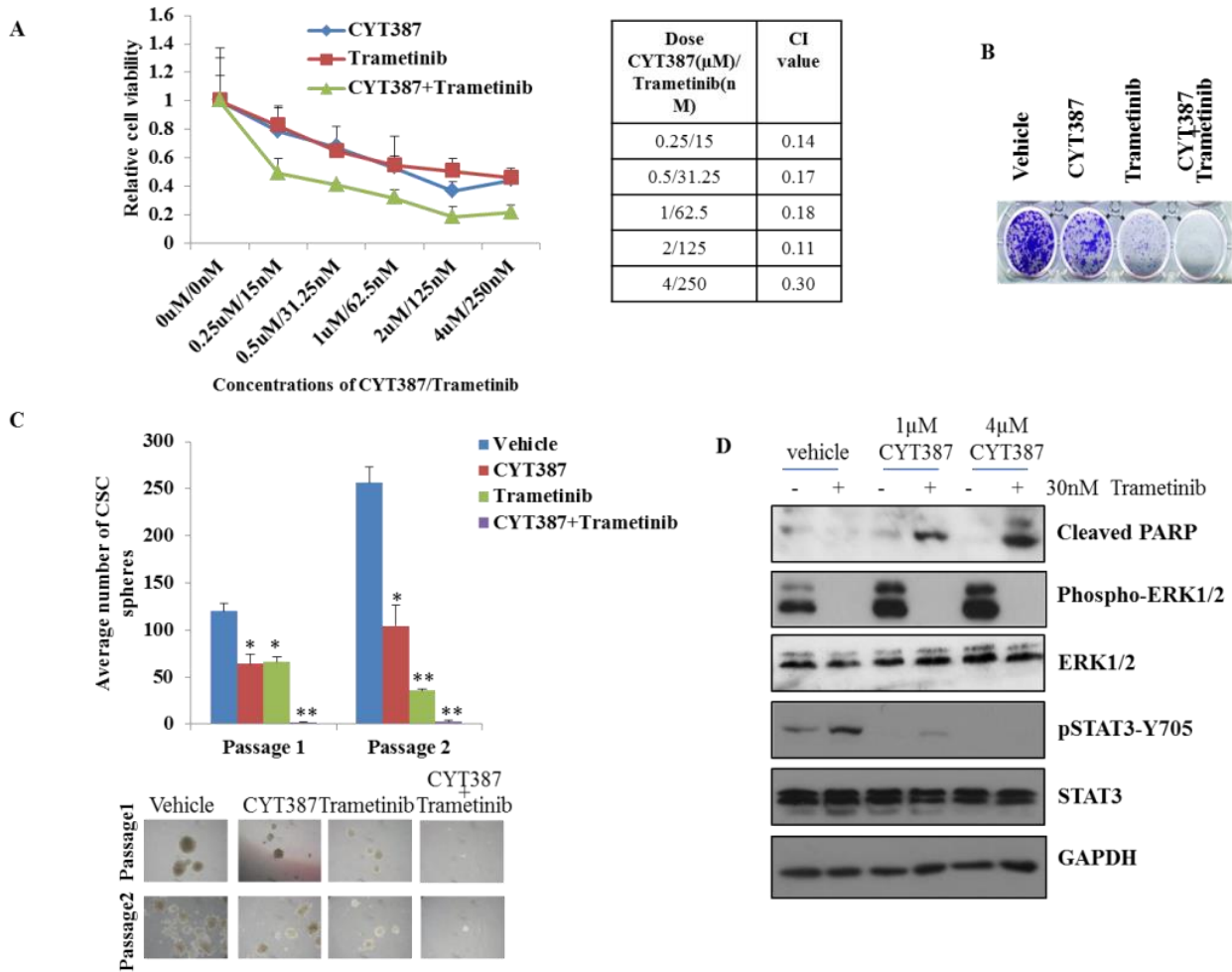
Figure 24: CYT387 synergizes with Trametinib in Pancreatic cells (A) MTT assay was performed on L3.6pl cells that were treated with indicated amounts of CYT387 and Trametinib for 72 hours. CYT387 and Trametinib show synergy in reducing cell viability. Combination indices are shown. (B) L3.6pl cells were cultured in 1μM CYT387 and 15nM Trametinib or the combination for 10 days and colonies were visualized by crystal violet staining. (C) L3.6pl cells were cultured under CSC culture conditions with 2μM CYT387 or/and 30nM Trametinib. After

Figure 24 (Continued)
10 days the number of colonies was counted. Two passages of cells were performed. Representative colony sizes are shown. (D) L3.6pl cells were treated with indicated amount of CYT387 and Trametinib as shown and western blot was performed using indicated antibodies. *p<0.05 and **p<0.002

Combination of IKBKE and MEK inhibitors inhibited PDAC tumor growth and metastasis. We further tested the *in vivo* efficacy of inhibition of IKBKE and MEK alone and their combination in orthotopic PDAC model. L3.6pl cells were orthotopically implanted in 6-week-old nude mice. Following a week of implantation, the mice were treated with CYT387 and trametinib individually and their combination as well as vehicle control for 4 weeks. Tumor growth was monitored by Xenogen weekly. Treatment with CYT387 alone showed a 37% decrease in tumor volume while trametinib had 45% decrease. The combination treatment resulted in 67% reduction in tumor volume (Figure 25A and 25B). After completion of 4-weeks treatment, liver metastasis was examined by Xenogen analysis of the removed liver and quantified (Figure 25C). Liver metastasis is significantly lower in combination treated mice than mice treated with either CYT387 or trametinib alone (Figure 25C). Similarly, IHC analysis showed an increase in apoptosis and decrease in proliferating cells and angiogenesis in the combination treatment (Figure 25D). In agreement with the *in vitro* observations, CYT387 induced activation of ERK1/2 *in vivo* (Figure 25D). Further analysis of the xenograft tissues by Western blot revealed that trametinib treatment led to a significant increase in pAKT and combination treatment abrogated these feedback activation events (Figure 25E). Interestingly, RT-qPCR analysis showed an increase in expression of ErbB3 and IGF-1R in CYT387 or/and trametinib treatment groups (Figure 25F), which support our findings (Figure 21) and previous report that inhibition of ERK1/2 led to increase in ErbB3 and IGF-1R expression [211].

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Together, these data indicate co-targeting IKBKE and its feedback activation pathway as a promising targeted therapy in PDAC.
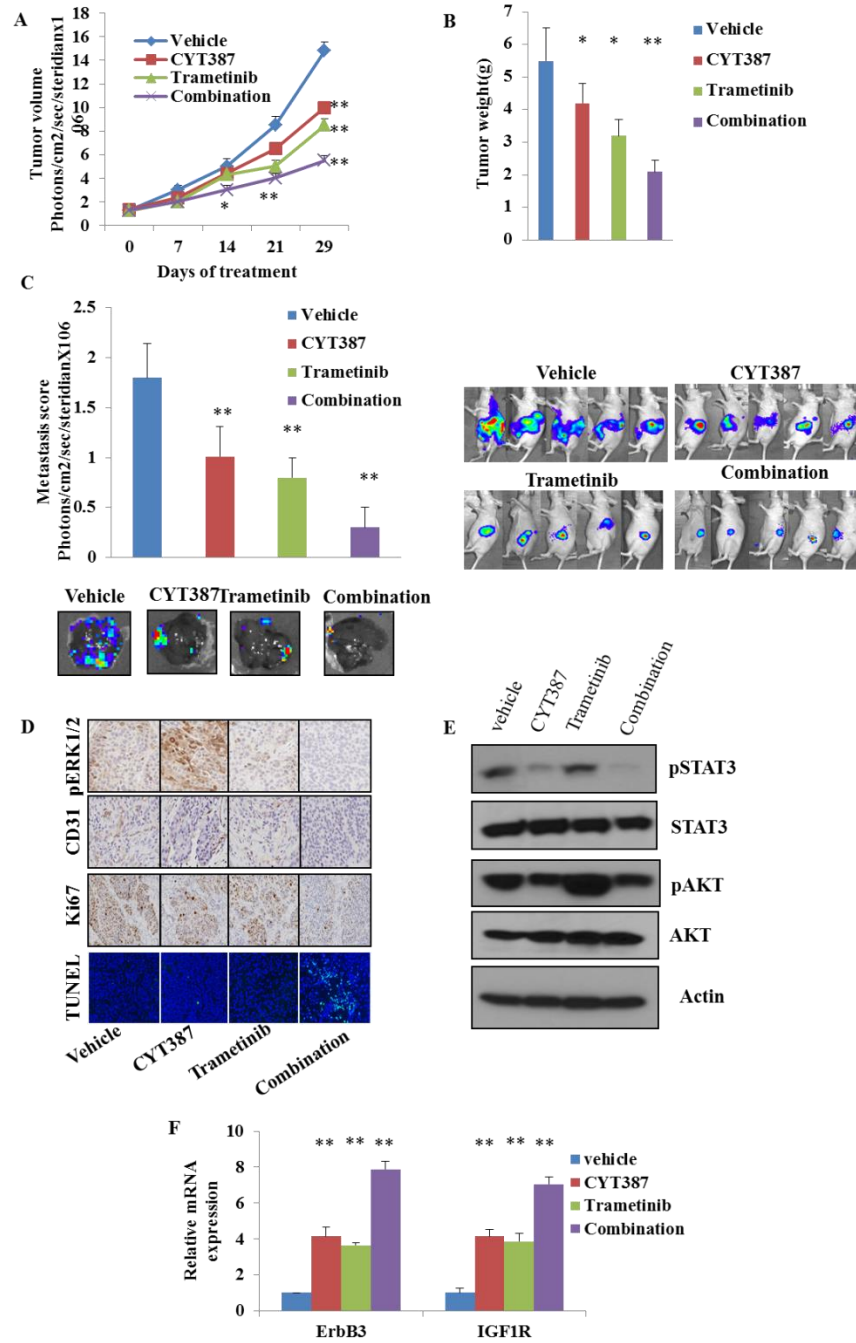
Figure 25: Combined CYT387 and Trametinib treatment synergistically decreased in vivo PDAC tumor growth and metastasis (A) Tumor volumes of the l3.6pl orthotopic tumors were measured once in a week for 4 weeks. (B) Tumor weights from mice bearing L3.6pl orthotopic

Figure 25 (Continued)
tumors and the representative images of mice with tumors at end point are shown. (C) Liver metastasis scores at the end-point. (D) IHC staining with pERK1/2, CD31, Ki67 and TUNEL of the orthotopic tissues. (E) Western blot analysis of mouse tissues were performed with indicated antibodies (F) Quantitative PCR with ErbB3 and IGF-1R primers was performed with RNA isolated from the mouse tissues. GAPDH was used as a reference. *p<0.05 and **p<0.002

Discussion

While KRAS mutations are observed in about 95% of PDAC patients, targeting KRAS has not been successful. Hence, it is important to understand and target the pathways downstream of KRAS signaling. In this study, we demonstrated that depletion of IKBKE reduces cell viability, cancer stem cell renewal and cell motility in PDACs. Mutant KRAS was previously shown to regulate IKBKE expression through Gli transcription factor [189]. But the efficacy of pharmacologic inhibitors of IKBKE were not evaluated in PDACs.
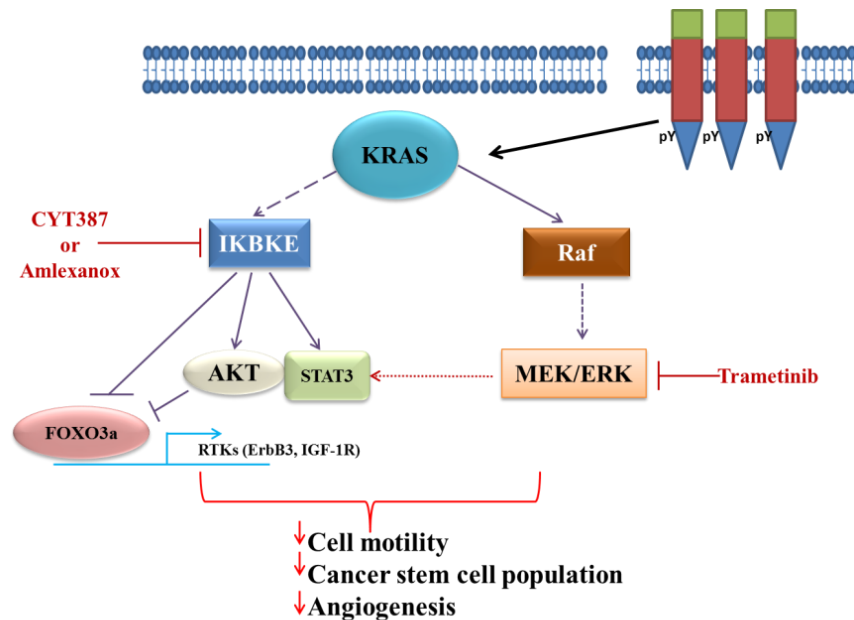
Figure 26: Schematic of IKBKE feedback circuit in pancreatic cancer. MEK/ERK and IKBKE are important mediators of KRas signaling. Inhibition of IKBKE leads to feedback activation of RTK expression. Release of FOXO3a inhibition is important for this feedback RTK expression. This leads to increased MEK/ERK signaling. Co-inhibiting both these pathways synergistically inhibit cancer stem cell population, *in vivo* tumor growth and metastasis.

Previous studies have shown that the MAPK and PI3K/AKT are major signal cascades to mediate KRAS oncogenic activity [225]. Inhibition of MAPK signaling leads to feedback activation of AKT[211] and STAT3 pathways[224] and inhibition of AKT leads to feedback activation of MAPK pathway [223]. Hence combined inhibition of both the MAPK and PI3K/AKT pathways are currently evaluated [226]. In this study we characterized that IKBKE is a targetable downstream effector of KRAS signaling. We also showed that CYT387, a JAK and IKBKE/TBK1 inhibitor that is in clinical trials for Myelodysplastic syndromes are efficient in inhibiting cell viability, invasion, migration and cancer stem cell population *in vitro.* While CYT387 showed moderate effect on tumor growth and metastasis *in vivo* and we found that inhibition of IKBKE led to feedback activation of ERK1/2 due to rapid up regulation of the expression of ErbB3 and IGF-1R (Figure 26). ErbB3 and IGF-1R upregulation was implicated in therapeutic resistance in several studies [211, 222, 223, 227-232]. Although this study has identified ErbB3 and IGF-1R as the major RTKs that mediated feedback activation of ERK1/2 with CYT387, there could be several mechanisms that lead to activation of ERK1/2 as shown previously in melanoma resistance to vemurafenib [233].

IKBKE regulates multiple oncogenic pathways such as AKT and STAT3. Activation of AKT pathway was shown as a resistance mechanism for MEK inhibition in KRas[mut] cancers [211], STAT3 pathway activation was shown to be responsible for increased metastasis of melanoma cells with MEK inhibition [224]. Moreover, activation of STAT3 pathway was implicated in resistance to several targeted therapies [230]. Hence targeting IKBKE in these scenarios may show better efficacy.

Cancer stem cells were previously shown to be important for pancreatic tumorigenesis and metastasis [97, 234]. It was also shown that the stem cell-like cells have an ability to differentiate into endothelial cells and promote angiogenesis [235, 236]. Interestingly we found that IKBKE expression can modulate CSC population. Although the mechanism by which IKBKE regulates CSCs is unknown, it was shown that TBK1, a homolog of IKBKE plays a role in cancer stem cell self-renewal and Tyrosine Kinase Inhibitor resistance in lung cancers [237]. Hence inhibitors targeting IKBKE/TBK1 are therapeutically significant.

Furthermore, we have shown that the IKBKE inhibitors synergize with a MEK inhibitor Trametinib to reduce cell viability and cancer stem cell population *in vitro* and tumor growth and metastasis *in vivo.* We have also shown that the combination treatment synergistically reduce *in vivo* cell proliferation and angiogenesis. Of note, Trametinib treatment *in vivo* led to an increase in phospho-AKT as shown before [211]. Hence, co-treatment with IKBKE inhibitors can increase the efficacy of Trametinib. This study has identified a novel targeted therapeutic strategy for pancreatic cancers.

CHAPTER 4

Activation of EphA2/IKBKE by PARP inhibitor: a mechanism and a critical target of PARP inhibitor resistance

Introduction

Ovarian cancer is the fifth leading cause of death in the United States with an estimated 14,180 cases of ovarian cancer-related deaths in 2015. Common treatment strategies include surgical resection, chemotherapy. Recently, PARP inhibitors have shown efficacy in BRCA-related ovarian cancers. Moreover it was shown that combined treatment of PARP inhibitor, Olaparib and cisplatin showed efficacy in BRCA deficient ovarian cancer patients [238]. Although the patients show good response, they become resistant to the therapy eventually. The proposed mechanisms of resistance to PARP inhibitors include loss of expression of PARP1, 53BP1, secondary mutations in BRCA genes [150, 151]. Another proposed resistance mechanism to Olaparib (AZD2281) is the overexpression of P-Glycoprotein that results in efflux of olaparib from the cells [239]. While past research on PARP inhibitor resistance focused on studying the role of Homologous recombination related genes, recent studies have identified growth factor signaling pathways such as PI3K, NFκB and c-Met as mediators of resistance to PARP inhibitors [240-242]. Feedback activation of these survival pathways were shown to be major contributors of resistance to targeted therapies. In this study we sought to identify novel feedback pathways activated by the PARP inhibitor olaparib/AZD2281.

EphA2 is a receptor tyrosine kinase with biphasic signaling potential. Tyrosine phosphorylation of EphA2 induced by the Ephrin ligand results in inhibition of the downstream

signaling pathways such as RhoA and FAK [128]. But in the absence of the ligand, EphA2 is phosphorylated by AKT, RSK1, Src in response to growth factors such as HGF and TNFα [129, 130, 243]. These phosphorylation events activate EphA2 downstream signaling pathways. EphA2 is overexpressed in ovarian cancers and its expression correlate with high grade cancers and poor survival [132]. It was also previously shown that co-treatment of EphA2 ligand with cisplatin reduced tumor weight and increased survival in murine ovarian cancer models. This synergistic effect was attributed to the ligand-mediated inhibition of angiogenesis [244]. EphA2 was shown to be activated by EGFR and BRaf inhibitors and it is implicated in resistance to these targeted therapies [245, 246]. Although vast amount of studies showed that feedback activation of RTKs in response to targeted therapies and chemotherapy can confer resistance, such mechanisms were not identified in the context of PARP inhibitors. Here we report that EphA2 is activated by AZD2281.

IKBKE is a non-canonical IKK-like kinase that activates AKT and NF-KappaB pathways. IKBKE was found to be overexpressed in ovarian cancers and its expression leads to resistance to chemotherapy [156]. Furthermore IKBKE was shown to induce ovarian cancer metastasis by the induction of p38 [187]. Although inhibitors of IKBKE have been elusive, CYT387, a known JAK1/2 inhibitor in Phase III clinical trials for Myelodysplastic syndrome was identified as an IKBKE/TBK1 inhibitor. CYT387 was shown to inhibit the autocrine cytokine production that led to inhibition of K-Ras dependent lung cancers and Triple Negative Breast Cancers [166, 215] but its efficacy in ovarian cancers is not known. In this study we report that olaparib/AZD2281 treatment led to activation of EphA2-IKBKE-AKT feedback loop. Inhibition of this feedback circuit by knockdown of EphA2 or IKBKE or by inhibition of IKBKE with CYT387 sensitized ovarian cancer cells to AZD2281.

Materials and methods

      Cell lines and reagents The ovarian cancer cell lines were a kind gift from Dr. Lin Zhang. All the cells were cultured in RPMI media supplemented with 10% fetal bovine serum and 1% Penicillin-Streptomycin. CYT387 and Olaparib were purchased from Selleckchem. Antibodies for AKT Ser473,AKT Thr 308, phospho STAT3 Y705, phospho EphA2 Ser897, EphA2 were from Cell Signaling technologies. siRNAs for EphA2 and EphB2 and control siRNAs were purchased from Sigma. Plasmids for EphA2 overexpression was a kind gift from Dr. Jennifer Isaacs. shRNA and overexpression plasmids for IKBKE were described before. sgRNA targeting EphA2 was obtained by oligo-cloning the guide RNAs into pLentiCRISPRv2 plasmid according to the manufacturer's protocol. Sequences for guide RNA is 5'-CTACTATGCCGAGTCGGACC-3'.

      Phospho-RTK array Phospho-RTK arrays were purchased from R&D biosystems and they were used according to manufacturer's protocol.

      ***in vitro* kinase assay of IKBKE** *in vitro* kinase assays were performed as shown before. GST-IκB was used as a substrate and the kinase reaction was immunoblotted with pIκB Ser32/36 antibody(Cell signaling Technologies).

      Cell viability assays and synergy studies For measuring cell viability, cells were plated at a density of 4000cells/well in a 96-well plate. After 24hours, the cells were treated with indicated amount of inhibitors and MTT assay was performed after 72 hours. Combination indices were calculated using Compusyn software.

Results

IKBKE is activated by olaparib treatment. We and others previously showed that overexpression of IKBKE results in resistance to chemotherapy [154-156]. Here we want to understand the precise mechanism regulated by IKBKE in therapy resistance. Since PARP inhibition was shown to result in accumulation of DNA damage and previously it was shown that IKBKE is activated upon DNA damage induction [193], we hypothesized that PARP inhibition will lead to IKBKE activation. Indeed, endogenous IKBKE purified from ovarian cancer cells treated AZD2281 showed higher kinase activity compared to IKBKE purified from vehicle treated cells (Figure 27A and B). Also, time course experiments showed an increase in IKBKE autophosphorylation at Serine 172 by 24 hours of AZD2281 treatment and it persisted for up to 48 hours. Interestingly, we also found that AZD2281 led to a significant increase in AKT and STAT3 activation that are known to be downstream of IKBKE (Figure 27C). Together these data confirm that IKBKE is activated in response to AZD2281.
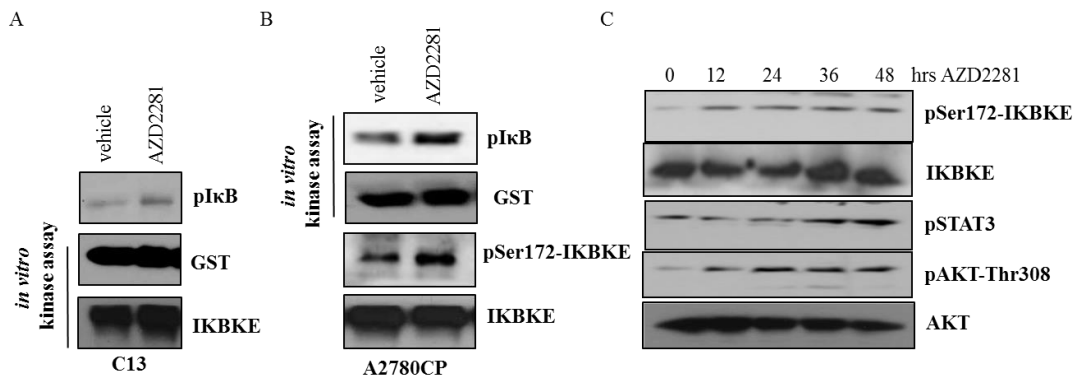
Figure 27: IKBKE is activated by olaparib treatment (A and B) C13 (in A) and A2780CP (in B) cells were treated with 20μM AZD2281 for 24 hours. IKBKE was purified from these cells and kinase assay was performed using GST-IκB as a substrate. (C) Immunoblot analysis of C13 cells treated with 20μM AZD2281 for indicated time points.

EphA2 is activated upon PARP inhibition. Since we have established that IKBKE is activated upon PARP inhibition, we sought to understand the mechanism of this activation. As we previously showed that IKBKE can be activated by EGFR [219] and upregulation of RTKs is a common resistance mechanism for both targeted therapies and chemotherapy [55, 247, 248], we hypothesized that IKBKE activation upon AZD2281 treatment could be mediated by RTKs. We performed a phospho-RTK array analysis to identify if any RTKs are activated upon PARP inhibition. Interestingly, we observed an increase in Tyrosine phosphorylation of EphA2, EphB2 and c-Met (Figure 28A). The increase in Tyrosine phosphorylation of EphA2 and EphB2 were further confirmed by immunoprecipitation of endogenous EphA2 or EphB2 from C13 cells and immunoblot with pan Tyrosine antibody (pTyr) (Figure 28B). Tyrosine phosphorylation of EphA2 can have a biphasic outcome on cell survival and motility. Although Ephrin-mediated EphA2 phosphorylation inhibits cell growth and invasion, Src mediated phosphorylation leads to activation of oncogenic signaling that enhance cell growth and invasion [128, 243]. EphA2 Ser897 phosphorylation by AKT was primarily studied as a ligand-independent mechanism of activation of EphA2 [129]. Since we have previously observed an increase in AKT activation by AZD2281, we tested the effect of AZD2281 on EphA2 Ser897 phosphorylation. Analysis of the time course lysates showed a significant increase in EphA2 Ser897 phosphorylation by 24 hours that continued to increase until 48 hours post-treatment (Figure 28C). Thus we conclude that AZD2281 treatment led to EphA2 activation by inducing Serine 897 phosphorylation.

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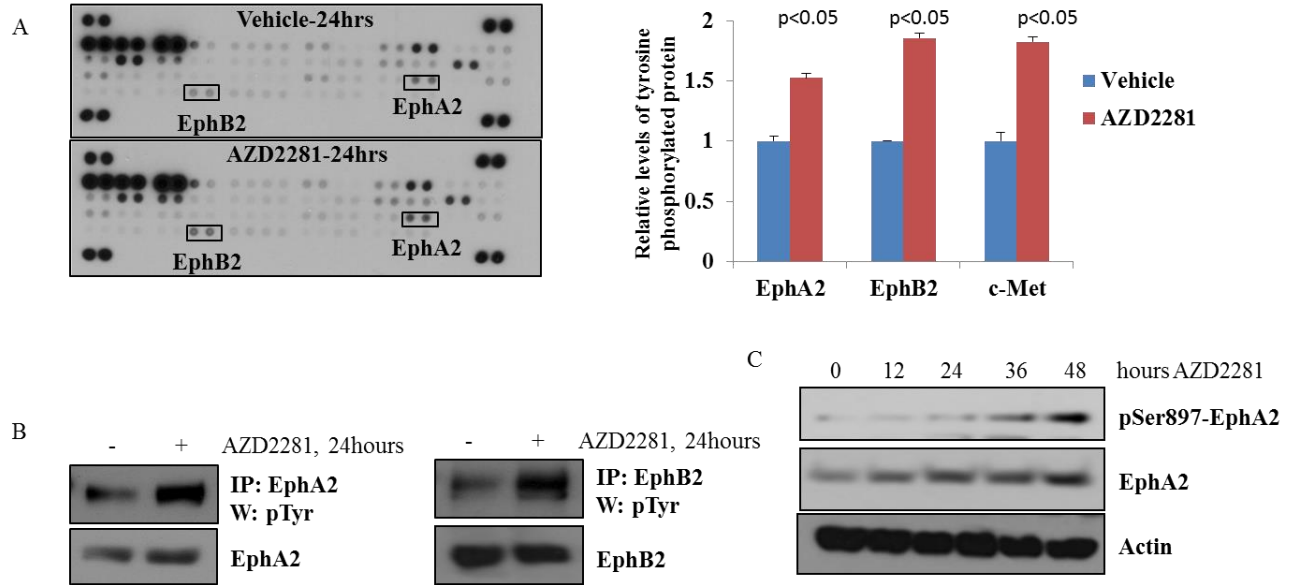
Figure 28: EphA2 is activated upon PARP inhibition (A) Phospho-RTK array with lysates from C13 cells treated with vehicle or 20μM AZD2281 for 24 hours. The dots corresponding to EphA2, EphB2 and c-Met were quantified and normalized to the control dots. (B) EphA2 and EphB2 were immunoprecipitated from C13 cells that were untreated or treated with 20μM AZD2281 for 24 hours. Immunoblot with pan-Tyrosine antibody was performed. (C) Lysates from C13 cells treated with 20μM AZD2281 for the indicated time were subjected to immunoblot analysis.

Recent studies showed that protein tyrosine phosphatases such as LMW-PTP and PTP1B regulate activation of EphA2 [249-251]. Dephosphorylation of EphA2 by PTP1B was shown to inhibit membrane localization of EphA2 leading to its lysosomal degradation [249]. Here we noticed that the cells treated with AZD2281 showed increased membrane localization of EphA2 (Figure 29A). Hence we hypothesized that PTPs might play a role in the activation of EphA2 upon olaparib treatment. Interestingly, we observed a significant inhibition of AZD2281 mediated EphA2-Ser897 activation when PTP1B was overexpressed (Figure 29B). Further investigation is required to understand the effect of olaparib on PTP1B and the precise mechanism of regulation of EphA2 by PTP1B in the context of olaparib treatment. Nevertheless,

these data show that olaparib treatment led to activation of Eph receptors in part by modulation

of PTP1B.
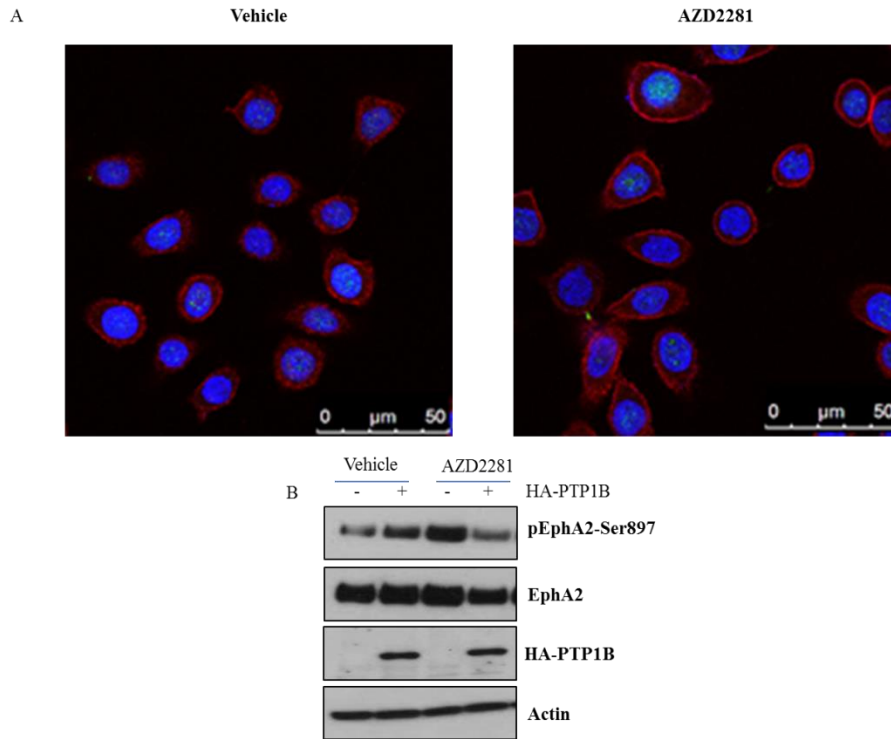
Figure 29: PTP1B is critical for AZD2281 mediated EphA2 activation. (A) C13 cells were treated with vehicle or AZD2281 for 24 hours and the cells are immunostained with EphA2 antibody. DAPI was used to stain the nuclei. (B) A2780CP cells were transfected with an empty vector or HA-PTP1B expressing vectors. 48 hours after transfection, the cells were treated with vehicle or 20μM AZD2281 for 24 hours and immunoblot analysis was performed for pEphA2-Ser897 and EphA2 (panel 1 and 2). Expression of HA-PTP1B was also shown (panel 3).

EphA2 is required for olaparib mediated IKBKE/AKT activation. Next we sought to

determine if EphA2 plays a role in activation of IKBKE and AKT pathways in the presence of

olaparib. Knockdown of EphA2 cells led to complete inhibition of IKBKE autoactivation

induced by AZD2281 in C13 cells (Figure 30A). Similarly, knockdown of EphA2 in C13 cells

resulted in decreased AKT activation (Figure 30B). This further confirms previous reports

suggesting that AKT is a target of EphA2 signaling [243]. To further understand the role of

EphA2 signaling in olaparib treatment, we generated EphA2 knockout A2780CP cells using

CRISPR-Cas9 system. We found that EphA2 knockout led to significant decrease in phospho AKT levels and that over expression of IKBKE could rescue this loss in AKT activation in the EphA2 knockout cells (Figure 30C). These data together show that EphA2 is required for the activation of IKBKE and AKT upon treatment with AZD2281 and EphA2 regulates AKT in part through IKBKE pathway.
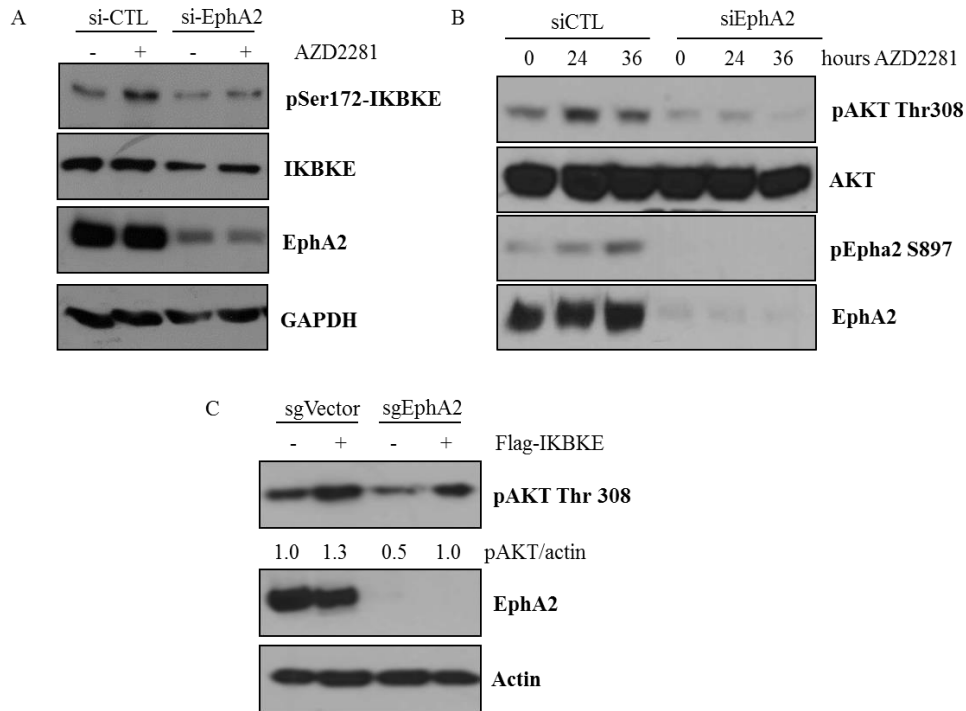
Figure 30: EphA2 is required for olaparib mediated IKBKE/AKT activation (A) C13 cells were transfected with siRNA targeting EphA2 or a control siRNA for 48 hours before treatment with 20μM AZD2281 for additional 24 hours. Immunoblot was performed with pSer172-IKBKE to show the autophosphorylation of IKBKE (panel 1) and EphA2 knockdown efficiency was shown in panel 3. (B) siRNA for EphA2 or control siRNA was transfected in C13 cells. 36 hours later the cells were treated with 20μM AZD2281 for indicated time points and immunoblot was performed as indicated. (C) Flag-IKBKE or Flag-vector was transfected into A2780CP-sgVector or A2780CP-sgEphA2 cells. 48 hours later the cells were lysed and immunoblot was performed with indicated antibodies.

EphA2 modulates Tyrosine phosphorylation of IKBKE and leads to activation of IKBKE We previously showed the importance of tyrosine phosphorylation of IKBKE in regulation of its kinase activity [219]. Since we also showed that EphA2 is able to activate

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IKBKE pathway, we tested if EphA2 can tyrosine phosphorylate IKBKE. Knockout of EphA2 led to significant decrease in both basal and AZD2281 induced IKBKE tyrosine phosphorylation and IKBKE kinase activity (Figure 31A). Furthermore, AZD2281 treated EphA2 knockout cells showed significant decrease in cell viability compared to the vector transfected cells (Figure 31B). To test the *in vivo* efficacy of EphA2 knockout on olaparib response, we injected the control or EphA2 knockout cells into athymic nude mice. But strikingly EphA2 knockout led to complete inhibition of tumor formation ability of these cells (Figure 31C). These data show that EphA2 is required for IKBKE activation and that EphA2 is an important oncogene in ovarian cancer.
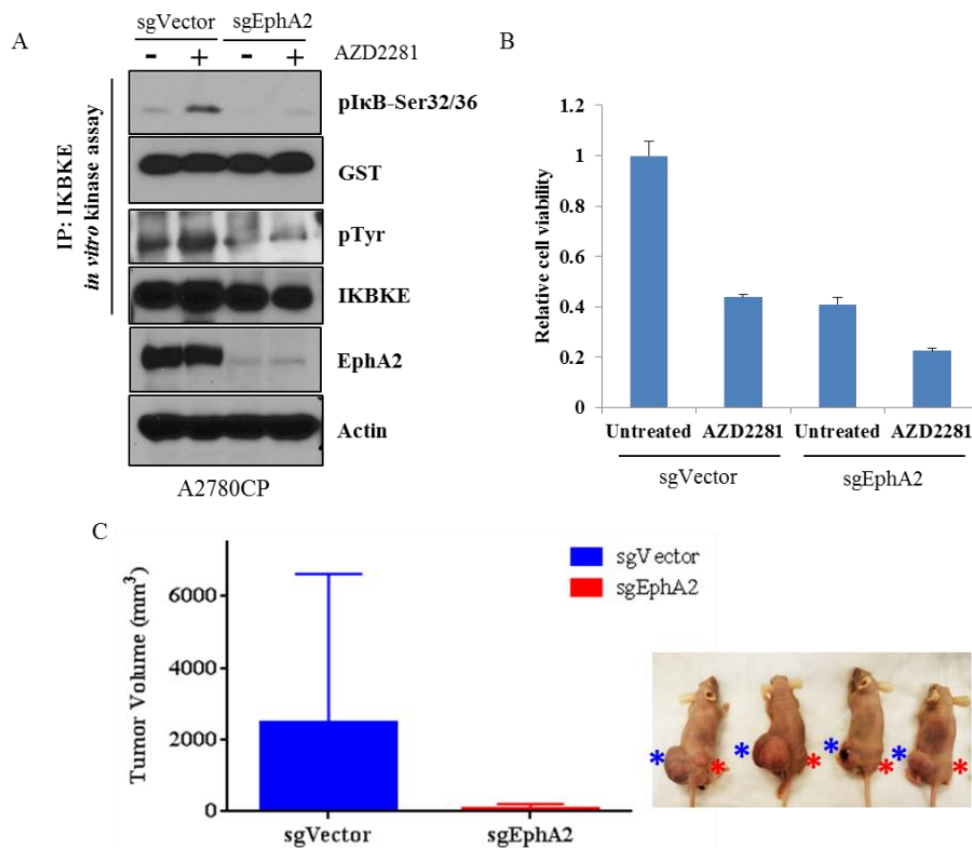
Figure 31: EphA2 modulates Tyrosine phosphorylation of IKBKE and leads to activation of IKBKE (A) sgVector or sgEphA2 expressing A2780CP cells were treated with 20μM for 24 hours. IKBKE was purified from these cells and in vitro kinase assay was performed using GST-IκB (panel 1). Immunoblot was performed with a pan-tyrosine antibody to show tyrosine

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Figure 31 (Continued)

phosphorylation of IKBKE (panel 3). EphA2 knockout efficiency was shown in panel 5. (B) The sgVector or sgEphA2 expressing cells were plated in 96-well format and treated with 20μM AZD2281. Cell viability was measured using MTT assay after 72 hours of AZD2281 treatment. (C) *in vivo* tumor growth efficacy of sgVector (left flank) and sgEphA2 (right flank) expressing A2780CP cells. Tumor volume was measured after 3weeks of cell implantation.

EphA2/IKBKE pathway is a mediator of olaparib resistance. Having showed that PARP inhibition leads to feedback activation of EphA2 and AKT pathways, we tested if this pathway is a mediator of resistance to AZD2281. Knockdown of IKBKE or EphA2 led to increased sensitivity (Figure 32A and 32C respectively) while overexpression of IKBKE or EphA2 led to decreased sensitivity of ovarian cancer cells to AZD2281 (Figure 32B and 32D respectively). Furthermore, increased pEphA2-ser897 levels and IKBKE levels tested across a panel of ovarian cancer cell lines correlated with increased IC50 for olaparib in these cells (Figure 33). Together, these data show that EphA2/IKBKE pathway activation leads to resistance to olaparib and this pathway can be used as a marker for evaluating olaparib response.
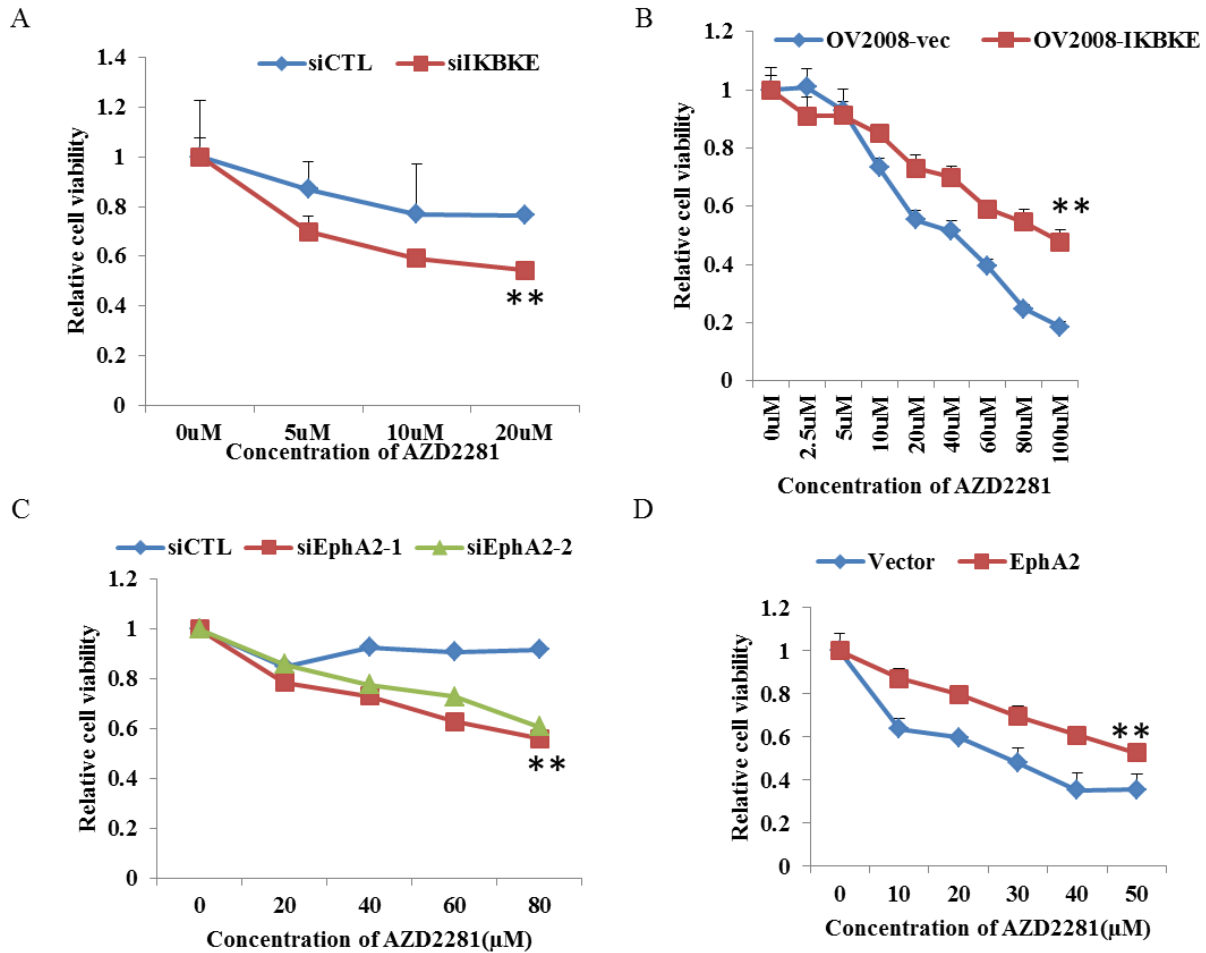
Figure 32: EphA2/IKBKE pathway is a mediator olaparib resistance. (A) siControl or siIKBKE transfected cells were plated in 96-well format and 24 hours later they were treated with indicated amount of AZD2281 for 72 hours and MTT assay was performed. (B) OV2008 cells were selected for overexpression of IKBKE or an empty vector. The cells were treated with increasing concentration of AZD2281 and MTT assay was performed after 72 hours. (C and D) Response to AZD2281 was tested as described before with control or EphA2 knockdown in C13 cells (C) and vector or EphA2 overexpressing OV2008 cells (D).
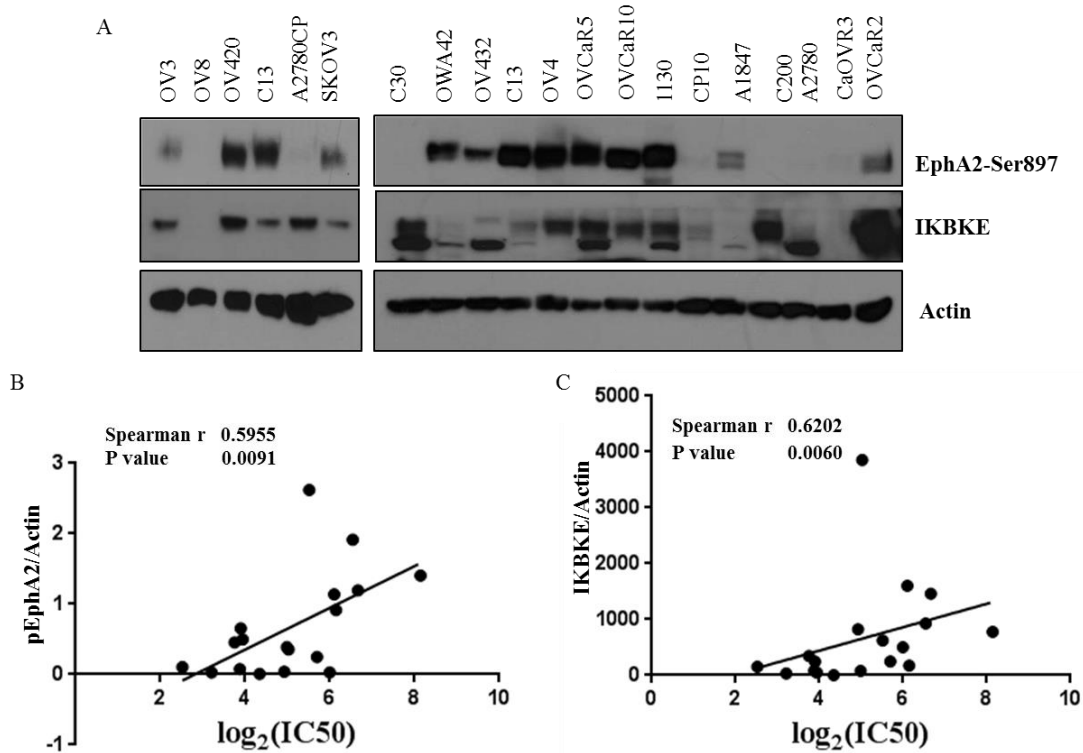
Figure 33: EphA2/IKBKE is a biomarker of olaparib resistance in ovarian cancer cells. (A) Immunoblot analysis for expression of pEphA2-Ser897 and IKBKE in a panel of ovarian cancer cells growing under steady state conditions. (Both the upper and lower bands were considered as IKBKE specific bands) (B and C) IC50 values for AZD2281 in the ovarian cells were calculated from dose response curves. The levels of pEphA2/actin (B) and IKBKE/Actin were calculated by quantifying the bands from panel A. The levels of pEphA2/actin (B) and IKBKE/actin (C) were plotted in correlation with the IC50 values measured.

Targeting IKBKE to increase the efficacy of olaparib. Although inhibitors of EphA2 are in early stages of development, IKBKE inhibitors are currently evaluated in the clinic. Hence we studied the efficacy of IKBKE inhibitors in sensitizing ovarian cancer cells to olaparib. Here we showed that IKBKE inhibitor CYT387 is able to inhibit olaparib mediated AKT activation and even more it was able to inhibit the activation of EphA2 (Figure 34A). We also found that CYT387 synergized with olaparib (Figure 34B) and combined treatment led to a significant increase is Caspase 3/7 activation (Figure 34C). Moreover, *in vivo* xenograft studies using A2780CP cells showed that the combined treatment of CYT387 and AZD2281 showed a

significant decrease in tumor growth while single agent treatment had no significant effect on *in vivo* tumor growth (Figure 34D and 34E). Together, these data show that inhibition of IKBKE pathway is a potential strategy to increase the efficacy of olaparib.
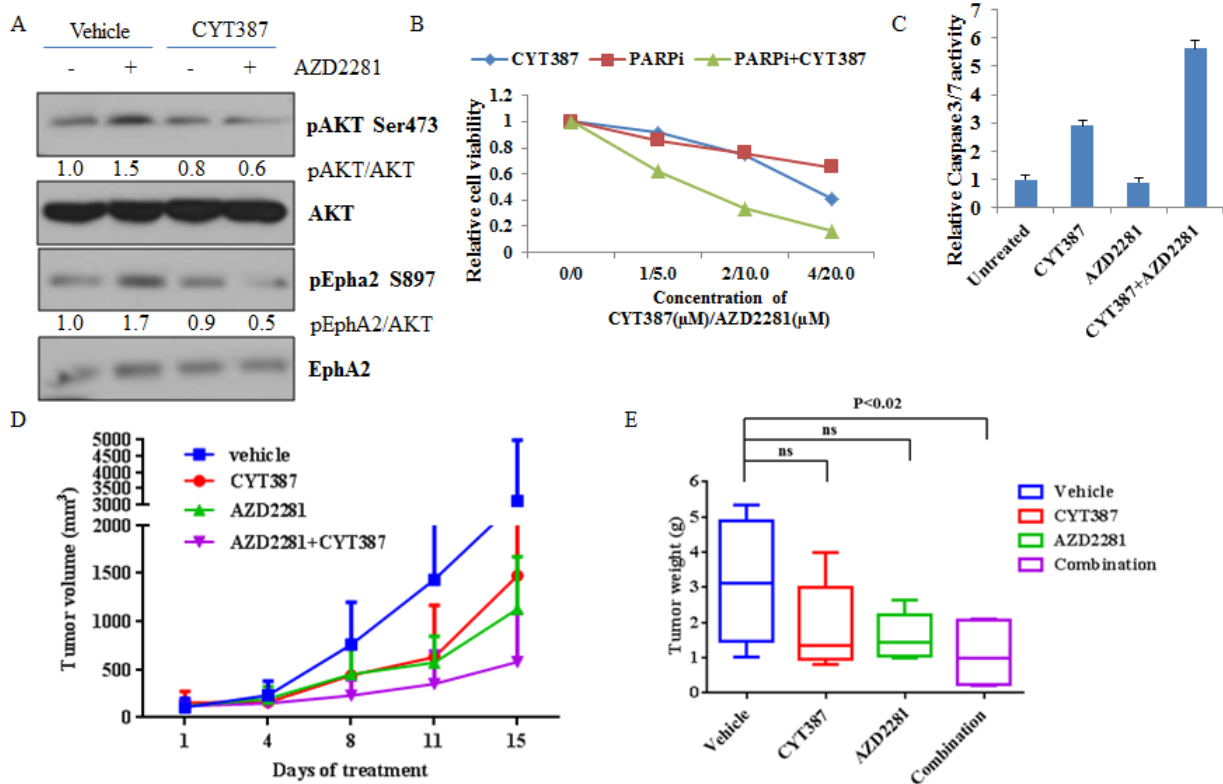
Figure 34: Targeting IKBKE to increase the efficacy of olaparib. (A) C13 cells were treated with 20μM AZD2281 or 2μM CYT387 as indicated for 24 hours and immunoblot analysis was performed. (B) Cell viability was calculated after 72hours of treatment with indicated amount of AZD2281 or CYT387 or the combination. (C) C13 cells were treated with 20μM AZD2281 or 2μM CYT387 or the combination for 24 hours and Caspase 3/7 activity was measured using Caspase-Glo assay. (D and E) Xenograft tumor growth (in D) and tumor weight at endpoint (in E) of A2780CP cells treated with vehicle or 50mg/kg AZD2281 or 10mg/kg CYT387 or the combination.

Discussion

Ovarian cancer is a challenging disease due to rapid acquisition of resistance to chemotherapy. Although several targetable signaling pathways in ovarian cancer are identified,

these targeted therapies show only moderate response as single agents in the clinic. Hence it is important to understand the alternative pathway activation that lead to reduced efficacy of these inhibitors. To this end, in this study we sought to understand the resistance pathways involved in PARP inhibition, specifically with olaparib treatment. Several unique PARP inhibitors are developed and these inhibitors are unique in their mechanism of action and hence different PARP inhibitors may show differential activation of resistance pathways. Nevertheless, in this study we identified that inhibition of PARP enzymes using olaparib led to an increase in activation of EphA2, IKBKE and AKT pathways (Figure 35). Furthermore we showed that inhibition of any nodes in this pathway (EphA2 and IKBKE) led to significant increase in response to olaparib. Interestingly, it was previously shown that AKT pathway is involved in olaparib resistance and hence PI3K/AKT pathway inhibitors in combination with PARP inhibitors are currently evaluated in clinic ([240, 252] and NCT01623349, NCT02338622).
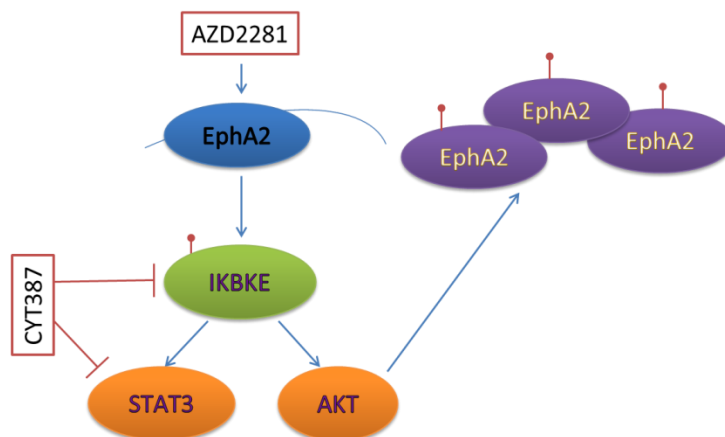
Figure 35: Schematic of EphA2/IKBKE feedback loop. AZD2281 treatment leads to EphA2 activation and activation of downstream IKBKE/AKT signaling. AKT is known to activate EphA2 by phosphorylation at Serine 897. This feedback loop can be disrupted by treatment with an IKBKE inhibitor that is capable of blocking activation of both AKT and EphA2-Ser897 in the presence of AZD2281.

The biology of Eph receptor family exhibit complex bidirectional signaling. Eph signaling induced by the Ephrin ligands were shown to inhibit cell transformation and cell migration. Ephrin-mediated Eph signaling was shown to inhibit the downstream signaling pathways such as Erk1/2, PI3K/AKT. But tumor cells circumvent this by suppressing the expression of Ephrin ligands or by alternative mechanisms of Eph activation [136, 251]. For example, EphA2 was shown to be activated by Src, RSK1/2 and AKT [129, 130, 243]. AKT and RSK1/2 were shown to directly phosphorylate EphA2 at Serine 897 leading to its activation and increased oncogenic signaling of EphA2. Activity of EphA2 can also be regulated by protein tyrosine phosphatases such as LMW-PTP and PTP1B. In this study we showed that PARP inhibition by olaparib led to significant activation of EphA2. We did not see a significant change in mRNA expression of Ephrin A1 by olaparib treatment (data not shown) showing that the mechanism of EphA2 activation is ligand-independent. Indeed, we saw a significant increase in the EphA2 Serine 897 phosphorylation by olaparib. Furthermore, we showed that overexpression of PTP1B led to significant decrease in olaparib mediated EphA2 activation. Although PTP1B resides on endoplasmic reticulum, it was shown to dephosphorylate EphA2 through vesicular recycling [249].

Several studies identified EphA2 as an important oncogene in ovarian cancers and higher expression of EphA2 correlated with poor patient survival and increased cell migration and angiogenesis [136, 138, 139]. Moreover EphA2 activation as a feedback response to targeted therapies was shown in melanoma and lung cancers [245, 246]. This study identified feedback activation of EphA2 upon PARP inhibition. These studies together show the clinical importance of EphA2 inhibitors, especially to reverse these observed drug resistance. We further showed that inhibition of the downstream IKBKE acts as a two edged sword by inhibiting the

downstream activation of AKT and also by inhibiting the AKT mediated phosphorylation of EphA2.

In addition to showing that we can increase the sensitivity of ovarian cancer cells to olaparib by inhibiting the EphA2/IKBKE axis, we also identified that EphA2 Serine 897 phosphorylation or IKBKE protein levels may be a useful biomarker for olaparib resistance. Although these observations warrant further clinical evaluation, this study provides a pre-clinical evidence to evaluate these findings in a clinical setting.

CHAPTER 5

Conclusions and future directions

The current challenges in treating cancer are due to lack of complete understanding of the signaling pathways involved in carcinogenesis. In addition, perturbation to the eco-system (i.e, tumor cells and the microenvironment) causes severe re-wiring of these signaling network thus imposing further challenges in finding the cure. Hence cancer treatment should not be perceived as a static entity but it should be considered dynamic and we need to further our understanding of these dynamic network changes. Although we made significant progress in identifying the targeted therapeutic agents for cancers having driver mutations, resistance mechanisms to these inhibitors is not completely known.

PI3K/AKT and MAPK/ERK pathways are known to be stimulated by the extracellular signals and these pathways are tightly controlled by positive and negative feedback mechanisms. PI3K/AKT pathway senses extracellular growth factors and nutrient availability and MAPK/ERK pathway senses cellular stress factors. Activation of either of these pathways leads to control of gene expression by modulation of transcription factors. While AKT activation leads to inhibition of FOXO3a, a transcription factor known to act as a tumor suppressor by inhibiting the cell cycle and anti-apoptotic gene expression. Similarly, activation of MAPK/ERK pathway leads to activation of the ETS and c-Fos transcription factors. Uncontrolled activation of PI3K/AKT and MAPK/ERK can be a consequence of specific driver mutations identified such as BRAF, KRAS, EGFR. Although inhibition of these driver pathways is expected to inhibit the

downstream AKT and ERK1/2 signaling, such inhibition of the driver pathway leads to paradoxical activation of the downstream signaling by utilizing alternate pathways. Understanding these pathways might help in identifying novel therapeutic targets to increase the response to these targeted therapies.

In this work, we focused on understanding the mechanisms of activation of the IKBKE pathway in the context of the oncogenic mutations and moreover in the context of drug resistance. Although IKBKE has been studied in the context of innate immunity, the precise mechanisms of activation of IKBKE are not known. Furthermore, the post-translational modifications, especially Tyrosine phosphorylation in IKBKE were not identified before. Since tyrosine phosphorylation of proteins lead to their activation, this dissertation work has identified an important regulatory mechanism in IKBKE.

In the first part of this study we identified IKBKE as an important downstream target of EGFR with activating mutations. We achieved this by showing that 1) IKBKE interacts with mutant EGFR 2) IKBKE is tyrosine phosphorylated by mutant EGFR at tyrosine 153 and tyrosine 179 residues 3) phosphorylation at these residues led to significant increase in IKBKE kinase activity 4) knockdown of IKBKE led to significant inhibition of AKT activation and tumorigenic phenotype in EGFR mutant lung cancer cells 5) pIKBKE-Y153 was high in NSCLC patients with EGFR mutation. We further explored the utility of IKBKE inhibitor amlexanox in the EGFR mutant lung cancer cells. This study showed strong synergy of amlexanox with a MEK inhibitor, AZD6244 as MAPK pathway is another arm in EGFR signaling. Feedback activation of MAPK with IKBKE inhibitor in the EGFR mutant NSCLCs was only moderate *in vitro* but this increase was more significant in the *in vivo* tumor models. It is interesting to note

that H1975 and H1650 cells that are resistant to EGFR TKIs responded well to the combined inhibition of IKBKE and MAPK signaling.

Another interesting observation in this study is that while wildtype and mutant EGFR could interact with IKBKE, only mutant EGFR is able to phosphorylate and activate IKBKE signaling. This could be partly due to wildtype EGFR being catalytically inactive, even when over expressed and EGF ligand is required to induce its kinase activity. Interestingly, EGF addition results in significant disruption of EGFR-IKBKE complex. Unlike wildtype EGFR, mutant EGFR is constitutively active and hence it could phosphorylate IKBKE in the absence of EGF.

The disruption of EGFR-IKBKE complex upon EGF treatment could be due to EGF-mediated sequestration of EGFR molecules from IKBKE or EGF bound EGFR might gain a different conformational change leading to its inability to interact with IKBKE. It is yet to be studied if other ligands of EGFR such as amphiregulin or TGFα also inhibit EGFR-IKBKE interaction. EGF induces endocytosis of EGFR receptor [253] and if this is affecting EGFR-IKBKE interaction, inhibition of endocytosis by other ways such as knockdown of clathrin should enhance EGFR-IKBKE interaction and restore IKBKE activation by EGFR. In addition, EGF treatment induces several post-translational modifications (especially phosphorylation) in EGFR, which alter EGFR dimerization and activation [253, 254]. If these phosphorylations disrupt EGFR-IKBKE interaction, treatment with a phosphatase should result in removal of these phosphorylations and enhanced interaction with IKBKE. Another important question that arises from these observations is the physiological relevance of IKBKE-wildtype EGFR interaction. Further studies are required to test the relevance of IKBKE- wildtype EGFR interaction; such as

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if IKBKE alters sensitivity of wildtype EGFR to EGF stimulation, does IKBKE expression alter localization of EGFR.

Furthermore, studies should focus on identifying the minimum peptide sequence in IKBKE that participates in EGFR-IKBKE interaction. Identification of this sequence will help in generation of peptides that can specifically disrupt EGFR-IKBKE interaction and IKBKE activation. These peptides will be a great therapeutic resource for EGFR mutated non-small cell lung cancers that are dependent on IKBKE for survival. Another important direction for these studies would be to evaluate the role of tyrosine phosphorylation of IKBKE on other post-translational modifications that were identified in IKBKE. This requires a comprehensive evaluation of the post-translational modifications in wildtype EGFR (with intact tyrosine residues) or the unphosphorylatable IKBKE (with tyrosine 153 and 179 residues mutated) treated with known activators of IKBKE pathway such as DNA damage, pro-inflammatory cytokine treatment and overexpression of activated EGFR, followed by immunoblot analysis with previously characterized ubiquitination and SUMOylation specific antibodies for IKBKE.

Since we observed moderate levels of feedback activation of ERK1/2 with IKBKE inhibitor in NSCLC, we tested this pathway in pancreatic cancers. Pancreatic cancers have driver KRAS mutation and IKBKE was shown to be an hyper-activated in mutant KRAS cells either by the transcriptional activation of Gli1 or by the autocrine activation of IKBKE due to production of IL1-β by mutant KRAS. Although previous evidence showed that IKBKE will be an important therapeutic target in pancreatic cancer, IKBKE inhibitors are not yet studied in the context of PDACs. Here we showed that IKBKE inhibition or knockdown of IKBKE led to a significant decrease in cell viability, cell migration and also cancer stem cell population.

Furthermore IKBKE inhibition led to a significant increase in ERK1/2 activation in KRAS mutant PDAC cells.

The levels of ERK1/2 activation upon IKBKE inhibition is much more significant in PDAC cells tested compared to the NSCLC cell lines. This prompts the possibility that this feedback ERK1/2 activation is highly dependent on tissue context and also might be affected by the driver pathways dysregulated in this context. It is important to expand these studies to a panel of cancer cells with different driver mutations to be able to define the precise context of signaling required for the feedback pathway activation. Moreover, the precise contribution of JAK pathway inhibition by CYT387 in mediating feedback ERK1/2 activation is yet to be shown. This can be achieved by immunoblot analysis with phospho ERK1/2 of JAK1, JAK2 or STAT3 knockdown cells treated with CYT387. The effect of JAK-STAT3 pathway knockdown on synergy of MEK inhibitor treatment with IKBKE knockdown should also be evaluated.

Here we found that inhibition of IKBKE led to feedback activation of RTKs such as ErbB3 and IGF1R through their transcriptional activation. Moreover, FOXO3a was found to be necessary for this feedback RTK activation. IKBKE was shown to inhibit FOXO3a by direct phosphorylation and hence inhibition of IKBKE led to a decrease in FOXO3a phosphorylation and stabilization. FOXO3a is very well studied for its role in aging and also for its tumor suppressive functions. Hence the FOXO3a mediated RTK expression is paradoxical to the function of FOXO3a under normal physiological conditions. Hence it is yet to be studied how FOXO3a acts as a transcriptional activator for these RTKs. One plausible explanation could be that unphosphorylated FOXO3a interacts with specific transcriptional co-activators to induce its transcriptional activity. Recently, BRD4 was shown to orchestrate the response to PI3K

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inhibitors by altering the RTK expression, it will be interesting to see if BRD4 has a role to play in IKBKE inhibitor-mediated RTK expression. This further opens up new therapeutic opportunities as BRD4 inhibitors have shown some success in cancers.

IKBKE was previously shown to be important in resistance to cisplatin in ovarian cancers and to chemotherapy resistance in NSCLC. In the present study we also report that IKBKE mediates resistance to PARP inhibitor, olaparib in ovarian cancers. Moreover, we also showed that olaparib treatment led to activation of EphA2 pathway and increased tyrosine phosphorylation of IKBKE. Here we also report that with olaparib treatment we see a positive feedback regulation of EphA2-IKBKE-AKT pathway which provided several opportunities for therapy. Interestingly, AKT pathway inhibitors are currently studied in combination with PARP inhibitors. Ovarian cancer cells with higher levels of pEphA2-Ser897 showed significant resistance to olaparib and increase/decrease of EphA2 or IKBKE expression in ovarian cancer cells showed resistance/sensitivity to olaparib respectively. Significantly, olaparib is currently FDA approved for ovarian cancer patients with BRCA mutations, it will be interesting to see if pEphA2-Ser897 can also be used a marker to predict olaparib resistance. This warrants a retrospective analysis of patient specimens for pEphA2-Ser897 levels and the correlation of this signal to tumor responsiveness to PARP inhibitors such as olaparib.

Hence together, this study identified new opportunities for combination therapies in ovarian cancer and further underlines the benefits of EphA2 inhibitors for ovarian cancer patients. Although in this study we focused on EphA2-AKT axis, it will be important to study the relevance of EphA2-FAK axis in olaparib response. FAK acts downstream of EphA2 and it was previously reported that co-targeting both EphA2 and FAK resulted in better tumor response and

higher inhibition of a plethora of downstream signaling networks [255]. Furthermore, the contribution of IKBKE pathway activation by EphA2 can also be further tested by evaluating the efficacy of activated IKBKE to restore resistance to olaparib in the context of EphA2 knockout.

Moreover, this study further confirmed that growth factor signaling such as receptor tyrosine kinase signaling is modulated as a response to PARP inhibitors. Hence it is important to perform a systems biological analysis of changes in pathway activation as a response to PARP inhibition. This can be achieved by performing a phosphoproteomics assay or by activity based protein profiling (ABPP) of cells treated with vehicle or AZD2281. Interestingly, in this study we observed that PTP1B overexpression abrogated AZD2281-mediated EphA2 activation. PTP1B was shown to reside on the endoplasmic reticulum (ER) and during endocytosis EphA2 was shown to come in close proximity to PTP1B resulting in dephosphorylation of EphA2 [249]. It will be important to perform an in-depth study of the effect of AZD2281 on endoplasmic reticulum and shuttling of protein between ER and plasma membrane by live cell imaging.

Another important direction for future studies will be to use immunocompetent mice for *in vivo* studies. Since IKBKE is extensively shown to regulate immune cells, it will be highly important to evaluate the efficacy of IKBKE inhibitors in immunocompetent mice. Since the current evidence shows that IKBKE knockout leads to enhanced T-cell activity, IKBKE inhibitors can be expected to have a two-way effect: 1) by inhibiting the growth of tumor cells and 2) by activation of T-cells to induce anti-tumor immunity. Especially for the olaparib study in ovarian cancers, olaparib was previously shown to synergize with PD1 blockade due to high accumulation of DNA damage resulting in elevated immunogenicity with olaparib treatment. Hence IKBKE inhibitors might have better synergistic effect with olaparib in immunocompetent

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mice. Due to inherent inter- and intra-tumoral heterogeneity, true efficacy of any of the combination strategies discussed here will require thorough clinical evaluation.

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