# EVALUATING THE THERAPEUTIC POTENTIAL OF THE PAK1 AND TBK1 KINASES IN PANCREATIC DUCTAL ADENOCARCINOMA

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## ABSTRACT

Nicole Marie Baker: Evaluating the therapeutic potential of the PAK1 and TBK1 kinases in pancreatic ductal adenocarcinoma
(Under the direction of Channing J. Der)

Pancreatic ductal adenocarcinoma (PDAC) is an extremely lethal cancer characterized by a high frequency (>95%) of activating mutations in the *KRAS* oncogene, which is a well-validated driver of PDAC growth. However, to date, no successful anti-KRAS therapies have been developed. Inhibitors targeting components of KRAS downstream signaling pathways, when used as monotherapy or in combination, have been ineffective for long-term treatment of KRAS-mutant cancers. Decidedly, the most studied and most targeted KRAS effector pathways have been the RAF-MEK-ERK mitogen-activated protein kinase (MAPK) cascade and the PI3K-AKT-mTOR lipid kinase pathway. The apparent lack of success exhibited by inhibitors of these pathways is due, in part, to an underestimation of the importance of other effectors in KRAS-dependent cancer growth. Additionally, compensatory mechanisms reprogram these signaling networks to overcome the action of inhibitors of the ERK MAPK and PI3K pathways. Consequently, the central hypothesis of my dissertation research is that a better understanding of the role of less studied KRAS effector signaling pathways may lead to more effective therapeutic strategies to block KRAS effector signaling and PDAC growth.

Although the TIAM1-RAC1 small GTPase effector pathway has been validated as a driver of KRAS-mutant cancer growth, how RAC1 mediates this role has not been established. My studies aimed to address a possible critical role for the p21-activated kinase

1 (PAK1) in this effector pathway. In support of this, I found that PAK1 protein levels are overexpressed both in a subset of pancreatic cancer cell lines and in primary patient tumor samples. Moreover, I determined that stable shRNA-mediated suppression of PAK1 protein expression inhibited the anchorage-dependent and -independent growth of PDAC cell lines *in vitro*. I also observed that a pharmacologic inhibitor of PAK1 recapitulated the reduced growth phenotypes observed upon genetic ablation of PAK1.

As *KRAS*-mutant tumors are known to upregulate certain cellular processes in order to support the increased metabolic demands of uncontrolled cellular proliferation, I sought to determine whether PAK1 signaling was partially accountable for ensuring that these metabolic needs were met. My studies confirmed a role for PAK1 in regulating macropinocytosis, a mechanism by which PDAC cells acquire macromolecules (e.g., proteins, polysaccharides, and lipids) from the extracellular environment as a source of nutrients. I found that both pharmacologic inhibition and genetic ablation of PAK1 resulted in markedly decreased macropinocytosis in PDAC cells. These data suggest inhibition of PAK1 in KRAS-mutant PDAC could interfere with PDAC metabolism and reduce tumor cell growth. I observed a further reduction in macropinocytosis upon inhibition of PAK1 together with concurrent ERK1/2 or PI3K inhibition. In summary, my results support PAK1 as a promising therapeutic target for pancreatic cancer.

My lab and others have provided strong evidence for the key role of a second, less studied KRAS effector pathway, the RalGEF-RAL small GTPase effector pathway, in the growth of pancreatic and other cancers. One critical effector of RAL is the Sec5 component of the exocyst complex. How Sec5 contributes to the role of RAL in cancer remains unresolved. White and colleagues initially identified a Sec5 function independent of exocyst regulation that involved the TANK-binding kinase 1 (TBK1). When a study that searched for synthetic lethal partners of mutant KRAS identified TBK1, these findings suggested that

TBK1 may be a critical mediator of RalGEF-RAL effector-driven cancer growth. However, a subsequent study questioned the role of TBK1 in the growth of KRAS-mutant cancers.

When my lab obtained a novel pharmacologic inhibitor of TBK1, I embarked on studies to determine whether inhibition of TBK1 kinase activity could be an efficacious treatment strategy for PDAC. My studies revealed that inhibition of TBK1 alone led to limited growth inhibition in PDAC cell lines. Furthermore, I found that concurrent inhibition of TBK1 did not enhance the growth inhibitory activity of an ERK inhibitor. However, loss of TBK1 protein via shRNA or pharmacologic inhibition prompted the development of large, intracellular vesicles that appeared to be swollen autolysosomes and the product of non-productive autophagy. This work suggests that TBK1 may play a role in PDAC autophagic flux and provides a rationale for pairing a TBK1 inhibitor with other targeted therapies or chemotherapies to drive these tumor cells towards death.

In summary, my studies support my hypothesis that concurrent inhibition of multiple KRAS effector pathways may provide more effective therapeutic strategies for PDAC. They emphasize that single agent therapies targeting KRAS effector signaling will not be effective, a reality that is emerging from ongoing clinical trials. While my studies took a rational approach to identifying these combinations, unbiased chemical library screens with PAK1 and TBK1 inhibitors will likely identify additional combinations of inhibitors for PDAC.

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| | |
|---|---|
| ARF | ADP-ribosylation factor |
| AID | Autoinhibitory domain |
| AKT | Protein kinase B |
| ATCC | American Type Culture Collection |
| BAD | Bcl-2-associated death promoter |
| Bcl-2 | B-cell lymphoma 2 |
| CDC42 | Cell division cycle 42 small GTPase |
| cDNA | Complementary deoxyribonucleic acid |
| C-terminus | Carboxyl-terminus |
| DMEM | Dulbecco's modified Eagle medium |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| EGF | Epidermal growth factor |
| EGFR | Epidermal growth factor receptor |
| Exo84 | Exocyst complex 84 kDa subunit |
| ERK | Extracellular signal-regulated kinase |
| FAK | Focal Adhesion Kinase |
| FBS | Fetal bovine serum |
| FITC | Fluorescein isothiocyanate |
| FTI | Farnesyltransferase inhibitor |
| GAP | GTPase-activating protein |
| GDP | Guanine diphosphate |
| GEF | Guanine nucleotide exchange factor |

| | |
|---|---|
| GFP | Green fluorescent protein |
| $GI_{50}$ | Growth inhibitory 50, concentration of drug to cause 50% reduction in proliferation |
| GPCR | G-protein coupled receptor |
| GST | Glutathione S-transferase |
| GTP | Guanine triphosphate |
| GTPase | Guanosine triphosphatase |
| h | Hour |
| HRAS | Harvey rat sarcoma viral oncogene homolog |
| $IC_{50}$ | Inhibitory concentration 50, concentration of drug to cause 50% reduction in protein signaling activity |
| IFR3 | Interferon regulatory factor 3 |
| IHC | Immunohistochemistry |
| IKK | IκB kinase |
| IPA-3 | (2,2'-dihydroxy-1,1'dinaphthyldisulfide) |
| kDa | Kilodalton |
| KRAS | Kirsten rat sarcoma viral oncogene homolog |
| LCCC | Lineberger Comprehensive Cancer Center |
| LPS | Lipopolysaccharide |
| MAPK | Mitogen-activated protein kinase |
| MEK | Mitogen-activated protein kinase kinase |
| MET | mesenchymal-epithelial transition factor receptor |
| mL | Milliliter |
| MM | Mis-match siRNA |
| mM | Millimolar |

| | |
|---|---|
| mRNA | Messenger ribonucleic acid |
| mTOR | Mammalian target of rapamycin |
| MTT | 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide |
| MYC | v-Myc avian myelocytomatosis viral oncogene homolog |
| NCI | National Cancer Institute |
| NF1 | Neurofibromin 1 |
| NF-κB | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| NIH | National Institutes of Health |
| nM | Nanomolar |
| NRAS | Neuroblastoma RAS viral oncogene homolog |
| NSCLC | Non-small cell lung cancer |
| NS | Non-specific shRNA |
| N-terminus | Amino-terminus |
| p90RSK | p90 ribosomal S6 kinase |
| PDAC | Pancreatic ductal adenocarcinoma |
| PAK | p21-activated kinase |
| PBD | p21-binding domain |
| PBS | Phosphate-buffered saline |
| PDEδ | Phosphodiesterase-delta |
| PDGF | Platelet-derived growth factor |
| PDK1 | Phosphoinositide-dependent protein kinase 1 |
| PI3K | Phosphatidylinositol 2-kinase |
| PIP2 | Phosphatidylinositol-4,5-bisphosphate |

| | |
|---|---|
| PIP3 | Phosphatidylinositol-3,4,5-triphosphate |
| PREX2 | Phosphatidylinositol-3,4,5-triphosphate-dependent RAC exchanger 2 |
| PTEN | Phosphatase and tensin homolog |
| PVDF | Polyvinylidene difluoride |
| RAB | Rat brain small GTPase |
| RAC | Ras-related C3 botulinum toxin substrate |
| RAF | Rapidly accelerated fibrosarcoma kinase |
| RAL | RAS-like small GTPase |
| RalBP1 | Ral binding protein 1 |
| RalGAP | Ral GTPase-activating protein |
| RalGDS | Ral guanine nucleotide dissociation stimulator |
| RalGEF | Ral guanine nucleotide exchange factor |
| RAN | RAS-related nuclear protein |
| RAS | Rat sarcoma viral oncogene homolog |
| RBD | RAS-binding domain |
| RHO | Ras homologous small GTPase |
| RNA | Ribonucleic acid |
| RNAi | RNA interference |
| RPPA | Reverse phase protein array |
| RTK | Receptor tyrosine kinase |
| Sec5 | Exocyst complex component 2 |
| SEM | Standard error of the mean |
| SDS | Sodium dodecyl sulfate |
| SFK | Src family kinase |

| | |
|---|---|
| SOS1 | Son of sevenless homolog 1 |
| shRNA | Short hairpin ribonucleic acid |
| siRNA | Short interfering ribionucleic acid |
| TANK | TRAF-associated NF-κB activator |
| TBK1 | TANK-binding kinase 1 |
| TBST | Tris-buffered saline containing Tween-20 |
| TCoB | Tubulin cofactor B |
| Tiam1 | T-cell invasion and metastasis gene 1 |
| TLR | Toll-like receptor |
| TMA | Tissue microarray |
| TNF | Tumor necrosis factor |
| TRAF | TNF receptor-associated factor |
| TRC | The RNAi Consortium |
| WT | Wild-type |
| μm | Micron |
| μM | Micromolar |
| μL | Microliter |

# CHAPTER 1: INTRODUCTION[1]

## RAS small GTPases in cancer

The RAS (rat sarcoma viral oncogene homolog) small GTPases comprise a family of proteins that are involved, to some degree, in nearly every known cellular process (1). The members of this monomeric G-protein family are bound to guanine triphosphate (GTP) when in an activated state and are able to bind to and regulate downstream effectors (e.g., RAF) (2). GTP is then hydrolyzed to guanine diphosphate (GDP), either by slow intrinsic hydrolysis within the RAS protein, or by interaction with GTPase activating proteins (GAPs; e.g., NF1), which provide critical amino acid residues and assist the GTPase in adopting a more favorable conformation to catalyze the hydrolysis of GTP to GDP. Subsequently, guanine nucleotide exchange factors (GEFs; e.g, SOS1) facilitate the exchange of GDP for GTP, thus continuing the GTPase activation-deactivation cycle (2).

The RAS superfamily is comprised of five distinct groups: the ARF, RAB, RAN, RHO, and RAS small GTPases (3). Of these families, the two with the strongest association with cancer are the RAS and RHO families (1, 4-6). In addition to RAS, the RAS family includes the RAL small GTPases (7, 8). Among the best-studied RHO family members is RAC1 (9, 10). In addition to sharing strong structural and biochemical similarities with RAS, as described below, RAL and RAC1 also function downstream of RAS as key effectors in

[1]Portions of this introduction are adapted from a previously published review of PAK1 in RAS mutant cancer. The author list is as follows: Nicole M. Baker, Hoi Yee Chow, Jonathan Chernoff, and Channing J. Der.

driving RAS biology (11-13). RAC1 mutations have also recently been identified in human cancer (14).

There are three genes that encode four isoforms of the founding members of the RAS protein family: HRAS, NRAS, KRAS4A, and KRAS4B. KRAS4A and KRAS4B are formed by alternative KRAS gene splicing (1). The sequence of these proteins is highly conserved, save for a C-terminal hypervariable domain, which contains a tetrapeptide CAAX-motif (Cysteine-Alanine-Alanine-terminal amino acid) that is differentially prenylated to facilitate proper RAS subcellular localization (15, 16). In 1982, multiple groups discovered that these proteins were mutationally activated in human cancers and act as oncogenes (17-22). Subsequently, extensive sequencing of many human cancers determined that approximately 25% of all human cancers contain activating mutations in one of the three RAS isoforms (COSMIC). KRAS mutations comprise 86% of all RAS mutations, followed by NRAS (10%), with HRAS mutations rarely seen in cancer (2). Though over 130 missense mutations of RAS proteins have been identified in human tumors, 98% of these mutations are found at three specific residues: G12, G13, and Q61 (2, 23, 24). Missense mutations at these residues leaves RAS proteins in a state that is GAP insensitive and, due to the low intrinsic rate of GTP hydrolysis, this results in RAS proteins that are essentially constitutively bound to GTP and actively signaling to downstream effectors.

Normal RAS proteins are a central signaling hub coordinating extracellular growth signals with downstream cytoplasmic signaling that promotes proliferation, survival, migration, adhesion, and gene transcription (25). In resting normal cells, RAS is inactive. Upon growth factor stimulation, normal RAS is transiently activated, rapidly returning to the inactive GDP-bound state in the absence of growth factors. In contrast, constitutively active RAS proteins found in cancer are persistently signaling and can drive every hallmark characteristic of cancer. The central role of RAS proteins in these diverse cellular processes, coupled with the exceptionally high rate of RAS mutations in human cancer, poise RAS

proteins as an important target for therapeutic intervention. With RAS mutations found in cancers that comprise the three top causes of cancer deaths in the US (lung, colorectal and pancreatic cancer), an effective anti-RAS therapy will have a significant impact on cancer deaths.

***Therapeutically targeting mutant RAS in human cancer***

Over three decades of intense effort have gone into attempting to drug RAS (1). Though directly targeting mutationally activated RAS for the treatment of human cancer sounds promising, and while impressive progress has been made, it remains to be determined whether direct inhibitors can be developed into clinically active and effective drugs. Since mutant RAS is persistently GTP-bound, by analogy to ATP-competitive inhibitors of protein kinases, one logical approach is the development of GTP antagonists. The primary reason these efforts have been confounded is due to the picomolar affinity of RAS for GTP (26). The especially high affinity of RAS for its natural activator leaves little room for pharmacologic intervention in the GTP-binding pocket. Therefore, attempts to directly target mutant RAS have been difficult. However, in recent years, moderate success has been seen with small molecules that directly bind RAS. For example, targeting specific mutant RAS proteins, such as KRAS-G12C, has been reported (27).

With the uncertain success in designing a direct inhibitor of RAS, the focus of most researchers has hinged on a multitude of indirect strategies to impair mutant RAS activity in tumor cells. The earliest research involved disrupting the posttranslational modifications of RAS that tether it to the plasma membrane. Unfortunately, inhibition of farnesyltransferase (28, 29), which is responsible for adding a C15 farnesyl lipid group to the CAAX motif of RAS, was unsuccessful due to the ability of KRAS and NRAS to be modulated by another enzyme when farnesyltransferase activity is blocked (30). Under these conditions, a related enzyme, geranylgeranyltransferase, which adds a C20 geranylgeranyl group to the CAAX

motif, restores RAS plasma membrane association. Another recent method for targeting KRAS membrane association involves inhibition of PDEδ, a RAS-binding protein that chaperones RAS to the plasma membrane (31, 32). However, more research is required to determine whether PDEδ inhibitors are a promising direction for therapeutic intervention (33).

A currently exciting alternative approach to inhibit RAS activity involves exploiting the roles RAS plays in cellular metabolism (34-36). Tumor cells often exist in a hypoxic, nutrient depleted environment, which directly opposes the elevated metabolic needs of these cells. Many studies have demonstrated that RAS mutant tumors exhibit increased rates of glycolysis and non-oxidative phosphorylation (37). Additionally, nutrient scavenging from both internal and external resource pools, via autophagy and macropinocytosis, respectively, has been validated as a critical component necessary to support RAS-mutant tumor growth. Autophagy is a process of "self-eating" whereas macropinocytosis is a process of taking up extracellular materials (38-40). Both processes culminate in the lysosomal degradation of captured cargo and the creation of free nutrients that tumor cells use to sustain their unremitting growth.

Another method for targeting mutant RAS signaling has been to directly target effector pathways of RAS with known roles in driving tumor growth. This strategy is so far the most promising and the most clinically advanced approach for disrupting RAS activity due to the multitude of small molecule inhibitors designed against RAS downstream effectors. RAS-GTP binds to a spectrum of functionally diverse downstream effectors, including many protein kinases that are pharmacologically tractable (41). Protein kinases have been the most successfully targeted protein class in oncology due to accessibility and druggability of the ATP-binding pocket. Genetic studies in mouse models and cancer cells have demonstrated the critical requirement for effectors in RAS-mutant driven cancer initiation, progression and maintenance (1, 42).

Given the complex nature of RAS signaling, whether targeting one or multiple RAS effector signaling pathways will be required for effective and long-term therapy is unresolved (41, 43). Currently, most efforts have centered on the two canonical RAS effectors, the RAF serine/threonine protein kinases and the class I lipid kinases, the phosphatidylinositol 3-kinases (PI3Ks) (44). Numerous inhibitors of each of these effector pathways are currently under clinical evaluation. Most attention has been focused on the RAF serine/threonine kinases. One RAF isoform, BRAF, is often mutationally activated in human cancers (45). Activated RAF phosphorylates and activates MEK1 and MEK2, which then go on to phosphorylate and activate the ERK1/2 mitogen-activated protein kinases (Figure 1.1).

PI3Ks comprise the second most studied RAS effector class. PI3K activation causes increased conversion of phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PIP3). Membrane-bound PIP3 can then regulate a diversity of signaling proteins, including the AKT1-3 serine/threonine kinases. One PI3K isoform, p110 alpha is frequently mutated in cancer and PI3K is considered essential for *RAS*-driven cancer development (46, 47).

There are currently at least 30 inhibitors of the RAF-MEK-ERK pathway and 50 inhibitors of the PI3K-AKT-mTOR pathway under clinical evaluation (clinicaltrials.gov) (43, 48-51). However, limited clinical efficacy has been observed with these inhibitors as monotherapy. Likely, combinations of these inhibitors will be required to achieve clinical success. A central hypothesis of my research is that inhibitors of the less studied RAS effector pathways - those leading to the activation of the RAC1 and RAL small GTPases - may address this need. However, while these pathways have been clearly validated in driving the growth of RAS mutant cancers, attractive pharmacologic approaches for blocking them remain to be identified. In Chapter 2, I propose that the PAK1 serine/threonine kinase may be a promising candidate for targeting RAC1 downstream signaling. In Chapter 3, I

propose that the TBK1 serine/threonine kinase may be a promising direction for targeting the RAL effector pathway.

Yet another method for indirectly targeting mutant RAS activity has involved the search for synthetic lethal partners of mutant, but not wild-type, RAS (52, 53). A gene is a synthetic lethal partner of mutant KRAS when its function is vital to the growth of only cancer cells containing mutant RAS, whereas its loss in RAS wild-type cells is inconsequential to cellular viability and function. Many unbiased functional genetic screens using large RNAi libraries against a multitude of gene products have sought to identify proteins whose loss sensitizes cells to loss of KRAS. Chapter 3 of my thesis includes the description of one such identified synthetic lethal partner of mutant KRAS, the TBK1 serine/threonine kinase (54). However, enthusiasm for the data generated in synthetic lethal studies is mixed due to some misgivings about the reproducibility of results from these reports (55).

***Targeting RAC-PAK signaling in RAS-driven cancers***

A somewhat overlooked RAS effector network results in activation of a RHO family protein, the RAC1 small GTPase. One mechanism that this can be mediated through is via RAS interaction with a RAS-GTP binding domain (RBD)-containing RAC-selective GEF, TIAM1 (56, 57) (Figure 1.1). Another mechanism involves PI3K-mediated formation of PIP3, which then activates other RAC-selective GEFs (e.g., PREX1/2, Vav) (58, 59).

The three RAC isoforms are members of the RHO branch of the RAS superfamily (3). They are best known for their regulation of actin organization, in particular to regulate lamellipodia induction and promotion of cell migration and pinocytosis. RAC also regulates the formation of reactive oxygen species (60). The recent identification of activated RAC1 mutants in melanoma supports an important driver role for RAC in cancer growth (61, 62).

Like RAS, RAC is a GDP-GTP regulated binary switch, with RAC-GTP engaging multiple effectors (63). While the precise effector(s) that drive RAC-dependent cancer

growth remain to be determined, the PAK protein kinases are intriguing candidates. Below, I summarize the evidence for the importance of the RAC-PAK effector signaling pathway in RAS-driven cancer development and growth.

***RAC and RAS in cancer***

Early studies identified upregulated RAC activation in HRAS-transformed rodent fibroblasts (57, 64, 65). These were followed by studies where dominant negative RAC1 mutants that sequester and inactivate RAC-GEFs, impaired the growth of HRAS-transformed rodent fibroblasts (13, 66, 67). Subsequent genetically engineered mouse model studies found that tissue-restricted genetic loss of *Rac1* impaired mutant *Kras*-driven lung (68) and pancreatic (69) cancer development. Furthermore, in a mutant *Kras*-driven model of papilloma development, tumor tissue exhibited increased levels of RAC-GTP, and loss of one *Rac1* copy alone was sufficient to reduce tumor growth and increase survival (70).

The key effectors that drive RAC-dependent cancer growth remain to be elucidated. In an early study utilizing effector-binding mutants of RAC1 to study the effectors important for transformation of NIH 3T3 mouse fibroblasts, PAK1 was found to be dispensable (71). These analyses suggested that RAC1 regulates at least four distinct effector-mediated functions and that multiple pathways may contribute to RAC1-induced cellular transformation. However, since subsequent studies identified cell type and species differences in the effectors involved in RAS-mediated transformation (72, 73), a reevaluation of the role of PAK1 in RAC1-dependent cancer growth in human cancers is clearly merited. Another RAC1 activity, upregulation of reactive oxygen species, in which PAK1 is also involved (74), has been suggested to contribute to RAC1-mediated growth regulation

***PAK activation in RAS mutant cancer***

PAKs comprise a family of six proteins divided into two sub-groups: group I comprises PAK1-3 and group II contains PAK4-6 (75-77). Since group I PAKs are RAC and CDC42 effectors, whereas group II PAKs are CDC42 only, my work will primarily focus on the group I PAKs. Although the group I PAKs share strong sequence identity in their kinase domains (92-95%), PAK1 is thus far the most studied family member, so I have focused primarily on PAK1 in my studies.

Though PAK1 activity can be deregulated by a diversity of mechanisms in cancer that include gene amplification and increased gene transcription (76, 78), here I focus on activation of PAK downstream of RAS, RACGEFs, and RAC. While in the inactive conformation within the cytosol, PAK1-3 form head-to-tail homodimers with the N-terminal autoinhibitory domain (AID) of one monomer inserted within the C-terminal kinase domain of another. Upon binding of RAC1-GTP to the GTPase binding domain of group I PAKs, a conformational change releases the AID from the kinase domain leading to autophosphorylation at multiple serine/threonines and activation of PAK catalytic activity, allowing phosphorylation of substrates (79). Additionally, plasma membrane-associated RAC binding facilitates PAK plasma membrane recruitment, where PAKs can interact with effectors.

***PAK effector signaling in human cancer***

Group I PAKs regulate a spectrum of catalytically diverse substrates (76, 80). The precise substrates critical for PAK-dependent cancer growth remain to be fully understood and the interplay of multiple substrates is likely involved. In particular, PAK1 facilitates cross-talk with both the RAF and PI3K effector signaling networks. PAK1 can enhance ERK signaling by phosphorylation of RAF-1 (S338) and MEK1 (S298) (81-84). PAK1 also regulates the PI3K-AKT-mTOR pathway, where PAK1 exhibits a kinase-independent

scaffolding function to facilitate PDK1-mediated recruitment of AKT to the plasma membrane to facilitate AKT activation (85). The physiologic relevance of PAK1 cross-talk with ERK and AKT signaling is supported by the observation that genetic or pharmacologic ablation of PAK1 impaired both ERK and AKT activation in *Kras*-driven skin tumors (86). Pharmacologic inhibitors of the RAF and PI3K pathways have been ineffective in *RAS* mutant cancer cells, in part, due to kinome reprogramming mechanisms that stimulate signaling activities that overcome inhibitor action (87-90). Consequently, combined targeting of PAKs and members of these pathways, such as MEK, ERK, PI3K or AKT, may help overcome these resistance mechanisms. However, PAK1 cross-talk with these RAS effector pathways can be context-dependent as PAK1 suppression in KRAS-mutant colon carcinoma cells impaired anchorage-dependent and -independent proliferation, but not ERK or AKT activation (91).

PAKs are also capable of influencing transcription of genes that promote cell cycle progression and cell survival. In breast cancer and colon cancer cell lines, PAK1 can phosphorylate β-catenin on S663 and S675, stabilizing it and promoting its nuclear translocation and transcriptional stimulation of TCF-responsive genes, including *CCND1* and *MYC* (92, 93).

PAKs enhance cell survival by phosphorylating proteins associated with apoptosis. PAK1 phosphorylates BAD on S111 to prevent Bcl-2 binding and induction of apoptosis (94). Additionally, PAK1 can phosphorylate and induce relocalization of RAF-1 to the mitochondria where it also inhibits BAD by phosphorylating it on S112 (94).

PAKs are also critical mediators of the cytoskeleton and cell motility. PAK1 and PAK2 phosphorylate LIM kinase on T508, and LIMK in turn phosphorylates cofilin to prevent actin depolymerization (95, 96). Additionally, PAK1 can phosphorylate the p41-ARC subunit of the Arp2/3 complex to promote actin nucleation and cell motility (97, 98). PAKs are also involved in microtubule reorganization through both tubulin cofactor B (TCoB), a protein

responsible for assembling tubulin heterodimers (99), and through the inactivation of stathmin, which is normally responsible for destabilizing microtubules at the leading edge of cells (100-102).

Metabolism is a critically important factor to the survival of cancer cells because of their high energy demands, and PAKs play a role in driving several metabolic processes that aid tumor cell growth and survival. Elevated macropinocytosis to facilitate increased extracellular protein and lipid uptake is one consequence of the high metabolic requirements of cancer cells (36). PAK1 was found to be necessary and sufficient for growth factor- and RAC-induced macropinocytosis in NIH 3T3 fibroblasts (103). RAC and PAK1 were found to be both necessary for bladder cancer cell macropinocytotic uptake of Bacille Calmette-Guerin (BCG), a strain of bacteria used in the treatment of bladder carcinoma (104). Additionally, bacterial uptake was also stimulated by activated KRAS or HRAS and this activity was blocked by pharmacologic inhibition of group I PAKs by IPA-3 (2,2'-dihydroxy-1,1'dinaphthyldisulfide). This study suggests that the activity state of PAKs in cancer cells could be a determinant of efficient uptake of cancer therapeutics. Similarly, in pancreatic cancer cells, KRAS-dependent stimulation of macropinocytosis and uptake of albumen (105) may provide a basis for the efficacy of albumen-bound (nab) paclitaxel for the treatment of this cancer. It will also be important to assess a role for RAC-PAK signaling in KRAS-dependent macropinocytosis to determine whether pharmacologic inhibition of PAK1 may be an effective approach to blocking cancer cell metabolism.

While PAKs are canonically thought of as functioning in the cytosol or at the plasma membrane, they do contain several nuclear localization signals (NLS) and play several roles within the nucleus. In zebrafish, PAK1 nuclear import is essential for development (106). In cancer cells, increased nuclear accumulation of PAK1 has been associated with advanced tumor stage in colorectal and breast tumors (107, 108). In breast tumors, increased nuclear PAK1 is capable of phosphorylating ERα at S305 and causing it to become active in a

ligand-independent manner, leading to tamoxifen resistance (109). Finally, PAK1 can translocate to the nucleus to drive transcription of fibronectin, which is crucial for supporting pancreatic cancer cell growth and migration (110).

The first evidence for a role for PAK1 in RAS-dependent growth transformation came from studies in model cell systems. Ectopic expression of a kinase-dead PAK1 dominant negative mutant impaired HRAS and RAC1 growth transformation of rat 3Y1 fibroblasts (111) or HRAS transformation of Rat-1 rat fibroblasts, but not NIH3T3 mouse fibroblasts (112, 113). Similarly, dominant negative RAC1 and kinase-dead PAK1 inhibited KRAS transformation of MT4H1 rat Schwann cells (114). Recently, in a mouse model of *Kras*-driven skin squamous cell carcinoma formation, genetic ablation of *Pak1* strongly impaired tumor initiation and progression (86). Together with the validated role of RAC1 in RAS-driven oncogenesis, these observations implicate the RAC-PAK effector pathway as a target for the development of anti-RAS therapeutic strategies. Like the RAS small GTPase, the RAC small GTPase is not considered a highly tractable drug target. Therefore, below we focus on the development of inhibitors of the PAK1 kinase for cancer treatment.

***Clinical-translational advances***

PAK inhibitor development is still largely at the preclinical stage, with only one PAK inhibitor evaluated in clinical trials (115). Due to the high sequence identity of the kinase domains, most attempts thus far have yielded molecules with a high affinity for all group I PAK members, and in some cases, inhibitory activity for both group I and II PAKs. Early stage ATP-competitive PAK inhibitors (e.g., staurosporine, A-FL172) lacked selectivity for PAK. The only PAK inhibitor to reach clinical trials was a pan-PAK inhibitor, PF-3758309 (116). This compound was identified originally as a hit in a screen for inhibitors of PAK4, but it proved to effectively inhibit all PAK family members, in addition to other protein kinases. Preclinical evaluation showed anti-tumor activity against multiple human tumor cell lines,

leading to Phase I evaluation in patients with solid tumors. Unfortunately, this trial was stopped in phase I due to pharmacokinetic issues. Subsequently, derivatives of PF-3758309 have been described with much improved pharmacologic properties, raising hope that this class of compound may yet have clinical utility (117).

More recently, Licciulli and colleagues described the discovery of a small molecule pyridopyrimidinone, FRAX59, that potently inhibits Group I PAKs by preventing ATP-binding and hydrolysis (118). FRAX597 exhibited high specificity and potency for Group I PAKs, although potent inhibition of other kinases was also seen. When evaluated *in vivo*, FRAX597 inhibited the tumorigenic growth of *NF2*-null Schwann cells. *NF2* loss causes RAC1 and PAK1 activation, indicating that this compound could be a viable therapeutic strategy for treating PAK-dependent tumors. FRAX597 treatment also phenocopied genetic loss of *Pak1* and impaired *Kras*-driven skin tumorigenesis (86). Interestingly, in this mouse model, both genetic and pharmacologic inhibition of PAK1 resulted in reduction of ERK and AKT activity, supporting the importance of PAK1 signaling cross-talk with these two RAS effector pathways.

Peterson and colleagues performed a screen to identify small molecule allosteric inhibitors of Cdc42 activation of Group I PAKs. The results of this screen led to the development of IPA-3, which interacts with the PBD/AID region of group I PAKs and prevents their activation by GTPase binding (119, 120). IPA-3 showed strong selectivity for Group I PAKs, with no inhibitory activity for Group II PAKs or more than 200 other protein kinases evaluated. However, the inability of IPA-3 to inhibit already activated PAK1, its micromolar $IC_{50}$ and its rapid metabolism to a toxic compound due to the reduction of the disulfide bond it contains, limit the ability to transition IPA-3 as a clinically useful chemical platform.

With increasing experimental evidence validating a driver role for PAKs in tumor growth and invasion, a key issue for the clinical advancement of PAK inhibitors will be

defining genetic and/or biochemical markers that identify those cancers that will respond to anti-PAK therapy. The position of PAK downstream of mutant KRAS and RAC, in addition to PAK signaling cross-talk with the key RAS effector pathways, support PAK inhibitors as a therapeutic strategy for RAS-mutant cancers. Given the involvement of multiple effectors in driving RAS-dependent cancer growth, PAK inhibition in combination with inhibitors of RAF or PI3K effector signaling will likely be required. Currently, pharmacologic inhibitors of PAK1 also inhibit other Group I PAKs; whether PAK1-selective inhibitors are more desirable and possible to develop are issues that remain to be resolved. Of the spectrum of PAK substrates, which substrate(s) will provide a reliable biomarker for PAK inhibitor anti-tumor activity also remains unclear. A survey of the patent literature indicates that more PAK inhibitors are in the pipeline (121, 122). As more potent and selective inhibitors become available, the answers to many of these unresolved questions will likely be addressed. In Chapter 2, I detail my studies with a novel, ATP-competitive and PAK1-selective inhibitor.

***The RalGEF-RAL pathway in RAS-mutant cancer***

Another less studied RAS effector pathway is the RalGEF-RAL pathway. The RAS-like (RAL) members of the RAS family are two highly identical proteins, RALA and RALB (8). These two small GTPases contain a C-terminal CAAX motif that specifies subcellular location, similar to other RAS family members. Both RALA and RALB interact with RalGEFs that facilitate the exchange of GDP for GTP. These RalGEFs, which include RalGDS (123-125) and RGL2/3 (126, 127), were identified in studies that sought proteins that interact with RAS family proteins, and thus comprise a direct link between RAS signaling and RAL activation. Like RAF and PI3K, RALGEFs also contain RBDs that promote their association with RAS-GTP (8). To catalyze hydrolysis of GTP to GDP and return RAL proteins back to an inactive state, there are two RalGAP proteins that consist of a heterodimer of RalGAPβ paired with either RalGAPα1 or RalGAPα2.

Our lab and others have demonstrated that RALA and RALB have divergent roles in promoting various traits of cancer growth. For example, in pancreatic cancer RALA is necessary for anchorage-independent growth in vitro and tumorigenicity *in vivo* but RALB is dispensable (128). In contrast, RALB, but not RALA, is required for invasion *in vitro* and metastasis *in vivo* (129). Moreover, loss of RALB is lethal for tumor cells, but not normal cells (128). These differential roles for two highly identical proteins, which diverge primarily in their C-terminal membrane targeting sequences and their posttranslational modifications (130), highlight the importance of subcellular localization and substrate engagement for promoting specific cellular outcomes in that RALA and RALB must engage with disparate downstream effectors in the correct time and place to regulate these RAL isoform-driven phenotypes.

***Targeting the RAL effector TBK1 in human cancer***

Like other RAS family members, RAL sits at the center of a diverse web of cellular effectors that mediate processes such as gene transcription, endocytosis, autophagy, and actin reorganization (8). The most well characterized effector signaling pathway of RAL proteins involves the exocyst complex, which facilitates cellular transport of vesicular cargo from the Golgi apparatus to the plasma membrane where it is released into the intercellular millieu (131). Two members of the exocyst complex that directly engage with RAL proteins are Exo84 AND Sec5. Interestingly, downstream of RALB, Sec5 is able to bind to and activate TANK-binding kinase 1 (TBK1) (132), a non-canonical IκB kinase (IKK), involved in regulation of NF-κB.

NF-κB signaling is a tightly controlled pathway that transcriptionally governs several aspects of cell physiology, most importantly, proliferation, survival, and the immune response to cellular pathogens (133). Misregulation of NF-κB has been heavily implicated in supporting many tumorigenic phenotypes in myriad cancer types, in part by upregulation of

genes that encode proteins important for growth and cell survival. Typically, complexes of different NF-κB family members are held inactive in the cytoplasm by inhibitors of NF-κB, known as IκB proteins. In response to various stimuli, such as inflammatory signals, the canonical IKKs, IKKα and IKKβ, become activated and promote dissolution of the IκB-NF-κB complex through phosphorylation and ubiquitylation of IκB proteins (134).

There are other IKK proteins besides IKKα/β that are responsible for activating NF-κB signaling. These non-canonical IKKs act downstream of diverse stimuli that converge on Toll-like receptors (TLR) and TNF receptor-associated factor (TRAF) proteins. These receptors mediate TANK binding to TBK1, which is one such non-canonical IKK (135). TBK1 can then go on to promote both survival and inflammatory signaling. TBK1 is the focus of my studies in Chapter 3.

Additionally, TBK1 is capable of phosphorylating the interferon regulatory factor proteins, IRF3 and IRF7, and inducing transcription of pro-inflammatory genes (55, 136). Therefore, the ability of the exocyst component Sec5 to induce activation of TBK1 provides a critical linkage between RAS-mediated proliferative signaling in cancer and NF-κB responsive inflammation (132). TBK1 also plays a role in pro-survival signaling through direct phosphorylation of AKT at both T308 and S473 (Figure 1.2) (137).

A study searching for synthetic lethal interactors of mutant RAS utilized a systematic siRNA screen and reported that TBK1 was deleterious to cancer cell lines harboring mutationally activated but not wild-type KRAS (54). The results of this screen were validated in non-small cell lung cancer (NSCLC). However, subsequent studies in other cell types reported that the relationship between KRAS and TBK1 was far more complex than initially imagined, and that not all KRAS-mutant tissue was exclusively dependent on TBK1 signaling for tumor cell growth and survival (55, 137).

One study screened a panel of PDAC cell lines for dependency on TBK1 using both genetic and pharmacologic approaches. Neither pharmacologic inhibitors of TBK1 nor

shRNA-mediated suppression of TBK1 expression had any measurable effect on the viability of the cell lines tested, though reduction in pIRF3 S386 was observed (55). Thus, conflicting observations have been made concerning whether TBK1 is a bona fide target for RAS-mutant cancers. In Chapter 3 I describe the application of a novel TBK1 inhibitor to focus on the study of TBK1 in KRAS-mutant PDAC. In my studies, I determined whether TBK1 inhibition could enhance the anti-tumor activity of an ERK1/2 inhibitor, as ERK1/2 is a vital member of the canonical RAF-MEK-ERK RAS effector pathway.

***Pancreatic ductal adenocarcinoma as a model to study KRAS effector signaling***

Pancreatic ductal adenocarcinoma (PDAC) is among the deadliest human cancers, with a 5-year survival rate of approximately 8% (138). In 2016, PDAC surpassed breast cancer and is now the 3$^{rd}$ leading killer among cancer deaths in the United States and is set to surpass colorectal cancer by 2020 (139, 140). The reasons for the dire and deadly state of PDAC are manifold. First of all, patients are often asymptomatic, affording the primary tumor time to disseminate and create extensive metastatic lesions in other areas of the body, such as the lymph nodes, lungs, liver and other abdominal compartments (141). Surgical resection of the primary tumor is the only option for long-term effective treatment, though most patients, at the time of diagnosis, are well past the stage where surgical resection is a viable option. This leaves radiation and chemotherapy as the only treatment strategies for the majority of PDAC patients.

Since 1997, gemcitabine, a nucleoside analog that disrupts DNA replication, has been the standard of care for PDAC (138, 141, 142). Recently, two new therapies emerged that have become standards of care for PDAC, both of which are drug cocktails comprised of traditional cytotoxic anti-cancer drugs. Approved in 2013, one of these treatments is gemcitabine in combination with nab-paclitaxel. Nab-paclitaxel is a nanoparticle formulation of an albumen-conjugated form of the mitotic spindle inhibitor, paclitaxel. Approved in 2011,

the second treatment is the four-drug cocktail FOLFIRINOX, comprised of folinic acid, fluorouracil (5-FU), irinotecan and oxaliplatin. 5-FU is a pyrimidine analogue and oxaliplatin is a DNA alkylating agent; both disrupt DNA synthesis. Irinotecan is a topoisomerase inhibitor that introduces strand breaks into DNA, also disrupting DNA synthesis. Folinic acid is a vitamin B analog that decreases some of the toxic side effects of 5-FU.

Despite the approval of these therapies for PDAC, most patients still succumb to this disease in less than a year (139). While our knowledge of the genetic basis of pancreatic cancer is quite extensive (143-147), unlike in other cancers where such knowledge has led to the development of effective targeted therapies, no truly effective targeted therapies are available for PDAC. One targeted therapy that is approved for PDAC is erlotinib, an inhibitor of the EGFR receptor tyrosine kinase, which is responsible for driving activation of RASGEF proteins that stimulate RAS signaling (148). The clinical trial that led to the approval of erlotinib in combination with gemcitabine in 2005 showed a statistically significant but clinically insignificant two-week improvement in patient survival (149). That this minor improvement in survival was enough for erlotinib to be approved for PDAC therapy speaks volumes about the dire need for new therapeutic interventions for this disease. In fact, erlotinib is no longer used in the clinic for PDAC. Clearly, the need for more effective treatments will become even more acute in the coming years as PDAC overtakes colon cancer as the number two cancer killer in the US.

PDAC is striking in that it harbors a KRAS mutation in nearly 100% of cases (2), and mutant KRAS is a well-validated driver of this disease. Although activating mutations in KRAS occur exceptionally early in the development of PDAC, appearing in the benign precursor lesions, pancreatic intraepithelial neoplasias (PanINs), cancer cell line and mouse model analyses show that ablation of KRAS expression dramatically impairs PDAC growth (42). Thus, KRAS is the key step in both PDAC initiation and maintenance (42, 150).

Aside from KRAS mutations, three other genes are mutated frequently in PDAC. These include the TP53, CDKN2A and SMAD4 tumor suppressors (141). Since pharmacologic inhibitors that antagonize an activated oncoprotein, such as KRAS, should be much easier to develop than small molecules that restore the function of lost tumor suppressors, KRAS is the most attractive target for PDAC drug discovery. While many other genes have been identified in PDAC, these occur at frequencies of less than 5% (1, 25, 43). Thus, for this and other reasons mentioned above, the development of anti-KRAS drugs is one of four major directions for the field identified recently by the National Cancer Institute. The high frequency of KRAS mutations in PDAC and their driver role in this cancer type make PDAC an exceptional model for studying KRAS signaling and for elucidating the downstream signaling mechanisms that KRAS employs to promote tumor development and maintenance.

***Rationale and objectives for the studies described in this document***

The aims of my thesis work described herein encompass evaluating the contribution and therapeutic potential of PAK1 and TBK1, which are downstream protein kinase components of two less studied KRAS effector pathways, the Tiam1-RAC and RalGEF-RAL pathways. In Chapter 2, I describe my studies of PAK1 in PDAC. I determined that PAK1 was necessary to maintain PDAC cell line proliferation and anchorage-independent growth. I show that PAK1 could be promoting the growth and survival of pancreatic cancer cells via macropinocytosis, wherein the cell non-specifically engulfs portions of the extracellular compartment as a means to scavenge nutrients and to fuel unrestrained growth. In Chapter 3, I demonstrate that, although TBK1 appears to be dispensable for maintaining PDAC cell viability, both *in vitro* and *in vivo*, TBK1 activity is intimately linked to autophagy, and this linkage could be exploited by pairing TBK1 inhibitors with other targeted therapies or with conventional cytotoxic drugs. Overall, my data suggest that, although PAK1 and TBK1 may

not represent exceptionally clinically useful targets on their own, both of these kinases are indelibly connected to cellular metabolism in PDAC and therefore warrant more research to assess their potential as therapeutic targets in combination with other agents.

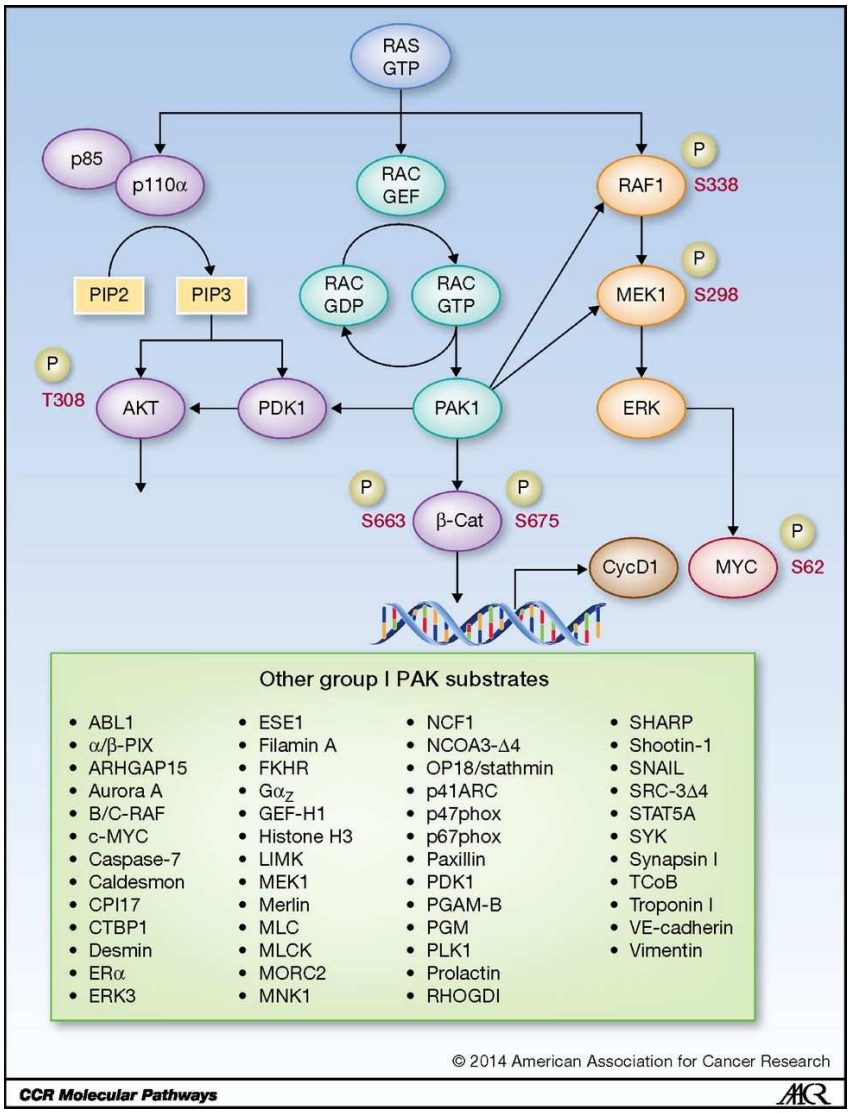



**Figure 1.1: RAS-RAC-PAK1 effector signaling.** The importance of the RAF-MEK-ERK and PI3K-AKT-mTOR effector signaling networks: both are well-validated drivers of mutant RAS-dependent cancer growth. RAC is activated by RAS through direct (e.g., via Tiam1) or indirect (via PIP3 formation) activation of guanine nucleotide exchange factors for the RAC small GTPase. The Group I PAKs comprise one key effector family of RAC. Over 50 substrates of PAK1 have been described. These substrates include components of the ERK MAPK cascade. Activated PAK1 can also function as a scaffold to facilitate AKT activation.

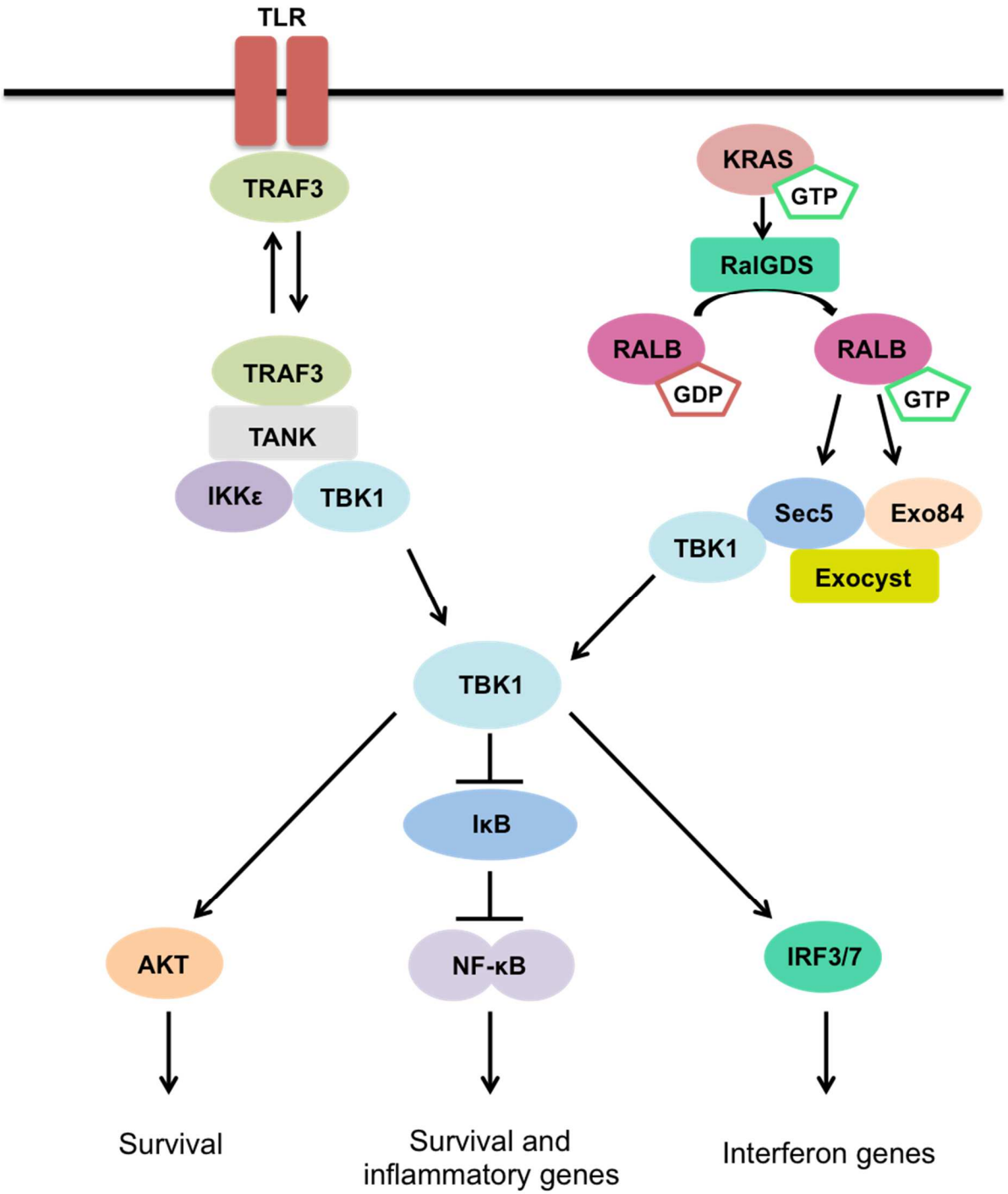



**Figure 1.2: TBK1 is activated downstream of both TLR signaling and RAS-mediated RALB/Sec5 signaling.** TBK1 is canonically activated downstream of inflammatory or immune signals from Toll-like receptors (TLRs), though it can also become activated downstream of RAS-mediated activation of RAL and the Sec5 component of the exocyst complex. TBK1 can then go on to modulate survival signaling via direct phosphorylation of AKT, or inflammatory signals via release of NF-κB inhibition and stimulation of IRF3/IRF7.

## CHAPTER 2: PAK1 REGULATES MACROPINOCYTOSIS IN PANCREATIC CANCER[2]

### *Overview*

Despite attempts to directly target mutant KRAS and to design inhibitors of validated KRAS effector pathways, to date, no clinically successful anti-KRAS therapies have been developed. The lack of success of these inhibitors is due, in part, to the importance of other effectors in KRAS-dependent cancer growth and to the upregulation of compensatory signaling programs that overcome inhibitor activity. We have determined that PAK1 serine/threonine kinase protein expression is elevated in a subset of primary patient pancreatic tumors and in pancreatic ductal adenocarcinoma (PDAC) cell lines. Both genetic silencing of *PAK1* and pharmacologic inhibition of PAK1 kinase activity by a novel, highly-selective ATP-competitive inhibitor, AZ13705339, impaired PDAC anchorage-independent growth. Additionally, as mutant KRAS has been linked to upregulation of metabolic processes, such as macropinocytosis, we examined the ability of PAK1 to regulate macropinosome formation in PDAC. Inhibition or genetic ablation of PAK1 resulted in a marked decrease in macropinocytosis. Surprisingly, PAK1-driven macropinocytosis is independent of KRAS-RAC1 signaling. Finally, we determined that concurrent inhibition of PAK and either ERK or PI3K synergistically reduced macropinosome formation in a subset of PDAC lines. Our findings validate PAK1 as a therapeutic target in PDAC.

[2]This chapter is currently under review for publication. The author list is as follows: Nicole M. Baker, ,Meagan B. Ryan, G. Aaron Hobbs, Kirsten L. Bryant, Tikvah K. Hayes, Campbell D. Lawson, Andrea Wang-Gillam, Janine LoBello, Haiyong Han, drenne D. Cox, and Channing J. Der. All experiments were performed by myself, except for Figure 2-1, panels C-D.

***Introduction***

Pancreatic cancer is a rapidly fatal disease with a 8% 5-year survival rate (138, 139). Despite the fact that nearly all pancreatic ductal adenocarcinomas (PDAC) are driven by activating mutations in the *KRAS* oncogene, no clinically effective anti-KRAS targeted therapies have been developed. KRAS acts as a major cell signaling hub that promotes multiple cellular processes required to maintain tumorigenic growth (1). Most notably, a plethora of inhibitors have been generated against the two canonical KRAS effector pathways, the RAF-MEK-ERK mitogen-activated protein kinase (MAPK) and the PI3K-AKT-mTOR lipid kinase pathways, with many currently under clinical evaluation (43). Disappointingly, as monotherapy, these inhibitors have shown limited to no clinical benefit. One known basis for their limited clinical efficacy is the process of dynamic kinome reprogramming, whereby pharmacological inhibitors induce signaling changes that compensate for and overcome inhibitor actions (88).

A second likely basis for the limited efficacy of inhibitors of the canonical effector pathways is an unreallized requirement to concurrently inhibit other effector pathways that are also essential for KRAS-dependent cancer growth and that can compensate, at least in part, for impaired signaling through the canonical effectors. One such less studied pathway includes the Tiam1 guanine nucleotide exchange factor (GEF) and the RAC1 small GTPase. *Tiam1*-deficient mice show impaired *Hras*-induced tumorigenesis (56). Other studies demonstrated that *Rac1* deficiency reduced mutant *Kras*-driven lung (68) and pancreatic cancer growth (69). However, the key effector(s) that are critical for Tiam1-RAC1-dependent oncogenesis remain to be established.

Like KRAS, RAC1 can interact with a spectrum of functionally diverse effectors. Of these, the PAK1 serine/threonine kinase is an excellent candidate effector for RAC1-dependent cancer growth. PAK1 substrates include proteins involved in regulating cellular processes that control cell proliferation, survival, cytoskeletal rearrangement, motility, and

epithelial-mesenchymal transition. PAK1 has been shown to act as a driver in breast (151, 152), colon (91, 93), lung (153, 154), and other cancers (79). A recent study implicated a requirement for PAK1 in initiating and driving tumor development in a mouse model of *Kras*-mutant skin squamous cell carcinoma (86). More importantly, studies of PAK1 in PDAC reveal that PAK1 is a key regulator of MET-regulated PDAC cell migration (155) and that PAK1 promotes PDAC cell growth through transcriptional regulation of NF-κB and fibronectin (110).

Additionally, PAK1 is known to regulate macropinocytosis (103), an actin-driven process by which cells engulf extracellular protein as a source of amino acids to sustain the increased metabolic demands of tumor growth. Mutant KRAS has been shown to drive macropinocytosis in PDAC (64, 105, 156) and inhibition of macropinocytosis impaired PDAC tumor growth. However, the critical KRAS effector signaling pathways that promote macropinocytic activity in pancreatic cancer have not been determined. Elucidation of how mutant KRAS drives macropinocytosis may identify a therapeutic target, such as PAK1, to enable effective blockade of macropinocytosis and PDAC growth.

In this study, we addressed a role for PAK1 in KRAS effector signaling, macropinocytosis, and pancreatic cancer growth. Unexpectedly, PAK1 activity and signaling were largely independent of KRAS and RAC1 in the PDAC cell lines tested. However, we did find elevated PAK1 protein expression in PDAC cell lines and patient tumors. Further, genetic and pharmacologic suppression of PAK1 impaired both anchorage-independent growth and macropinocytosis. Thus, PAK1 may be a relevant therapeutic target in KRAS-driven pancreatic cancer, albeit not directly via KRAS itself.

### *Materials and Methods*

#### Cell lines, tumor tissue lysates, and tissue microarray

Authenticated PDAC cell lines were obtained from ATCC and maintained in either DMEM (HPAC, MIA PaCa-2, and PANC-1) or RPMI-1640 (AsPC-1, HPAF-II, and CFPAC-1) supplemented with 10% fetal bovine serum, and were not maintained in continuous culture for longer than two months after receipt from the source. Detergent buffer lysates generated from patient primary pancreatic tumor and adjacent non-tumor tissues were kindly provided by Dr. Jen Jen Yeh (UNC-Chapel Hill). Pancreatic tumor tissue was acquired and tissue microarrays (TMAs) were generated by the Translational Genomics Research Institute (TGen) (Phoenix, Arizona) from freshly cut sections of fixed, embedded, de-identified primary pancreatic cancer tumor tissues.

#### Plasmids and reagents

Lentiviral plasmids encoding shRNA against *PAK1* were obtained from the University of North Carolina Lenti-shRNA Core Facility. All shRNAs are in the pLKO.1 backbone and contain a puromycin resistance gene. All hairpin sequences are based on those deposited in the TRC RNAi Consortium of the Broad Institute, and are as follows: NS (non-specific): (5'-CCTCTTGATGAACCATCTATT-3'), shPAK1-1: (5'-CTTCTCCCATTTCCTGATCTA-3'), and shPAK1-2: (5'-GCTGTGGGTTGTTATGGAATA-3').

Silencer Select siRNA targeting KRAS (#s s7939 and s7940) and RAC1 (#s s11711 and s11713) were obtained from Thermo-Fisher. Mismatch control siRNA was obtained from Dharmacon (#D-001210-05).

Primary antibodies used for TMA staining and for western blot analysis were obtained from Cell Signaling Technologies: PAK1 (#2602), pMEK1 S298 (#9128), MEK1 (#9124); Sigma-Aldrich: β-actin (#A5441), GAPDH (#G8795), and Vinculin (#CP74); and

Millipore: anti-KRAS (#OP24) and anti-RAC1 (#05-389). Secondary HRP-conjugated anti-mouse (#31432) and anti-rabbit (#31460) antibodies were obtained from Thermo-Fisher.

**Immunohistochemistry**

Pancreatic TMAs were stained with anti-PAK1 antibody (Cell Signaling Technology, #2602). Samples were blinded and the intensity of PAK1 staining was scored by a pathologist (J. LoBello) and binned as none (+0), low (+1), medium (+2), or high (+3).

**Lentiviral transduction to silence *PAK1***

Lentivirus was generated in HEK-293T cells transfected with the pLKO.1 shRNA expressing plasmids and the psPAX2 and pMD2.G packaging vectors (Addgene). Virus was harvested 48 h post-transfection. PDAC cell lines were seeded at a density such that they would reach approximately 70% confluence the following day, and then were transduced with NS or shPAK1 virus in the presence of 8 μg/μL polybrene. Stably transduced cells were selected for 2-3 days in puromycin at a concentration determined empirically for each cell line.

**siRNA transfection to silence KRAS and RAC1**

All siRNA knockdown experiments were performed via reverse transfection to maximize transfection efficiency. Briefly, 10 μM siRNA was complexed with Lipofectamine RNAiMax (Life Technologies) per the manufacturer's instructions, and cells were seeded in 6 well dishes at a density of $3x10^5$ cells/well in the presence of this complex. Knockdown was allowed to proceed for 48 hours before cells were used in assays.

**Growth assays**

For anchorage-dependent proliferation assays, cells in complete growth medium were seeded in 96-well plates at cell densities varying from $1x10^3$ to $5x10^3$ per well depending on the cell line. To monitor cell viability after 72 h, 3-(4,5-dimethylthiazol-2-yl)-

2,5-diphenyltetrazolium bromide (MTT) was added to each well at a final concentration of 5 μg/ml. Cells were incubated in the presence of MTT for 4 h, media was aspirated, cells were lysed in 100 μL DMSO per well, and the absorbance at 590 nM was measured.

Soft agar colony formation assays were performed as we have described previously (157). Briefly, 0.6% bacto-agar dissolved in complete growth medium was allowed to solidify in a 6- or 12-well plate. Cells were seeded on top at densities ranging from $5x10^3$ to $2x10^4$ cells per well, depending on the growth properties of each cell line, in 0.3% bacto-agar in complete growth medium. Cells were fed with complete growth medium supplemented with vehicle alone (DMSO) or with PAK inhibitor AZ13705339 for 7-14 days. Colonies were stained with 2 mg/mL MTT, visualized with a Typhoon FLA 7000 Scanner, and quantified with ImageJ (NIH) (158).

**Inhibitor treatment**

The PAK1-selective ATP-competitive inhibitor, AZ13705339 (manuscript in preparation, McCoull et al., 2016) and the PI3K inhibitor, AZD8186 (159), were synthesized and provided by AstraZeneca. The ERK1/ERK2-selective ATP-competitive and allosteric inhibitor SCH772984 (160) was provided by Merck. Inhibitors were dissolved in DMSO to yield stock concentrations of 10-100 mM, aliquotted, and stored at -80°C.

PDAC cell lines were seeded and allowed to adhere for 24 h before inhibitor treatment. Compounds were dissolved in DMSO and serially diluted to attain the desired treatment concentrations. The amount of DMSO was held constant in all samples. $GI_{50}$ and $IC_{50}$ values for PDAC cells treated with AZ13705339 were determined with Prism Graphpad 6 software.

**Western blot analyses**

Cells were lysed in NP-40 buffer containing 1 M Tris pH 7.5, 1 M $MgCl_2$, 5 M NaCl, 10% NP-40, 10% glycerol, 0.25% sodium deoxycholate, and phosphatase and protease

inhibitors. Protein concentration was determined by Bradford Protein Assay (Bio-Rad). Lysates were resolved by SDS-PAGE and transferred to PVDF membranes that were then probed with the appropriate antibodies. Following application of Amersham ECL Prime Western Blotting Detection reagent (GE Healthcare Life Sciences #RON2232), chemiluminescence was visualized with a BioRad ChemiDoc, and images were analyzed for quantitation of bands with ImageJ.

**Macropinocytosis assay**

The macropinocytic index of cells was determined as described previously (161). Briefly, cell were seeded into glass-bottom MatTek dishes at densities such that they would reach approximately 30-40% confluence within 24 h. Cells were treated with vehicle or inhibitor at the indicated concentrations in complete growth medium for 24 h, then in serum-free medium for 16 h. The treated cells were then transferred to serum-free medium containing 1 mg/mL 70 kDa dextran conjugated with FITC (Thermo-Fisher, #D1823), for 30 min, washed with PBS, and fixed in 4% paraformaldehyde. Nuclei were stained with DAPI and dishes were stored in the dark at 4°C in PBS containing 1 mg/mL BSA and 0.01% sodium azide until they were imaged. Cells were visualized on a Zeiss LSM 700 Confocal Laser Scanning Microscope at 63x magnification. Ten discrete fields were collected per sample and ImageJ was used to quantitate the area of FITC-dextran signal per total cell area of each image, as detailed previously (161).

**RAC1 activation assay**

The level of active RAC1-GTP was determined by a GST-PAK-PBD affinity assay as described previously (162). Briefly, 48 h post transfection with KRAS or RAC1 siRNA, whole cell lysates were collected, protein concentration was determined, and lysates were rotated at 4°C for 30 min in the presence of Sepharose beads covalently linked to GST-PAK-PBD,

which were kindly provided by Dr. Keith Burridge. Western blot analysis was performed on pulldown samples and whole cell lysates as described above.

**Statistical analysis**

All data were imported into GraphPad Prism 6 software and statistical analyses were performed using a One-way ANOVA using multiple comparisons. Significance values were designated as follows: *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$.

***Results***

***PAK1 protein levels are elevated in PDAC cell lines and tissue***

Previous studies established a role for the RacGEF-RAC1 pathway in cancer growth (56, 57, 68, 69). However, the critical RAC1 effector(s) important for RAC1-dependent pancreatic cancer growth have not been established. Here we addressed a possible role for the RAC1-activated protein kinase, PAK1, in pancreatic cancer. We observed elevated levels of PAK1 protein in a subset of PDAC cell lines (8 of 11) compared to HPNE immortalized human pancreatic ductal epithelial cells (Figure 2.1A). Elevated protein levels did not correlate with *KRAS*-mutation status, since there were high levels of PAK1 in the *KRAS*-wild type PDAC cell line BxPC-3, but not in KRAS-transformed HPNE cells. Additionally, PAK1 total protein did not always correlate with pMEK S298 levels, a validated marker of PAK1 activity (163) (Supplemental Figure 2.1). This could imply either that PAK1 protein is not necessarily hyperactive simply because it is overexpressed, or that pMEK1 S298 is not an ideal biomarker for PAK1 activity.

We also detected high levels of PAK1 in lysates of primary tumor samples from PDAC patients (Figure 2.1B). Immunohistochemical analysis (IHC) of three separate primary PDAC tumor microarrays (TMAs) revealed that, although PAK1 staining was present in both primary tumor samples and adjacent normal tissues, typically the tumor exhibited more

intense staining (Figure 2.1C-D). We found no correlation of PAK1 levels with patient survival rates (data not shown).

### *Genetic suppression of PAK1 impairs anchorage-independent but not anchorage-dependent growth*

To determine whether PAK1 serves a driver role in PDAC, we evaluated the consequences of genetic suppression of PAK1 on PDAC growth. We utilized two shRNA sequences targeting *PAK1* to stably suppress PAK1 protein expression (Figure 2-2A). We then assayed the ability of cells to proliferate in anchorage-dependent and -independent growth assays. We observed limited reduction in anchorage-dependent proliferation (Figure 2-2B). In contrast, PAK1 suppression significantly reduced anchorage-independent growth as determined by colony formation in soft agar (Figure 2-2C). The level of growth suppression correlated with the level of knockdown achieved by each individual shRNA. We observed a reduction in soft agar colony formation in all six cell lines evaluated, albeit with a range of sensitivity to PAK1 knockdown. In 5 of 6 cell lines, we observed at least a 50-70% impairment of colony formation ($p<0.01$ to $p<0.001$). We conclude that PAK1 is critical for PDAC anchorage-independent cell proliferation. Unexpectedly, levels of pMEK1 S298, the best validated marker of PAK1 activity, were not reduced upon knockdown of PAK1 expression, possibly due to cellular reprogramming of compensatory signaling pathways (Supplemental Figure 2.2).

### *AZ13705339 effectively impairs anchorage-independent growth in PDAC cells*

To complement our genetic suppression studies of PAK1, we next utilized a novel ATP-competitive, highly selective small molecule inhibitor of PAK1. AZ13705339 was isolated from a kinase subset screen and displays selectivity for PAK1, with limited potency against PAK2 and SRC family kinases (manuscript in preparation, McCoull et al., 2016). We first evaluated the ability of AZ13705339 to block PAK1 activity as measured by a reduction

in phosphorylation of the well-validated PAK1 substrate, MEK1, at residue S298 (pMEK1) (163). We observed a dose-dependent decrease in pMEK1 S298 in all cell lines evaluated (Figure 2.3A-B), although the $IC_{50}$ for pMEK1 reduction varied significantly among them. MIA PaCa-2 cells were the most sensitive to the PAK1 inhibitor with respect to target inhibition, displaying the lowest $IC_{50}$ for pMEK1 reduction (2.2 μM), whereas CFPAC-1 cells were the most resistant (>10 μM). Additionally, although *in vitro* specificity analyses revealed that AZ13705339 exhibits limited potency against SRC family kinases (SFKs) (manuscript in preparation, McCoull et al., 2016), we did not observe detectable inhibition of SFK activity at concentrations that fully suppressed MEK1 phosphorylation at S298. Instead, inhibition of SFKs, as measured by phosphorylation of focal adhesion kinase (FAK), was seen only at a concentration approximately 10-fold greater than that required for inhibition of PAK1 activity (Supplementary Figure 2.3). This result indicates that AZ13705339 displays good selectivity for PAK1 in cells.

We next determined if pharmacologic inhibition of PAK1 signaling impaired the growth of PDAC cell lines on plastic. We observed a range of sensitivity that correlated generally with reduction in pMEK1. However, the concentrations for inhibition of anchorage-dependent growth ($GI_{50}$) were considerably higher than the $IC_{50}$ values for inhibition of PAK1 signaling (Figure 2.3C). The $GI_{50}$ values for sensitive cell lines ranged between 2 and 10 μM, with two cell lines, HPAF-II and CFPAC-1, demonstrating no sensitivity at 10 μM. Thus the growth inhibition seen at these high concentrations is likely due to off-target activities of AZ13705339. These results are consistent with our PAK1 shRNA analyses (Figure 2.2B), indicating that PAK1 is not essential for anchorage-dependent growth.

In contrast to its very small effect on anchorage-dependent growth, pharmacological inhibition of PAK1 with AZ13705339 caused significant reduction in anchorage-independent growth and colony formation in agar of several cell lines (Figure 2.3D). That both pharmacologic inhibition and genetic suppression of PAK1 caused similar growth

consequences supports a role for PAK1 kinase activity in driving anchorage-independent but not -dependent growth of PDC cells.

Interestingly, the two cell lines that demonstrated resistance to AZ13705339 when evaluated for viability on plastic, HPAF-II and CFPAC-1 (Figure 2.3C), exhibited enhanced colony formation with increasing doses of AZ13705339 (Figure 2.3D). This could be due to compensatory signaling pathways or due to the scaffolding function of PAK1 that couples PDK1 to AKT and induces phosphorylation of AKT at T308 to drive survival signaling, which treatment with AZ13705339 would not block.

***PAK1 inhibition reduces macropinocytosis***

We next addressed a possible mechanism for PAK1 support of PDAC growth. Recent studies found that PDAC cells exhibit increased macropinocytosis as one mechanism to fulfill their increased metabolic needs (105, 156). Since previous studies also described a role for PAK1 in macropinocytosis (103, 104, 164), we sought to determine whether PAK1 is important for macropinocytosis in KRAS-mutant PDAC cells. First, we used shRNA to suppress PAK1 expression in a panel of PDAC cell lines. Depletion of PAK1 markedly reduced macropinocytotic uptake in three out of four PDAC cell lines (AsPC-1, PANC-1, and HPAF-II, but not MIA PaCa-2) (Figure 2.4 A-C). In these lines, the impairment of macropinocytosis correlated with the level of PAK1 suppression.

To determine whether pharmacologic inhibition of PAK1 kinase activity can also reduce macropinocytotic activity, we treated cells with AZ13705339 in a 4-fold range of concentrations around the $IC_{50}$ for pMEK1 inhibition. Inhibition of PAK kinase activity with AZ13705339 diminished macropinocytotic uptake in the same cell lines in which macropinocytosis was disrupted upon PAK1 knockdown (Figure 2.4D-E). Therefore, we conclude that PAK1 activity is crucial for regulating macropinocytosis in PDAC cells.

***KRAS and RAC1 drive macropinocytosis in only a subset of PDAC cell lines***

PAK proteins are often activated downstream of the small GTPase RAC1 (165), and RAC1 has been previously implicated in driving macropinocytosis (166, 167). Additionally, both PAK1 and RAC1 are thought to be activated downstream of KRAS. We therefore determined whether KRAS and RAC1 regulate PAK1 activity and signaling. We used shRNA to stably suppress either *KRAS* or *RAC1* expression (Figure 2.5A) and observed that pMEK1 levels were not reduced. Thus, current evidence indicates that neither KRAS nor RAC1 activity regulate PAK1-dependent MEK1 signaling in PDAC cell lines, though it is possible that another marker of PAK1 activity may better reflect modulation of PAK1 activity downstream of KRAS or RAC1.

Next we performed macropinocytosis assays on our panel of PDAC cell lines following suppression of KRAS or RAC1 (Figure 2.5B). Loss of KRAS expression significantly reduced macropinocytosis in two PDAC cell lines, AsPC-1 and MIA PaCa-2, supporting previously published observations that KRAS is necessary for macropinocytic uptake in these and other PDAC cells (105) (Figure 2.5C). Two other PDAC lines, HPAF-II and PANC-1, displayed either no change in macropinocytosis or increased macropinocytotic uptake, respectively. This could be due to insufficient KRAS knockdown in the case of HPAF-II cells, or compensatory signaling mechanisms in PANC-1 cells. Alternatively, it is possible that KRAS regulates macropinocytosis in some but not all PDAC cell lines.

Knockdown of RAC1 was less potent at inhibiting macropinocytosis, with only AsPC-1 cells proving sensitive to loss of RAC1 (Figure 5 B-C). Additionally, in a standard RAC1-GTP pulldown assay, only AsPC-1 cells exhibited loss of RAC1-GTP levels following loss of KRAS expression (Supplemental Figure 2.4). Collectively, these results suggest that KRAS and RAC1 play a role in macropinocytosis in a subset of PDAC lines, but are not necessary for this process in all PDAC cells, and are not always linked within the same effector pathway.

***Dual inhibition of PAK1 and ERK or PI3K further reduces macropinocytic uptake in PDAC***

Although PAK inhibition alone caused a significant reduction in the macropinocytic index, we endeavored to determine whether combining inhibition of PAK1 with other targeted therapies could further reduce the ability of PDAC cells to undergo macropinocytosis. Furthermore, we sought to overcome the resistance to AZ13705339-mediated inhibition of macropinocytosis exhibited by MIA PaCa-2 cells that were nevertheless quite sensitive to AZ13705339-mediated inhibition of PAK1 kinase activity as measured by anchorage-independent growth. We propose that a blockade of extracellular nutrient scavenging could prove to be a viable strategy for the treatment of pancreatic cancer.

We treated a panel of PDAC cell lines with 450 nM SCH772984 (160), an ERK1/2 inhibitor, and 1μM AZD8186 (168), a PI3Kβ/δ inhibitor, either alone or in combination with 200 nM AZ13705339 for 24 hours and then performed a macropinocytosis assay as described above (Figure 2.6A). We probed for markers of PAK1 (pMEK1 S298), ERK1/2 (pRSK), and PI3K (pAKT S473) activity via western blot to verify that we achieved target inhibition at the indicated concentrations of inhibitor (Figure 2.6B). PI3K is a known regulator of macropinocytosis (167, 169), and we observed a significant reduction in macropinocytosis upon combination of AZ8186 with AZ13705339 in all cell lines tested compared with cells treated with these compounds as single agents. Additionally, dual ERK/PAK1 inhibition impaired macropinocytosis in all cell lines with the exception of PANC-1 cells, which have previously demonstrated resistance to SCH772984 (170). Moreover, combination treatment was the only method to significantly reduce inhibition of macropinocytosis in the relatively resistant MIA PaCa-2 cell line. These data suggest that multiple KRAS-driven pathways contribute to extracellular scavenging through macropinocytosis, and that combinatorial

inhibition of PAK1 and other validated KRAS effectors could lead to a macropinocytic blockade.

***Discussion***

Despite more than three decades of effort, an effective anti-KRAS therapeutic strategy has not reached the clinic. Currently, the most comprehensive efforts being pursued involve inhibitors of KRAS effector signaling, with many compounds currently under clinical evaluation (43). Disappointingly, inhibitors of components of the two canonical KRAS effector pathways, the RAF-MEK-ERK and PI3K-AKT-mTOR cascades, have not been effective when applied as single agents. One likely basis for the ineffectiveness of these compounds is that KRAS utilizes additional effectors to drive cancer growth. In this study, we addressed the role of PAK1 as a component of KRAS effector signaling and as a key driver of PDAC growth. Although we found that PAK1 activity is not directly linked with KRAS and RAC1, we did find that PAK1 is critical for anchorage-independent growth and for elevated extracellular nutrient scavenging. We conclude that pharmacologic inhibition of PAK1, in combination with inhibitors of other KRAS effector pathway components, may be an effective therapeutic approach for PDAC treatment.

The data in support of PAK1 as a necessary component of KRAS signaling in tumors (86, 110, 155) led us to survey a panel of PDAC cell lines and patient tumors. We observed that PAK1 protein expression is elevated in nearly all tumor cells and patient tumor tissues compared to controls. However, the expression of PAK1 did not directly correlate with mutant KRAS status, as a normal pancreatic ductal epithelial cell line (HPDE) transformed by mutant KRAS displayed lower PAK1 expression than untransformed HPDEs. Additionally, BxPC-3 cells, which are homozygous for wildtype KRAS but mutant for BRAF (171), exhibited one of the highest levels of PAK1 protein expression of any PDAC cell line.

Furthermore, levels of PAK1 protein did not correlate with an increase in phosphorylation of MEK1 at serine 298, a validated marker of PAK1 activity (163). However, we also observed that pMEK1 S298 was not an accurate marker of PAK1 protein loss. This is likely due to compensatory signaling mechanisms or the presence of other Group I PAK proteins in PDAC cell lines that can also phosphorylate MEK1 S298. Additionally, PAK1 possesses a scaffolding function whereby it can couple PDK1 to AKT in order to phosphorylate AKT on T308 and promote survival signaling (85). Phosphorylation of MEK1 at S298 is a good measure of PAK1 kinase activity, but does not take into account the scaffolding function of PAK1, which may play a large role in PDAC cell viability. Overall, these data, while establishing PAK1 overexpression in both conventional tumor cell lines and in patient tumor tissue, speak to the inherent heterogeneity of tumor tissue (145, 172) and the necessity for experimentation to be performed in a large number of samples in order to determine the role of any given protein in supporting pancreatic cancer growth.

One recent study focused on the role of PAK1 in mediating MET-induced PDAC cell migration (155) and found that PAK1 was a critical regulator of MET-driven cell motility and metastasis in mice, and that PAK1 activity drives resistance to MET inhibition. Yet another study identified a role for PAK1 promoting downstream transcription of fibronection via NF-κB activation, and proposed this was a mechanism that could support PDAC growth (110). Neither study thoroughly examined a role for PAK1 in relation to KRAS signaling, which is the ultimate driver of PDAC tumor growth. We chose to a complementary focus to these studies and examined KRAS-mediated activation of PAK1 and the ability of PAK1 to sustain the heightened level of PDAC cell proliferation and metabolism via macropinocytosis. We showed that either genetic or pharmacologic suppression of PAK1 function impaired anchorage-independent proliferation.

We then sought to extend the role of PAK1 in PDAC beyond these studies by evaluating its potential as a therapeutic target. We employed a novel ATP-competitive small

molecule inhibitor of PAK1, AZ13705339, which exhibits high selectivity for PAK1 and more limited affinity for PAK2 and other kinases. Though a definitive biomarker of PAK1 activity in tumors remains to be identified, we used pMEK1 S298 (163) as a reliable measure of PAK1 inhibition by AZ13705339. The majority of PDAC cell lines responded to AZ13705339 and loss of PAK1 expression similarly, in that anchorage-independent growth was impaired. However, two cell lines, HPAF-II and CFPAC-1, displayed some resistance to AZ13705339. It is possible that PAK1 kinase activity is not critical for maintaining growth in these two cell lines, and that the scaffolding function of PAK1 is more important for driving AKT activity. In this case, inhibition of kinase activity may stimulate the scaffolding function of PAK1 in an effort for cells to compensate for loss of PAK1 kinase activity. This hypothesis, if correct, would account for the seemingly opposing growth phenotypes observed in these cell lines upon knockdown of PAK1 protein and PAK1 inhibition. Furthermore, compensatory upregulation of signaling pathways and cell growth is often observed following pharmacologic inhibition. This has been observed with a BRAF V600E inhibitor, vemurafinib (173), and reprograming of the kinome in response to inhibitors is well validated (88). These studies, and ours, support the notion that multifaceted, combinatorial inhibitor strategies will be necessary to prevent tumor cells from subverting inhibition and continuing to proliferate. That a subset of PDAC lines is not dependent on PAK1 for this growth property likely reflects the genetic heterogeneity of PDAC. As a case in point, even with respect to what is arguably the most critical effector pathway for KRAS-dependent growth, we recently found that only ~50% of KRAS-mutant PDAC cell lines were sensitive to pharmacologic inhibition at certain nodes of the ERK MAPK cascade (170).

To begin to assess the cellular processes that PAK1 might regulate in PDAC, we first examined macropinocytosis, a mechanism by which cells uptake extracellular nutrients, such as proteins, and use lysosomes to break down these nutrients in order to use the byproducts to support catabolic processes (36). PAK1 has been previously implicated in this

process, though predominantly as a driver of endocytosis of viral particles or of phagocytosis in cells of the immune system (174-177). Additionally, PAK1 plays a role in the macropinocytosis of Bacille Calmette-Guerin (BCG), which is a type of bacterium, into bladder cancer cells (104). Commisso and colleagues found that mutant KRAS was capable of driving macropinocytosis as a means to scavenge extracellular amino acids for PDAC, but did not identify the mechanism by which KRAS mediates this process (105). That PAK1 was independently verified to contribute to macropinocytosis (103, 104, 164) and that it is a therapeutically tractable kinase thought to function downstream of mutant KRAS gave us reason to assess the role of PAK1 in promoting macropinocytosis in KRAS-mutant PDAC. Indeed, we observed that two out of the four PDAC cell lines surveyed depended on the expression of mutant KRAS to maintain levels of macropinocytosis. Surprisingly, all cell lines were dependent upon PAK1 activity to some degree despite our initial hypothesis that it would be KRAS mutation status, and not PAK1 activity alone, that would determine the macropinocytic potential of PDAC cells. These data suggest that, while PAK1 is a central contributor to macropinocytosis in PDAC, there are mechanisms independent of mutant KRAS that drive PAK1-mediated macropinocytosis in some KRAS-mutant tumors.

Finally, we addressed the utility of concurrent inhibition of PAK1 together with pharmacologic inhibitors of the canonical KRAS effector pathways. It is widely accepted that the best treatment strategies will likely exploit the effects of inhibitors against two or more signaling axes that are critical for tumor growth. In our study, we combined a PAK1 inhibitor, AZ13705339, with a dual allosteric and ATP-competitive inhibitor of ERK1/2, SCH772984 (160), or with AZD8186, an ATP-competitive inhibitor of PI3Kβ/δ (159, 168). In every PDAC line we tested, we saw a greater reduction in macropinocytosis with combined inhibition of PAK1 and ERK or PAK1 and PI3K, than with any inhibitor alone. Though in some cases the reductions were modest, especially when the compounds were used alone, combined inhibition of PAK1 and PI3K significantly reduced macropinocytosis in every cell line tested.

The resistance of MIA PaCa-2 cells to macropinocytic inhibition with PAK1 or PI3K inhibitor alone was markedly overcome upon combination treatment. This study establishes the groundwork for successful combined approaches to impair PDAC metabolism, and potentially, PDAC viability.

In summary, our data solidify a role for PAK1 in driving PDAC growth, potentially through macropinocytic scavenging of extracellular nutrients to support elevated PDAC cell metabolism. We demonstrate that both genetic loss and ATP-competitive inhibition of PAK1 kinase activity reduce both PDAC growth and macropinocytosis, though these activities are unexpectedly dependent upon KRAS and RAC1 signaling in only a subset of PDAC cells. Furthermore, we show that dual treatment of PDAC cells with PAK1 inhibitor and compounds targeting either ERK or PI3K have a combinatorial effect in blocking macropinocytosis. Overall, our findings suggest that small molecules targeting PAK1 activity, in combination with other inhibitors, may be an effective strategy for reducing PDAC tumor cell growth.

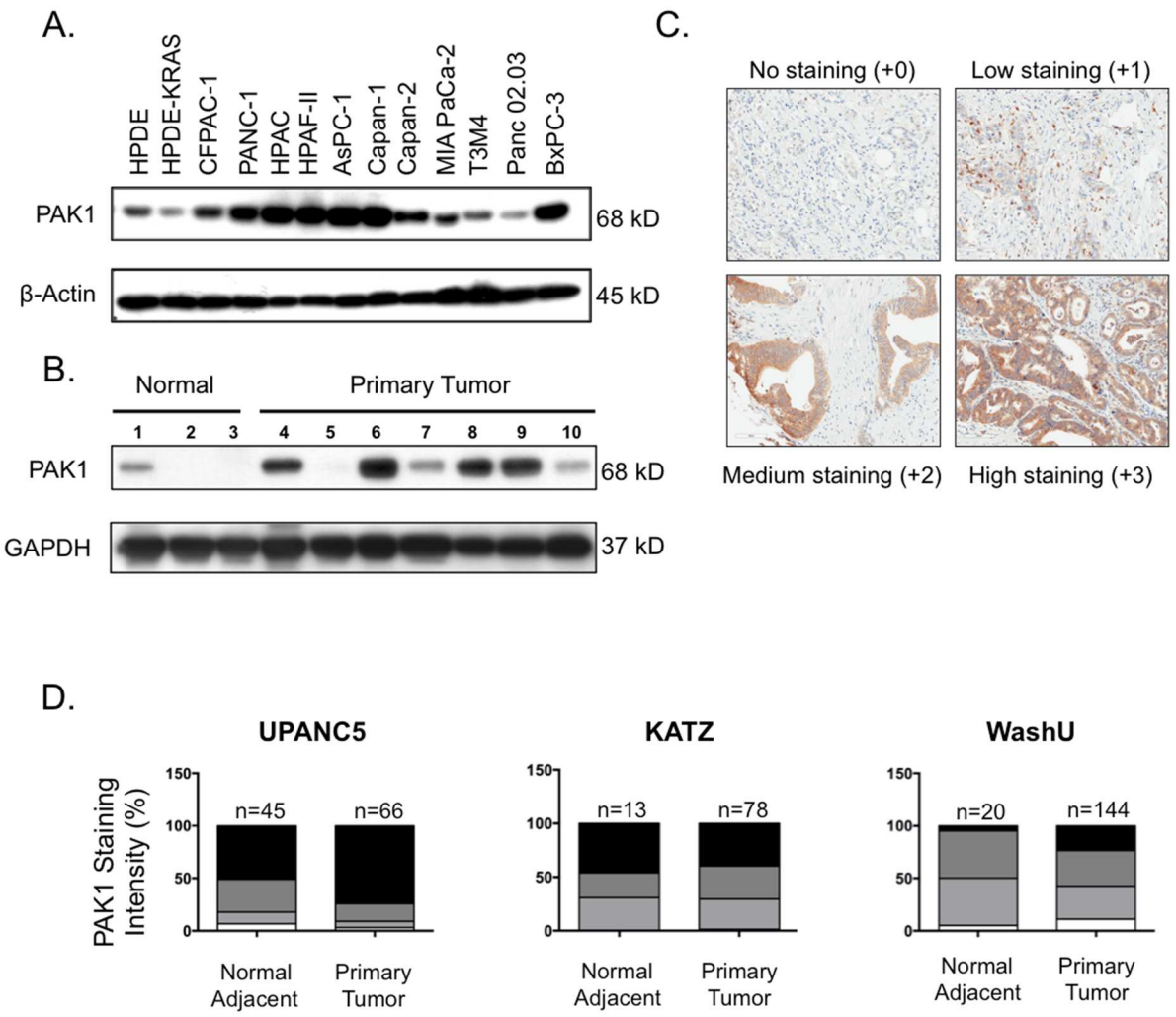



**Figure 1.1: PAK1 is overexpressed in a subset of PDAC cell lines.** A, Basal levels of PAK1 protein were evaluated via western blot in a panel of PDAC cell lines and in normal human pancreatic ductal epithelial (HPDE) cells with or without ectopic expression of mutant KRAS. B, Western blot of PAK1 protein levels in lysates from patient primary PDAC tumor tissues (lanes 4-10) and unmatched normal pancreatic tissues (lanes 1-3). C, TMAs containing human tissue samples from primary PDAC tumors and adjacent normal tissues were subjected to IHC staining for PAK1. Samples were blinded and evaluated by a pathologist, who scored them as follows: 0=no staining, 1=low staining, 2=moderate staining, 3=high staining. Shown are representative images of PAK1 staining intensity. D, Quantitation of PAK1 staining in three distinct TMAs of primary patient PDAC tumor tissue and adjacent normal tissue.

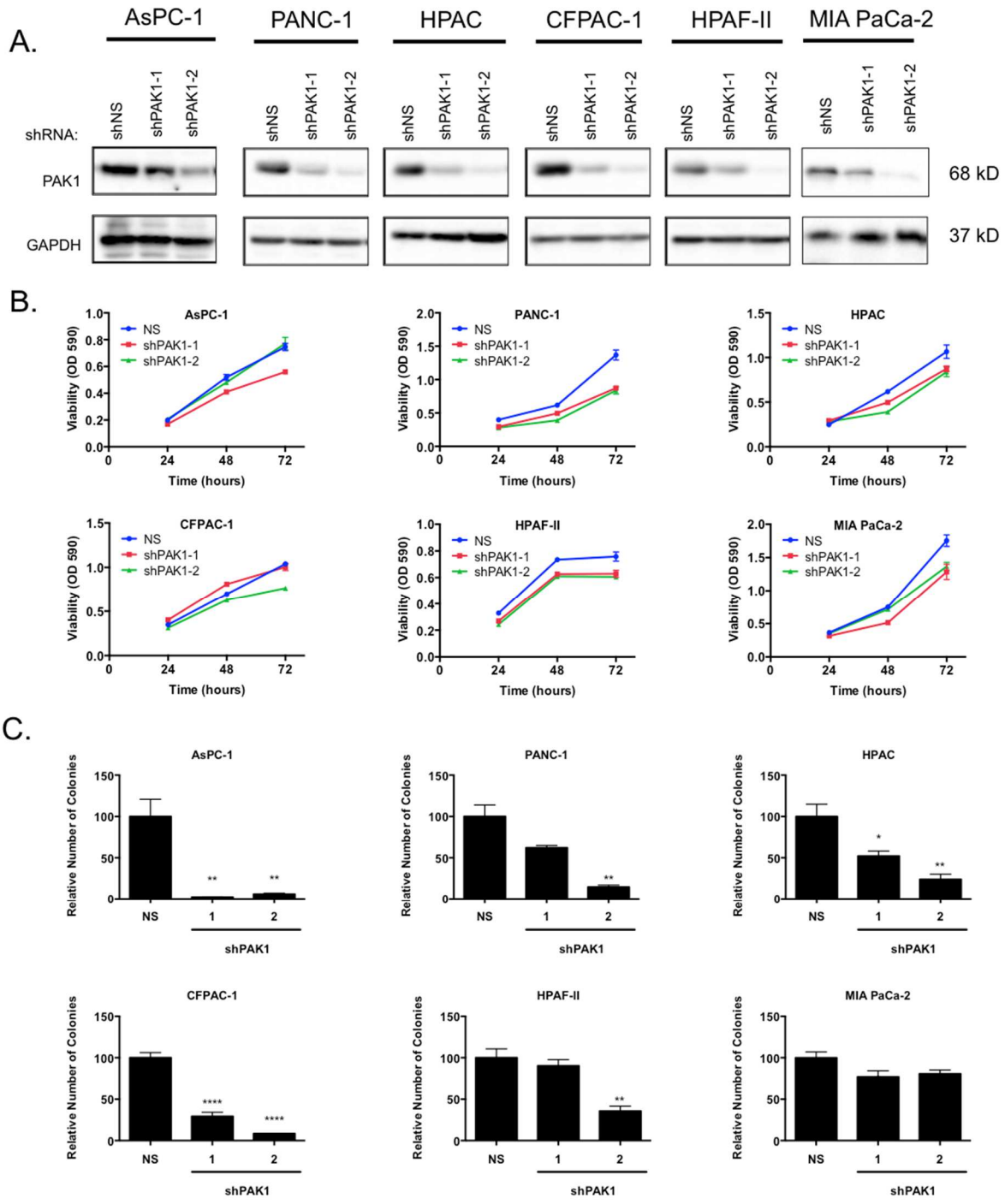



**Figure 2.2: Genetic suppression of PAK1 reduces cellular proliferation in KRAS-mutant PDAC cell lines.** A, A panel of PDAC cell lines was transduced with one of two distinct shRNA sequences against human PAK1 or with non-specific (NS) control shRNA. Loss of PAK1 was confirmed by western blot; GAPDH served as a loading control. B, Cell viability in 2D culture was measured with a standard MTT assay at the specified time points following PAK1 suppression. C, A 3D soft agar colony formation assay was performed in PAK1 knockdown PDAC cells. After 7-14 days, colony number was determined by MTT

staining and analysis in ImageJ. Data are represented as mean ± SEM and normalized to the NS control. Statistical analysis was done with a one-way ANOVA using multiple comparisons where *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$.

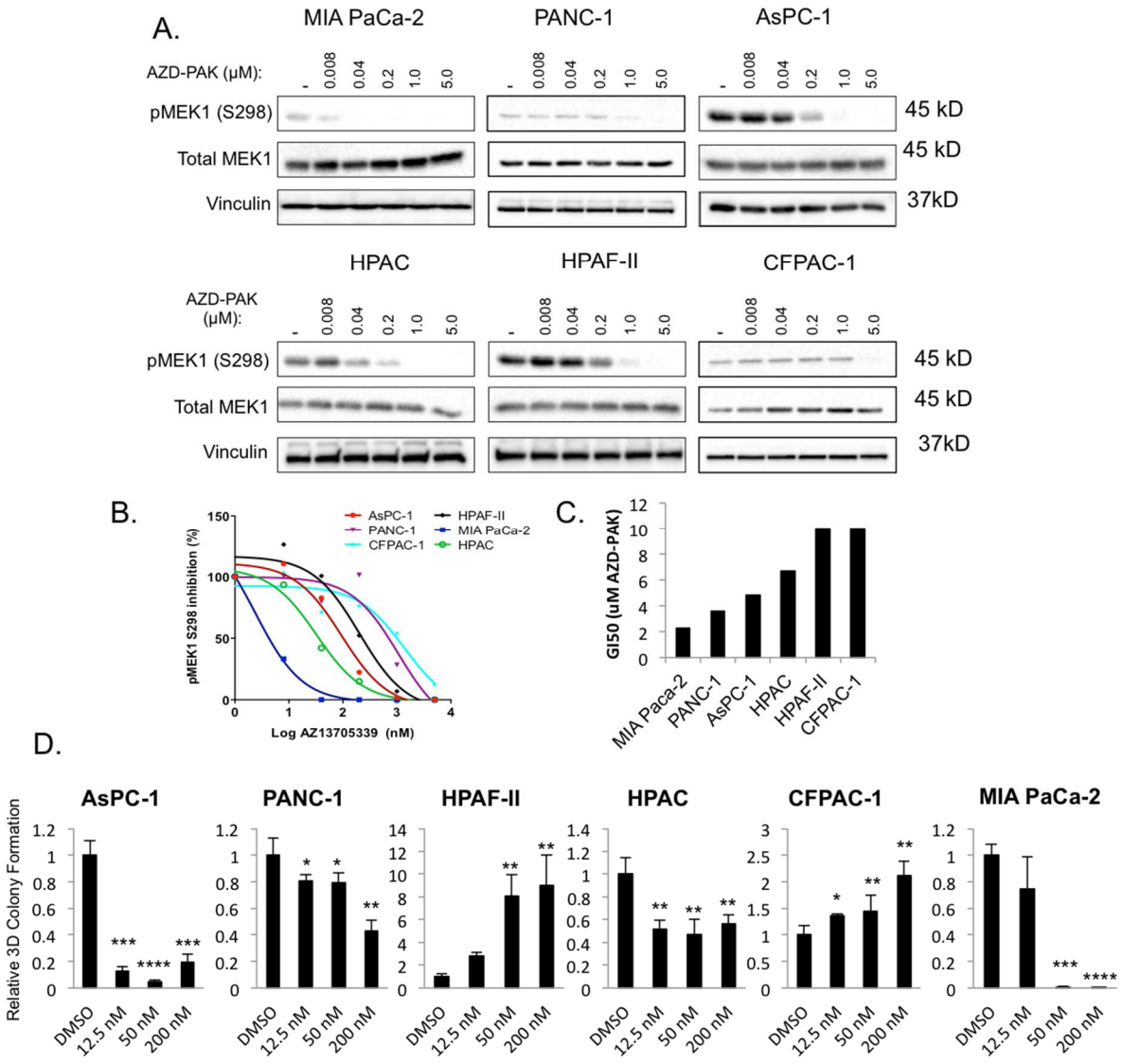



**Figure 2.3: Pharmacologic inhibition of PAK1 phenocopies the proliferative defect seen upon genetic suppression.** A, PDAC cells were treated for 24 h with the indicated concentrations of AZ13705339, and the $IC_{50}$ for inhibition of PAK-dependent phosphorylation of MEK1 at serine 298 was evaluated via western blot. B, Western blot data from panel A were subjected to densiotometric analysis and plotted as a ratio of pMEK1 to total MEK1. GraphPad Prism was used to generate dose response curves for each PDAC cell line. $IC_{50}$ values cluster in the mid nanomolar range. C, The $GI_{50}$ was determined by MTT viability assays using 12-point dose-response curves. $GI_{50}$ values cluster in the low micromolar range. D, Quantitation of 3D soft agar colony formation assay following treatment with AZ13705339. Data are represented as mean ± SEM and normalized to the NS control.

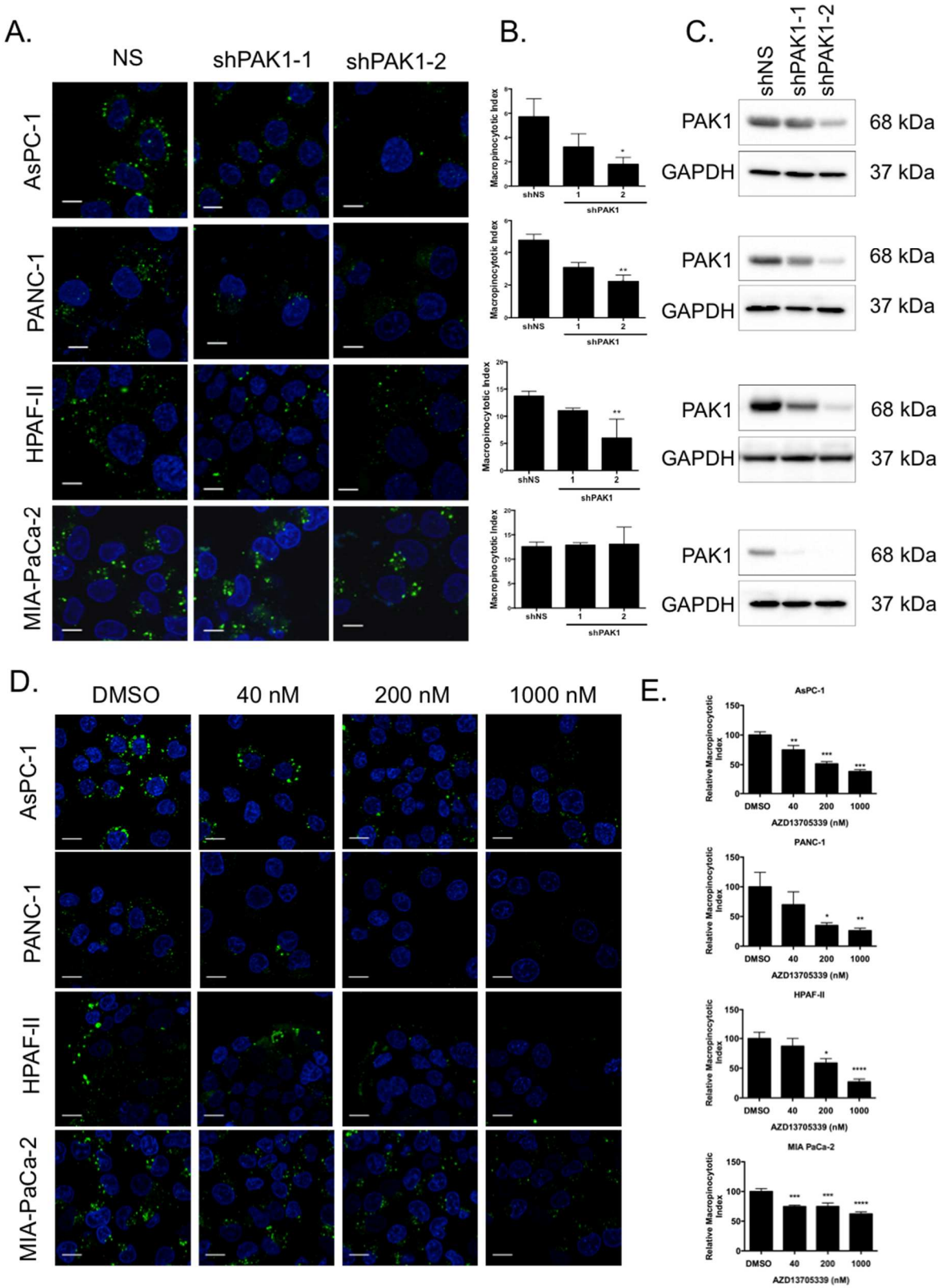

A.
NS
shPAK1-1
shPAK1-2
AsPC-1
PANC-1
HPAF-II
MIA-PaCa-2
B.
Macropinocytotic Index
shNS
shPAK1
C.
shNS
shPAK1-1
shPAK1-2
PAK1
GAPDH
68 kDa
37 kDa
D.
DMSO
40 nM
200 nM
1000 nM
E.
Relative Macropinocytotic Index
AZD13705339 (nM)
DMSO
40
200
1000

**Figure 2.4: Genetic suppression and pharmacologic inhibition of PAK1 significantly reduce macropinocytotic uptake in PDAC cells.** A, Confocal microscopy images of a panel of PDAC cell lines following stable PAK1 shRNA-mediated knockdown. Cells were starved overnight and transferred to growth medium containing fluorescent dextran before being fixed and stained for imaging. Green: 70 kDa TMR-dextran; Blue: DAPI. Scale bar represents 10 μm. B, Relative macropinocytic index was calculated in ImageJ for 10 fields, as a ratio of the area of green signal to the cell area. C, Western blot analysis of shRNA-mediated PAK1 knockdown. D, Confocal microscopy images of PDAC cell lines treated with a range of AZ13705339 concentrations spanning the IC50 for pMEK1 (S298) inhibition. Scale bar represents 15 μm. E, Quantitation of macropinocytic index for cells treated with AZ13705339 in panel D. Data are represented as mean ± SEM and normalized to the NS control. Statistical analysis was done with a one-way ANOVA using multiple comparisons where *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$.

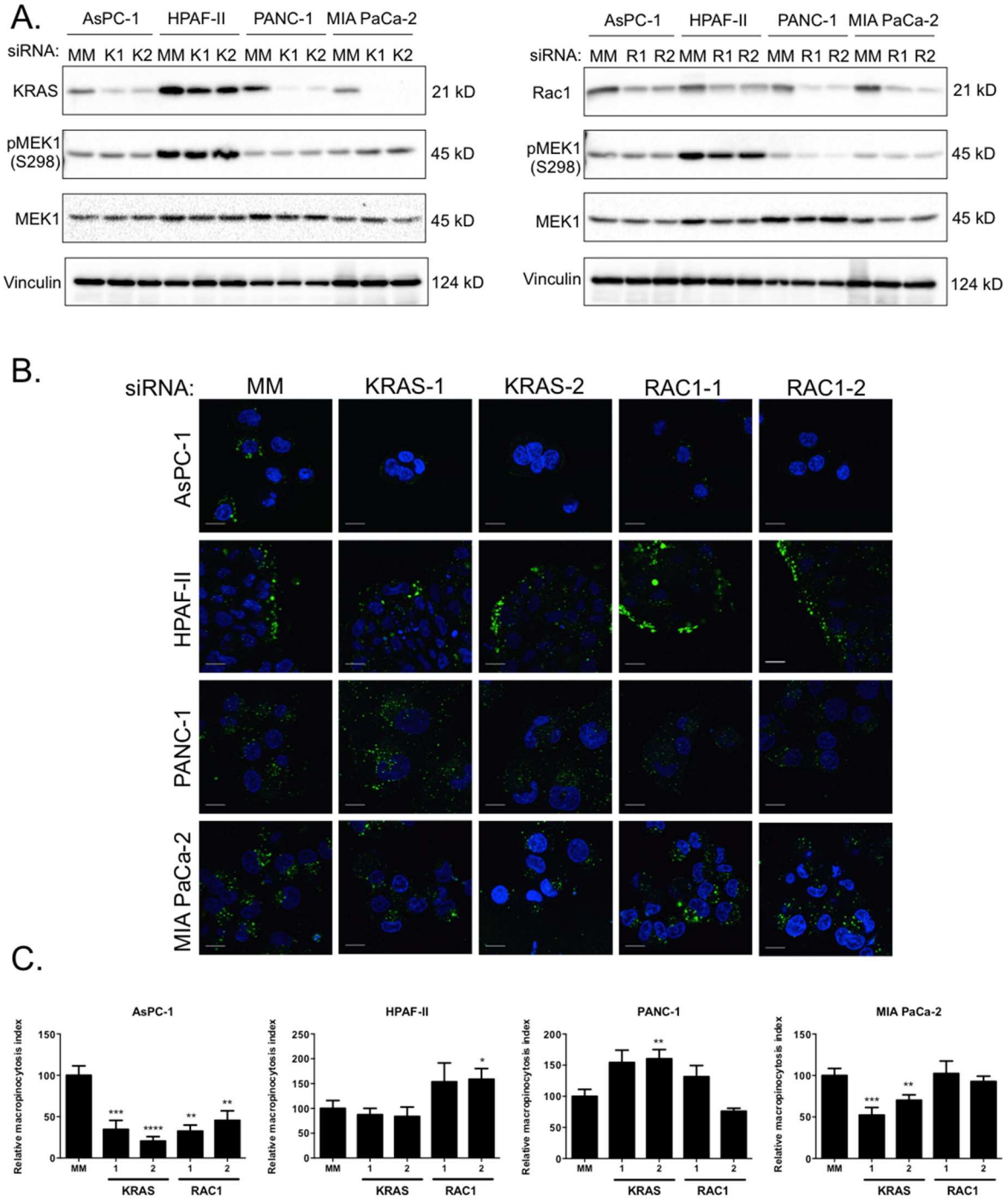



**Figure 2.5: A KRAS-RAC1-PAK1 signaling axis does not drive macropinocytosis in most PDAC cell lines.** A, KRAS and RAC1 were genetically suppressed via siRNA in a panel of PDAC cell lines, and pMEK1 (S298) levels were examined via western blot to determine PAK1 activity. B, Following knockdown of KRAS and RAC1, cells were treated with inhibitors and imaged via confocal microscopy. The macropinocytotic index was quantitated as before. Scale bar represents 15 μm. C, Quantitation of macropinocytic index from panel B. Data are represented as mean ± SEM and normalized to the NS control.

Statistical analysis was done with a one-way ANOVA using multiple comparisons where *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$.

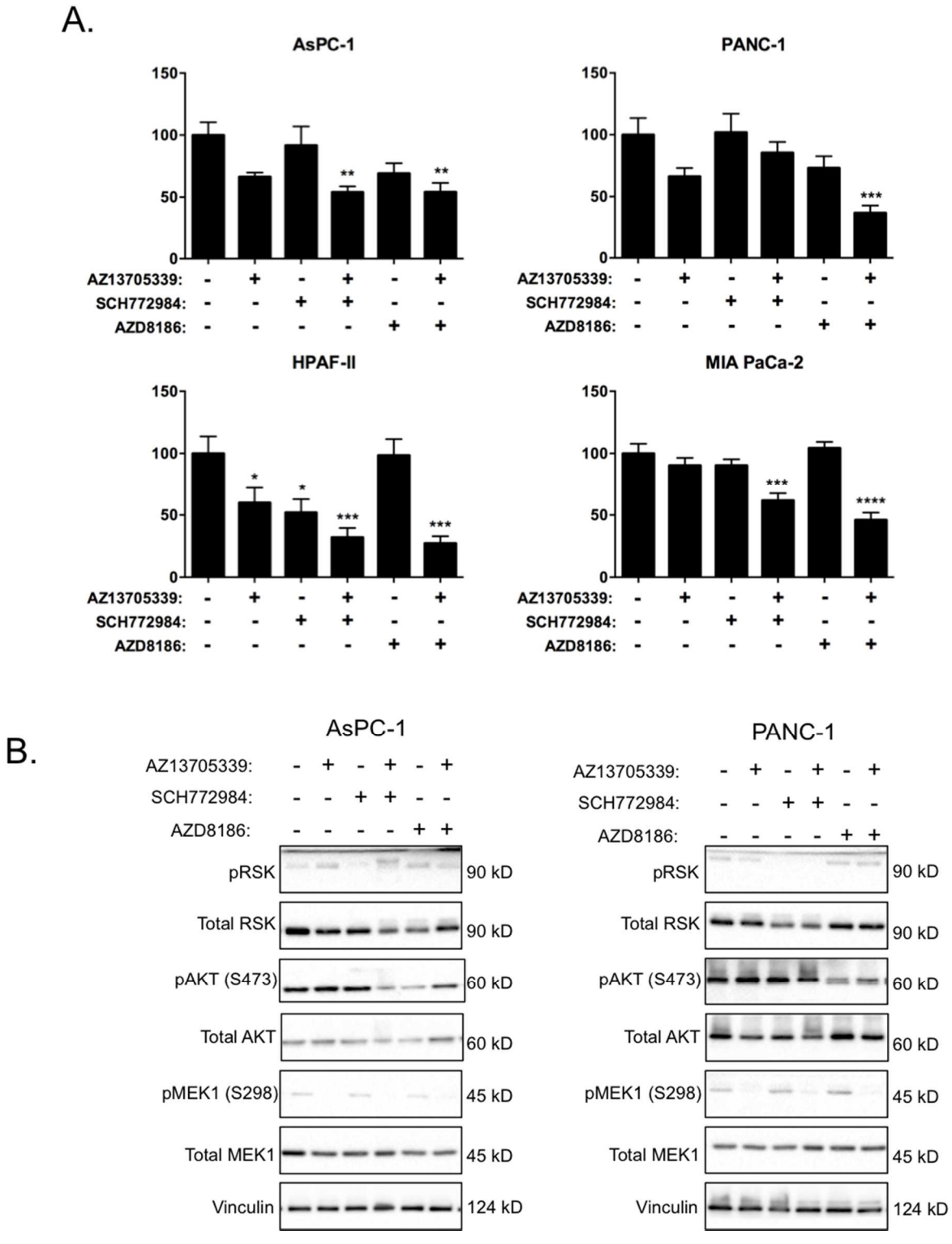
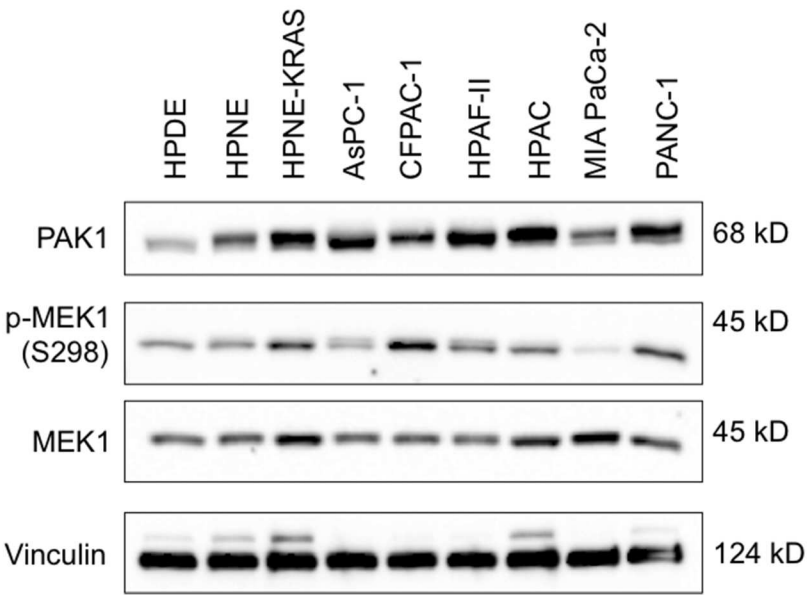
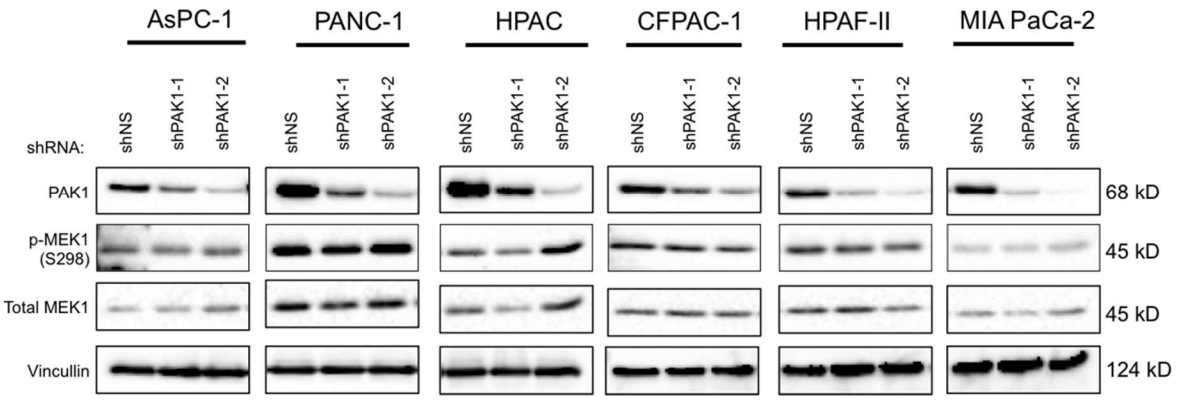



**Figure 2.6: Combinatorial inhibition of PAK1 with ERK1/2i or PI3Ki further reduces macropinocytosis in some PDAC cell lines.** A, Macropinocytosis assays were performed on a panel of PDAC cell lines with AZ13705339 (200 nM), SCH772984 (450 nM), and

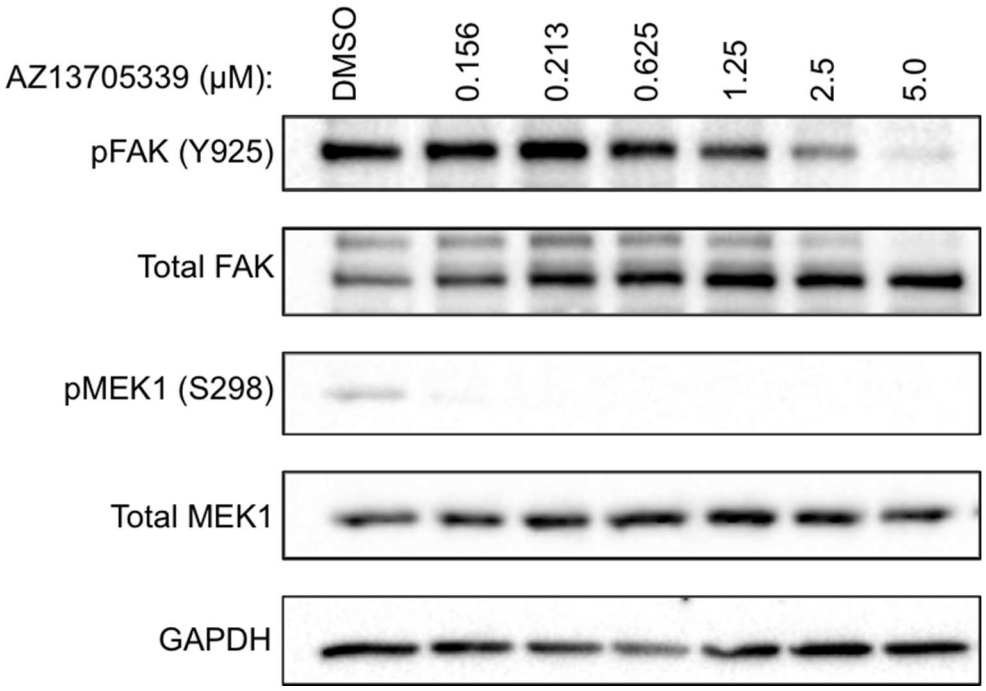
AZD8186 (1000 nM) alone or in combination with AZ13705339 (200 nM). The macropinocytic index was determined as before and data are represented as mean ± SEM and normalized to the NS control. Statistical analysis was done with a one-way ANOVA using multiple comparisons where *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$. B, Representative western blots demonstrating reduced phosphorylation of PAK1, ERK1/2, and PI3K substrates upon inhibitor treatment.



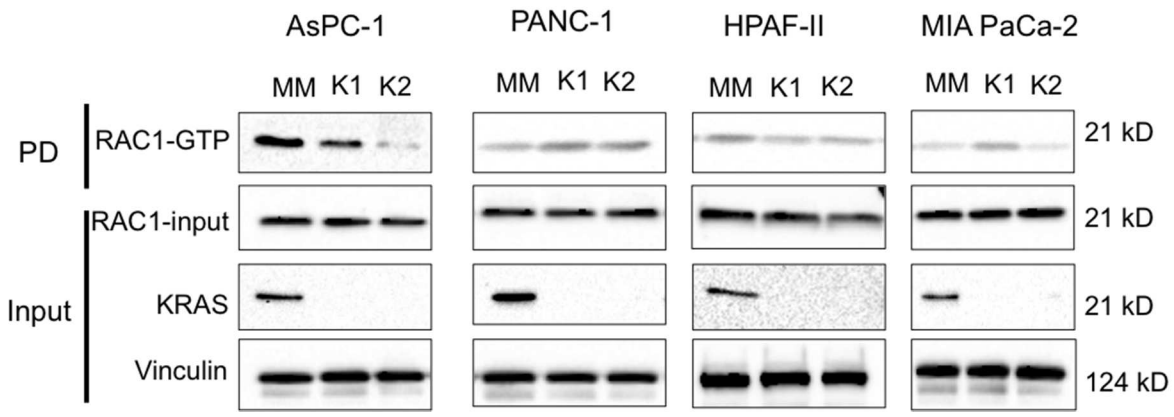

**Supplemental Figure 2.1: Levels of phospho-MEK1 at S298 do not correlate with levels of PAK1 in pancreatic cell lines.** Lysates of pancreatic cell lines including 6 PDAC lines and two non-tumor pancreatic lines (HPDE and HPNE) were probed by western blot for total PAK1 and phospho-MEK1. Vinculin served as a loading control.




**Supplemental Figure 2.2: Phospho-MEK1 levels are not reduced following stable knockdown of PAK1 in PDAC cell lines**. PDAC cell lines were transduced with one of two distinct shRNAs against PAK1 or a control non-specific (NS) shRNA. PAK1 knockdown and pMEK1 (S298) levels were evaluated via western blot. Vinculin served as a loading control**.**




**Supplemental Figure 2.3: PAK activity, but not SRC family kinase activity, is inhibited at sub-micromolar concentrations of AZ13705339.** AsPC-1 PDAC cells were treated with increasing concentrations of AZ13705339 for 24 h and cell lysates were subjected to western blot analysis. Phospho-MEK1 served as a marker of PAK activity and phospho-FAK served as a marker of SRC family kinase activity. GAPDH served as a loading control.




**Supplemental Figure 2.4: RAC1 activity is not dependent on KRAS in all PDAC cell lines.** Cells were transfected with two distinct siRNAs targeting KRAS (K1 and K2). Forty-eight h post transfection, cells were harvested and a RAC1-GTP pulldown assay was performed with GST-PAK1-PBD. Active RAC1-GTP and total RAC1 (input from total lysate) were analyzed via western blot.

## CHAPTER 3: TARGETING THE TBK1 PROTEIN KINASE IN KRAS-MUTANT PANCREACTIC DUCTAL ADENOCARCINOMA[3]

### *Overview*

Despite the critical importance of mutant KRAS in numerous cancers, including pancreatic ductal adenocarcinomas (PDAC), direct targeting of this small GTPase has not yet proven successful. To overcome this challenge, considerable effort has been devoted to identifying more druggable downstream targets, such as protein kinases, whose activities are required by mutant KRAS in order to exert its oncogenic effects. TANK-binding kinase (TBK1) has been proposed to be both a synthetic lethal partner of KRAS and a critical downstream component of the KRAS-RALB-effector pathway. However, whether TBK1 is required for KRAS-mediated transformation and tumor maintenance, and is therefore a useful therapeutic target in KRAS-mutant cancers, has been unresolved owing to conflicting results from several independent studies. To address this issue, we utilized a novel small molecule inhibitor of TBK1, LSN3090279, to examine the consequences of impaired TBK1 kinase activity in more than 300 cancer cell lines, 47 of which harbor KRAS mutations. Treatment with LSN3090279 led to growth suppression in a small subset of cell lines, but this suppression was not correlated with KRAS-mutation status. One possible explanation for this result is that TBK1 is not a critical effector of KRAS-mediated transformation. To

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further resolve this issue, we therefore focused on *KRAS*-mutant PDAC, arguably the most KRAS-dependent cancer type. We found that inhibition of TBK1 alone was ineffective at impairing the transformed growth phenotypes of most PDAC cell lines despite effective disruption of TBK1 signaling. To bolster the therapeutic potential of TBK1 inhibition, we chose a combinatorial strategy with an inhibitor of a key KRAS signaling component, ERK1/2. Although PDAC cell lines are sensitive to inhibition of ERK1/2 MAP kinases, a key node in the RAF-MEK-ERK kinase cascade that is the most critical effector of KRAS-mediated transformation in PDAC, even concurrent inhibition of TBK1 and ERK did not enhance TBK1 inhibitor activity against these cells, either in vitro or in vivo. We conclude that targeting TBK1 has limited therapeutic potential in PDAC unless effective combinations can be identified. Interestingly, we observed that LSN3090279 treatment induced autophagy and caused formation of enlarged autolysosomes, indicative of an unresolved autophagic process. TBK1 has a known role in regulating autophagosome formation, so it is possible that LSN3090279 will serve as a useful tool to aid further exploration of the complex and context-dependent roles of TBK1 in autophagy.

***Introduction***

Mutational activation of *RAS* family oncogenes is among the most frequent genetic alterations found in cancer (2), with high frequencies found in the cancers that comprise the top three causes of cancer deaths in the US (139). Despite the undruggable nature of RAS proteins, there is renewed interest and effort in identifying novel anti-RAS therapies for cancer treatment. Among the approaches currently being pursued, inhibitors of RAS effector signaling hold great promise. However, since RAS utilizes a diverse spectrum of downstream effectors to drive cancer growth (1), which effectors are best to target and whether concurrent targeting of multiple effectors will be required remain unresolved issues. Currently, most of the emphasis on effector targeting has centered on the two canonical

RAS effector pathways, the RAF-MEK-ERK mitogen-activated protein kinase cascade and the PI3K-AKT-mTOR lipid kinase pathway, with many inhibitors currently in clinical trials (43).

Among the less studied RAS effectors are the RAL guanine nucleotide exchange factors (RalGEFs) and the the RAL small GTPases, RALA and RALB (8). Both RALA and RALB have been implicated as critical effectors of RAS-driven cancer development and growth. However, the effectors that mediate RAL-dependent cancer growth remain to be fully elucidated. Additionally, despite their strong sequence identity, RALA and RALB commonly serve distinct roles in cancer growth (128, 129). For example, in pancreatic cancer we determined that RALA is required for tumorigenesis whereas RALB is required for invasion and metastasis (129). One promising candidate for a unique effector of RALB, but not RALA, is the TANK-binding kinase 1 (TBK1), a noncanonical IκB kinase (IKK) that is activated by RALB through the exocyst component Sec5, which regulates exocyst-independent activation of NF-κB (nuclear factor κB) and subsequent transcription of survival genes (132, 135, 178). That TBK1 may be a critical effector of a KRAS-RALB pathway in cancer is supported by the independent identification of TBK1 as a synthetic lethal interactor of mutant KRAS (54). TBK1 has also been found to be overexpressed in pancreatic, colorectal, breast, and lung cancer, and its aberrant activation has been shown to play a role in proliferation and survival of cancer cells with or without KRAS mutation (132, 135, 179, 180).

While it has been demonstrated that TBK1 is important in certain cancer types, like non-small cell lung carcinoma (54, 137), the exact mechanisms governing the dependence of cancer cells on TBK1 activity remain unclear. Some studies have shown that certain cancer cell lines harboring KRAS mutations require TBK1 for survival while other studies demonstrated that TBK1 is dispensable in KRAS-mutant cell lines (54, 55, 137). Thus,

whether TBK1 is a viable therapeutic target for KRAS-mutant cancers, and if so, under what conditions, remains unresolved.

Here, we seek to further understand the role of TBK1 activity in promoting cancer growth and survival, and specifically to address its function in PDAC. Since PDAC, a highly lethal cancer with limited treatment options (141), is characterized by an oncogenic KRAS mutation in nearly 100% of cases (2), inhibition of TBK1 has been considered as one approach to treat this disease. Inhibitors of the major effector pathways of RAS, including the RAF-MEK-ERK and PI3K-AKT pathways, have thus far proven ineffective in reducing PDAC tumor burden *in vivo*, leading to the desire for an alternative approach of targeting the RalGEF-RAL pathway, which has been implicated in PDAC tumorigenesis and metastasis (129, 181). TBK1 is one of two kinases defined downstream of the RalGEF-RAL signaling axis and therefore is considered one of the most tractable targets in this pathway (8).

The emerging evidence of a role for TBK1 in human cancers, as well as continued interest in its better characterized role in the autophagic and immune response to infections and inflammation, has led to the development of small molecule inhibitors targeting TBK1 kinase activity. The first such small molecule, BX795, was originally discovered as a PDK1 inhibitor, and its amino-pyrimidine component is a pharmacophore also used in newer compounds that have enabled probing the role of TBK1 in cancer and other diseases (137, 182-184). BX795 and a less promiscuous inhibitor, AZ909, were shown to inhibit clonogenic colony formation of NRAS-mutant melanoma cell lines (185). However, not all cancer types are sensitive to TBK1 inhibition. One study showed that neither genetic nor pharmacologic inhibition of TBK1 activity, as measured by disruption of IRF3 phosphorylation, was sufficient for growth inhibition of PDAC cells, suggesting that combination therapy may be necessary (55). Utilization of more recently developed inhibitors has begun to define TBK1-dependent mechanisms of cell survival and viability in cancer cells (136, 185, 186).

Here, we used a novel TBK1 inhibitor, LSN3090729, to further explore the effect of inhibiting TBK1 in PDAC and other cancer cells. We first surveyed a large panel of cancer cell lines derived from myriad tumor types, including 10 PDAC tumor cell lines, and found that, despite inhibiting TBK1 enzymatic activity, treatment with LSN3090729 had a minimal effect on anchorage-dependent proliferation that was often uncorrelated with RAS mutation status. A minimal impact on tumor cell proliferation was also observed in a panel of lung cancer cell lines as well as tumor xenografts of skin, lung, and pancreatic cancer. We found that only a small subset of the PDAC cell lines tested was sensitive to LSN3090279. Previous studies have demonstrated that single-agent inhibition of a target is ineffective in PDAC, and this holds true in the context of TBK1 inhibition, indicating that combination approaches of targeted therapies will be necessary (55). However, combined inhibition of ERK1/2, a validated and crucial RAS effector, and TBK1 produced no synergistic decrease in anchorage-dependent proliferation. However, we did see a negative effect on growth in a single cell environment in clonogenic cell growth assays, and these data suggest that examining the anchorage-independence of TBK1 inhibition in a 3D context may reveal greater sensitivity to LSN3090279. Aside from these results, we did observe an interesting metabolic phenotype upon TBK1 inhibition that resulted in non-productive autophagy. This observation, combined with previous evidence implicating TBK1 in autophagy (187-189), warrants further study of the role of TBK1 in PDAC cell metabolism and cell survival, and suggests that combining a TBK1 inhibitor with inhibitors of cellular metabolic pathways may be a viable therapeutic strategy.

### *Materials and Methods*

#### Cell lines and plasmids

PDAC cell lines were obtained from ATCC and maintained in either DMEM or RPMI-1640 supplemented with 10% fetal bovine serum. All cell lines were maintained in

continuous culture for two months or less. A lentiviral vector encoding a short hairpin RNA (shRNA) sequence, shT15, for human TBK1 was kindly provided by Dr. Paul Kirschmeier (Dana-Farber Cancer Institute) and was validated for TBK1 specificity as previously described (55). The pBabe-puro mCherry-EGFP-LC3 plasmid was obtained from Addgene (#22418).

**Inhibitor treatment assays**

Sensitivity of PDAC cells to the TBK1 inhibitor LSN3090279, alone or in combination with the ERK inhibitor SCH772984, was determined by MTT assay. Briefly, LSN3090279 was serially diluted from 30 μM to 0.0005 μM and added to a 96-well plate in the absence or presence of a constant concentration of SCH772984 (450 nM). Cells were seeded at a density of 3 x $10^3$ cells per well and allowed to proliferate at 37°C and 5% CO2 for 72 h. Then, cells were treated with 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 4 h at 37°C, formazan crystals were solubilized in DMSO, and the resulting absorbance at 590 nM was recorded.

Single cell clonogenic growth assays were performed by seeding cells in 6-well plates at 1,000 cells per well. Cells were allowed to adhere for 24 h and were then treated with LSN3090279 (2 μM) for 10 days. Colonies were stained with crystal violet (2 mg/mL) in 4% paraformaldehyde for 10 min and quantified using ImageJ (158).

**Western blot analyses**

Cells were lysed in NP-40 lysis buffer supplemented with protease and phosphatase inhibitor cocktails. Protein concentration was determined by a Bradford Protein Assay (Bio-Rad). Proteins were separated by SDS-PAGE, transferred to PVDF membranes, and probed with the following antibodies (all from Cell Signaling Technology): anti-AKT (#9272), anti-phospho-AKT S473 (#4060) anti-TBK1 (#3504), anti-phospho-TBK1 S172 (#5483), anti-IRF3 (#D83B9), anti-phospho-IRF3 S396 (#4D4G), anti-LC3B (#3868), anti-RSK (#9355), or

anti-phospho-p90RSK S380 (#9344). Anti-GAPDH was obtained from Sigma-Aldrich (#G8795). Blots were imaged with the BioRad ChemiDoc and analyzed using BioRad Image Lab and ImageJ (158) software.

**Autophagic flux assay**

Cells were transduced with lentivirus expressing pBabe-puro mCherry-EGFP-LC3 (112) and selected in puromycin for 48 h. Cells were then seeded into glass-bottom MatTek dishes, allowed to adhere overnight in complete growth medium, then transferred. to complete growth medium lacking phenol red and imaged live on a Zeiss 700 confocal microscope. Treatment with chloroquine (12.5 μM) was used as a positive control to visualize both autophagosomes and autolysosomes. Images were quantitated in ImageJ as the ratio of red (autolysosomes) to green (autophagosomes).

**Transmission electron microscopy**

Ultrastructural analysis of cells was performed as we previously described (190). Briefly, HPAC cells were collected by trypsinization, washed with PBS, fixed for TEM using 1.5% glutaraldehyde, and post-fixed using 1% osmium tetroxide in cacodylate buffer. Post-fixed cells were dehydrated through an acetone series prior to embedding in Spurr's epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and then imaged using a Hitachi H-600 transmission electron microscope. Images were acquired using an SIA digital camera.

***Results***

***LSN3090279 is a selective TBK1 inhibitor***

We synthesized LSN3090279, a 4-aryl-2-aminopyrimidine derivative (Figure 3.1A), and characterized the compound as a TBK1 kinase inhibitor with an *in vitro* $IC_{50}$ ranging from 19 nM to 73 nM in an *in vitro* kinase assay using IRF3 as a substrate (data not shown).

We next evaluated LSN3090279 for selectivity in a panel of over 100 protein kinases and found minimal off-target effects *in vitro* (Figure 3.1B). Thus, LSN3090279 is a moderately potent and selective inhibitor of TBK1.

We further characterized LSN3090729 by evaluating its ability to inhibit TBK1 signaling to known phosphorylation targets IRF3 and AKT in cells. First, we stably expressed TLR4 and IRF3 in HEK-293 cells to ensure that the known upstream (TLR4) and downstream (IRF3) components of the TBK1 signaling pathway were intact in our model system. We then stimulated TBK1 activity by treatment with lipopolysaccharide (LPS) and used an Acumen-based assay to measure the ability of LSN3090729 to block LPS-induced TBK1-mediated phosphorylation of its direct substrate IRF3 at S386 (Figure 3.1C). LSN3090729 blocked phosphorylation of IRF3 with an $IC_{50}$ of 125 nM.

To extend our cellular analysis, we compared pharmacologic inhibition of TBK1 kinase activity to genetic depletion of TBK1 and examined downstream TBK1 signaling to AKT. We either stably suppressed TBK1 expression by shRNA or inhibited TBK1 kinase activity with LSN3090729 for 1 h in PANC-1 pancreatic cancer cells starved of FBS (Figure 3.1D-E). We then treated cells with 100 ng/mL EGF for 5-8 min and confirmed that the TBK1-dependent phosphorylation of AKT S473 (pAKT) was also reduced in the presence of LSN3090729 (Figure 3.1E). Collectively, these results show that LSN3090279 potently inhibits TBK1-dependent AKT signaling in PDAC cells.

***LSN3090279 has a minimal effect on cancer cell proliferation in vitro and in vivo***

The effect of TBK1 depletion or inhibition on cell proliferation across different cancer types may be highly context-dependent. In one study, TBK1 was shown to be dispensable for the survival of a panel of PDAC cell lines that harbor mutant KRAS (55) whereas another study showed that TBK1 was required for KRAS-dependent lung cancer cell proliferation (54). Furthermore, a third study provided conflicting evidence regarding whether TBK1

inhibition is lethal to mutant KRAS-dependent cancer cell lines, demonstrating that some were sensitive to TBK1 inhibition, while others were resistant, even though all most cell lines tested had a KRAS mutation (137). To investigate the TBK1 dependence of RAS-mutant cancers more definitively, we sought to determine the impact of TBK1 inhibition by LSN3090729 on cancer cell proliferation, and whether this type of dependence on TBK1 was dictated by the presence of an activating RAS mutation. To that end, we assessed the effect of LSN3090279 (0 to 20 μM) on anchorage-dependent growth in a panel of over 300 cancer cell lines of varying RAS mutation status. This panel included cancer cell lines of lung, colon, and pancreatic origin, as well as a wide range of other tissue types. We found a large range of $GI_{50}$ values (where $GI_{50}$ is the concentration required for 50% growth inhibition), from 1.37 μM to over 20 μM (Figure 3.2A). The majority of cell lines were insensitive to LSN3090279, displaying a $GI_{50}$ >20 μM. There was no correlation between $GI_{50}$ and RAS mutation status (Figure 3.2A). We extended this study to examine the effects of LSN3090729 on anchorage-independent growth in a more select panel of KRAS-mutant cancer cell lines (Figure 3.2B). Again, we found that sensitivity to LSN3090729 did not correlate with RAS mutation status across this panel. Overall, LSN3090279 alone had only a minimal effect on cancer cell proliferation *in vitro*. However, there may be cell context-dependent differences in the requirements for TBK1 in proliferation. Therefore, we next set out to examine TBK1 inhibition in the context of PDAC cell lines known to exhibit strong KRAS-dependent growth.

***PDAC cell lines show varying sensitivity to pharmacologic TBK1 inhibition***

Since the RAS dependency of most of the cell lines analyzed in Figure 3.2 has not been established, we next focused our analyses specifically on KRAS-mutant pancreatic ductal adenocarcinoma (PDAC) cell lines. Though several mutant KRAS lines were initially screened and many were found insensitive to TBK1 inhibition (Figure 3.2A-B), our studies

focus in on a cancer type highly dependent upon mutant KRAS signaling. Additionally, due to the ambiguous findings described by Ou, Muvaffak, and colleauges regarding whether KRAS mutation status truly dictates TBK1 dependence, we screened a greater number of KRAS-mutant pancreatic cancer lines than the rest of these studies. We showed recently that these lines exhibit strong KRAS-dependent growth as determined by transient or stable si/shRNA silencing of KRAS expression in both anchorage-dependent and -independent assays (170).

We determined the impact of LSN3090729 on the anchorage-dependent proliferation of eight KRAS-mutant PDAC cell lines and two normal immortalized pancreatic epithelial cell lines (Figure 3.3A). We found that the growth of only two PDAC cell lines, PANC-1 and HPAC, was sensitive to TBK1 inhibition ($GI_{50}$ of 2.59 μM and 3.41 μM, respectively) whereas six other PDAC cell lines, including the two normal pancreas cell lines, were insensitive ($GI_{50}$ >8 μM). We also applied a single cell clonogenic growth assay to assess the effects of LSN3090729 activity. At 5 μM, there was near-complete inhibition of growth of two sensitive cell lines, HPAC and PANC-1, with partial growth suppression in two other lines, Capan-1 and SW1990 that were resistant under normal growth conditions in a 72 h MTT viability assay (Figure 3.3B). We conclude that sensitivity to LSN3090729 does not strictly correlate with mutant KRAS-dependency.

To determine whether the minimal effect on cell proliferation was due to insufficient inhibition of TBK1 signaling, we next compared the ability of LSN3090729 to suppress TBK1 signaling in sensitive and resistant cell lines. While TBK1 has an autophosphorylation site at serine 172, the phosphorylation status of this site is not indicative of TBK1 activation, contrary to what is often found in other kinases (184). Instead, it was previously determined that IRF3 phosphorylation levels are elevated chronically in PDAC cells and that phosphorylation of IRF3 at serine 396 is a reliable marker of TBK1 inhibition (55). We sought to determine if inhibition of phosphorylation of IRF3 or TBK1 by LSN3090729

correlated with sensitivity to growth inhibition using two sensitive and two resistant PDAC cell lines. We found that reduction in phosphorylation of IRF3 tracked closely with increasing doses of LSN3090729, whereas varying effects on TBK1 phosphorylation were observed (Figure 3.3C), which is also consistent with previous studies (55). Furthermore, in our two sensitive cell lines, PANC-1 and HPAC, we observed that 50% inhibition of TBK1 as measured by phosphorylation of IRF3 ($IC_{50}$) correlated with $GI_{50}$. Conversely, the $IC_{50}$ of LSN3090729 in two resistant cell lines, SW1990 and Capan-1, was much lower than that of the $GI_{50}$. These results suggest that, while IRF3 phosphorylation is a reliable marker of TBK1 inhibition by LSN3090729, inhibition of this phosphorylation is not always sufficient to block cell proliferation, and that when cells display an LSN3090729-resistant phenotype, it is because their proliferation is genuinely not TBK1-dependent. Furthermore, in contrast to the stimulus-induced TBK1 activation assays (Figure 3.1C), we found that significantly higher concentrations were needed to effectively reduce the basal, steady state levels of pIRF3 (Figure 3.3C).

Unexpectedly, two cell lines identified as resistant to TBK1 inhibition in standard 2D culture MTT assays, Capan-1 and SW1990, exhibited a response to inhibitor treatment in single cell clonogenic assays (Figure 3.3B), although only Capan-1 responded to genetic knockdown using shRNA (Figure 3.4A-B) These results may indicate that TBK1 has a specialized role in promoting specific aspects of cellular proliferation in certain contexts.

***TBK1 inhibition leads to non-productive autophagy***

Interestingly, upon treatment with LSN3090729, we observed a rapid formation of large vacuoles that remained unresolved after 72 h (Figure 3.5A). These vacuoles also formed to a lesser degree following depletion of TBK1 by shRNA (data not shown). Because of the known roles of TBK1 in autophagy (187-189) and the resemblance of these vacuoles to autolysosomes (191, 192), we sought to determine if autophagy was misregulated in

LSN3090729-treated cells. We observed an increase in total LC3BI and LC3BII as well as an increasing ratio of LC3BII to LC3BI, indicating that autophagy was induced and that lysosomal clearance was disrupted (112) (Figure 3.5B). To elucidate the identity of these vesicles, we performed transmission electron microscopy (TEM). Their ultrastructural properties revealed induction of autophagosome formation. The large vacuoles, while not autophagosomes, appeared to be lysosomes or autolysosomes containing cell debris (Figure 3.5C). TEM images following a time course of treatment with LSN3090729 (5 μM) revealed that these endocytic vesicles did not appear to form from the endoplasmic reticulum, nor from the plasma membrane, as there was an absence of coated pits. These observations ruled out various intracellular trafficking events and also macropinocytosis. Therefore, we sought to determine whether these vesicles were of another origin, and whether they were possibly enlarged autophagosomes or autolysosomes.

To confirm that autophagy was altered following treatment with LSN3090729, we used an mCherry-EGFP-tagged LC3 construct as previously described (112) to measure autophagic flux in HPAC cells. EGFP is sensitive to the acidic environment of the autolysosome and degrades whereas mCherry is not. Therefore, an increase in red signal over that of both red and green together indicates that autophagy is functioning and autolysosomes are generated following autophagosome formation. In HPAC cells treated with LSN3090729 (5 μM) for 24 h, there was an increase in the ratio of red puncta (autolysosmes) to green puncta (autophagosomes) relative to that ratio in vehicle-treated cells (Figure 3.5D). These data indicate that autophagic flux is increased in TBK1 inhibitor-treated cells. Further investigation is required to determine the effect of this LSN3090729-dependent regulation of autophagy on cancer cell proliferation and survival.

### *TBK1 inhibition has no effect on tumor growth in vivo*

Mounting evidence suggests that disparate effects of inhibitor treatment are often observed in cancer cells *in vitro* versus *in vivo* due to influences of the tumor microenvironment and inter- and intra-tumor heterogeneity (193-195). Therefore, we examined the effect of LSN3090729 on tumor growth to determine if the sensitivity observed in selected PDAC cell lines was recapitulated in a xenograft mouse model. Pharmacokinetic analysis showed that LSN3090279 has over 70% oral bioavailability and an acceptable half-life of 1.7 h in rats (Figure 3.1F). Initial observations in an HCT116 colorectal cancer-derived subcutaneous xenograft mouse model showed a modest decrease in tumor growth upon treatment with either of two different doses of LSN3090729 compared to vehicle control (Figure 3.6A). We extended this to PANC-1 pancreatic cancer (Figure 3.6B) and lung cancer A549 (Figure 3.6C) mouse xenograft models. In the pancreatic cancer model, a slight decrease in tumor growth was observed at a lower dosing regimen than in the colorectal cancer model (Figure 3.6B). In the lung cancer model, treatment with LSN3090279 caused only a minimal tumor growth delay, whereas, for comparision, the PI3K/mTOR inhibitor, BEZ-235, produced a dramatic decrease in tumor size (Figure 3.6C).

### *Combined TBK1 and ERK inhibition do not synergize to reduce PDAC cell proliferation*

Small molecule inhibitors against TBK1 in cancer have proven ineffective as single agents (55), and concurrent inhibition of other cancer-promoting pathways is being explored as a new approach. One study demonstrated that combined TBK1 inhibition with MEK inhibition in NRAS-mutant melanoma cells induced apoptosis, as shown by increased PARP cleavage and annexin V staining (185). However, this study did not address the impact of the combination treatment on cell proliferation. Previously, we have shown differential growth sensitivity to MEK and ERK inhibition in PDAC cells, with ERK inhibition proving

slightly more effective, although the majority of cells remained resistant to both inhibitors (170). We sought to determine if simultaneous inhibition of TBK1 and a validated KRAS effector, ERK, impacted PDAC cell growth.

We have previously determined that the growth of Capan1, SW1990, and PANC-1 cells is resistant to treatment with ERK inhibitor SCH772984 ($GI_{50}$ >4 μM) despite inhibition of ERK1/2 kinase activity, as measured by phosphorylation of RSK, at almost 10-fold lower concentrations ($IC_{50}$ = 450 nM) (170). We treated Capan1, SW1990, PANC-1, and HPAC cells with the ERK inhibitor, SCH772984, and the TBK1 inhibitor, LSN3090729, and measured the effect on anchorage-dependent growth over 72 h (Figure 3.7 A-D). Compared with ERK inhibitor treatment alone, we observed no significant synergistic effect of combined TBK1 and ERK inhibition. This combination was also tested *in vivo* in mice bearing subcutaneous SW1990-derived tumors. Here, we saw that, while ERK inhibition effectively reduced tumor growth rates, TBK1 inhibition, did not, and that TBK1 inhibition also did little to enhance the growth defect seen with ERK inhibition alone (Figure 3.7E) despite robust evidence that these compounds inhibited their targets (Supplemental Figure 3.1) We conclude that simultaneous inhibition of TBK1 and ERK does not enhance the therapeutic potential of either of these inhibitors in PDAC.

### *Discussion*

Mutationally activated KRAS is a driver in over 90% of PDAC cases (144). Despite an increased understanding of effector pathways mediating KRAS-dependent cancer growth, treatment options remain limited (141). With the exception of the EGFR inhibitor erlotinib, there are no approved targeted therapies for PDAC patients, and conventional cytotoxic drugs remain the standards of care. This is in contrast to other cancers including lung, skin, and breast, for which multiple approved targeted therapies options have contributed to prolonging patient survival (196-198). While inhibitors of PI3K, AKT, and MEK

have exhibited moderate effects on PDAC cell proliferation, the overall efficacy of these inhibitors observed in patients has been minimal (43), and other possible targets should be considered. Given the increasing evidence for TBK1 activity in promoting cancer (54, 55, 137, 199), we utilized a novel TBK1 inhibitor to address a role for of this less studied RAS effector in PDAC. Our results suggest a limited therapeutic value of targeting TBK1 alone in PDAC.

Given the implication of TBK1 overexpression and aberrant activation in promoting numerous cancers, several small molecule inhibitors targeting TBK1 have been developed. Inhibition of TBK1 with LSN3090279 had a minimal growth inhibitory effect on the majority of large panel of cancer cell lines from diverse tissue origins in both anchorage-dependent and -independent growth assays. Similarly, only a small subset of KRAS-mutant lines showed sensitivity to TBK1 inhibition. In both cell line groups, sensitivity did not correlate with RAS mutation status. Our findings agree with previous findings that TBK1 inhibition alone is not sufficient to reduce growth in most PDAC cell lines (55, 137). However, we do not believe that this finding alone argues that TBK1 will not be a useful therapy for RAS-mutant cancers. In our recent assessment of ERK inhibitors, we also found that only approximately 50% of KRAS-mutant PDAC lines demonstrated sensitivity *in vitro* and *in vivo* (170). Thus, to advance TBK1 inhibitors, future studies will need to define a biomarker(s) that are indicative of TBK1 sensitivity.

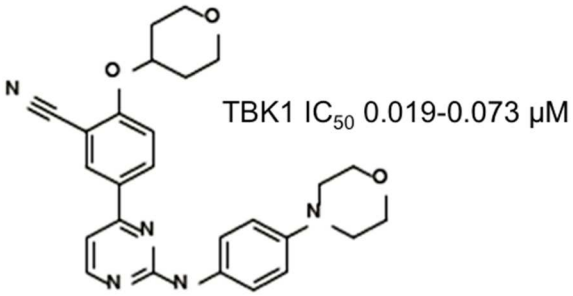
With TBK1 inhibition alone ineffective in most cell lines, we reasoned that concurrent inhibition of other key KRAS effector pathways may enhance TBK1 anti-proliferative activity. However, we found that combining ERK1/2 and TBK1 inhibition yielded no significant synergistic inhibitory activity beyond that seen with ERK inhibition alone, both *in vitro* and *in vivo*. Though inhibition of TBK1 has been shown to increase phosphorylation of ERK through feedback signaling in lung cancer cells, providing one explanation for the synergy seen with dual inhibition of these kinase pathways (180, 200), we did not observer this in

PDAC. These data highlight the importance of pancreatic cancer genetic heterogeneity. Our findings differ from another study that found combined TBK1 and ERK MAPK inhibition in a mouse model of KRAS-driven lung cancer caused tumor regression and demonstrated that the disruption of autocrine signaling by a TBK1 inhibitor led to sensitivity in lung cancer cells (200). Thus, exploring cytokine production by PDAC cells both sensitive and resistant to TBK1 inhibition may shed light on the TBK1-dependent mechanisms dictating PDAC cell proliferation.

One significant result of our study revealed that both genetic and pharmacologic inhibition of TBK1 led to upregulation of autophagic flux and the formation of large autolysomal vesicles in every PDAC cell line that we treated. Though these cells did not appear to be undergoing apoptosis or necrosis as they were still able to metabolize MTT and remained attached to tissue culture flasks, we hypothesize that this phenotype may contribute to the slight reduction in cell growth observed upon TBK1 inhibition or depletion. However, since this was seen in both sensitive and resistant cell lines, clearly this cellular change alone cannot explain the TBK1-dependency of sensitive cell lines. While combination treatment with an ERK1/2 inhibitor did not significantly reduce cell viability, it is possible that other inhibitors, either targeted therapies or general chemotherapies, may synergize with LSN3090729 and exploit a situation in which the cell health appears to be compromised.

In summary, while our study is in agreement with other recent studies that TBK1-dependency is not strongly correlated with RAS-mutation status, a subset of KRAS-mutant cell lines were sensitive. Future studies that apply unbiased chemical library screening will be needed to identify combinatorial TBK1 inhibition strategies to overcome the resistance of the majority of RAS-mutant cancer cells to TBK1 inhibition. Genetic screens to identify mechanisms of *de novo* resistance will also be needed to contribute to these efforts.

A

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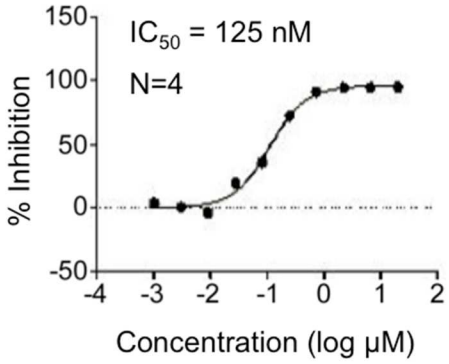


B

| Targets | IC50 (μM) |
|---|---|
| PDGFRα | <0.0667 |
| JAK2 | 0.0723 |
| MKNK2 | 0.108 |
| IKBKE | 0.138 |
| JAK3 | 0.154 |
| IRAK1 | 0.265 |
| JAK1 | 0.284 |
| MAP3K9 | 0.33 |
| TYK2 | 0.41 |
| CHUK | 0.564 |
| FLT4 | 0.627 |
| ULK1 | 0.665 |
| FLT3 | 0.669 |
| IKBKB | 0.838 |
| KDR | 0.863 |
| CDK2 | 0.974 |
| MAPK10 | 0.99 |

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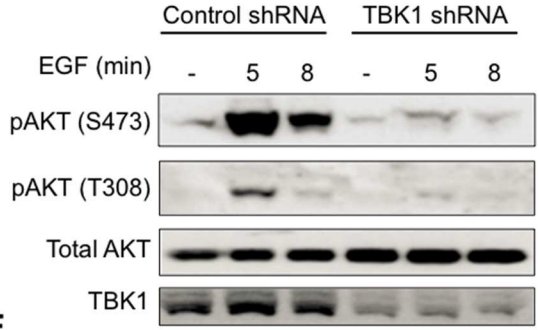


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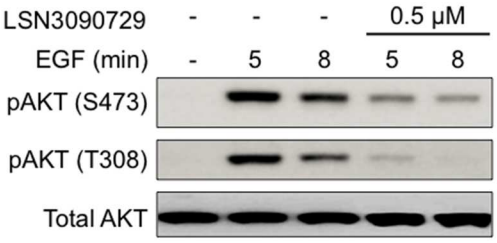


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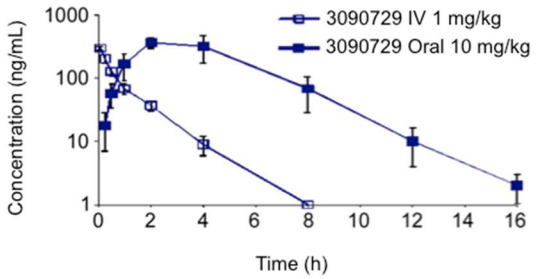



**Figure 3.1: LSN3090729 is an inhibitor of TBK1 *in vitro* and *in vivo.*** A, LSN3090729 was discovered as a TBK1 inhibitor with $IC_{50}$s ranging from 0.019 to 0.073 μM for inhibition of recombinant TBK1 *in vitro*. Other kinases are inhibited to a lesser extent. B, Evaluation of LSN3090729 inhibition in a panel of more than 100 protein kinases. Summarized are the activities detected under 1 μM. C, LSN3090729-mediated inhibition of LPS-induced

phosphorylation of IRF3 was evaluated in HEK-293 cells. D, The ability of TBK1 shRNA and E, LSN3090729 treatment to inhibit EGF-stimulated AKT phosphorylation at S473 was evaluated in PANC-1 cells. F, The pharmacokinetic profile of LSN3090729 delivered orally or intravenously (IV) was determined in rats.

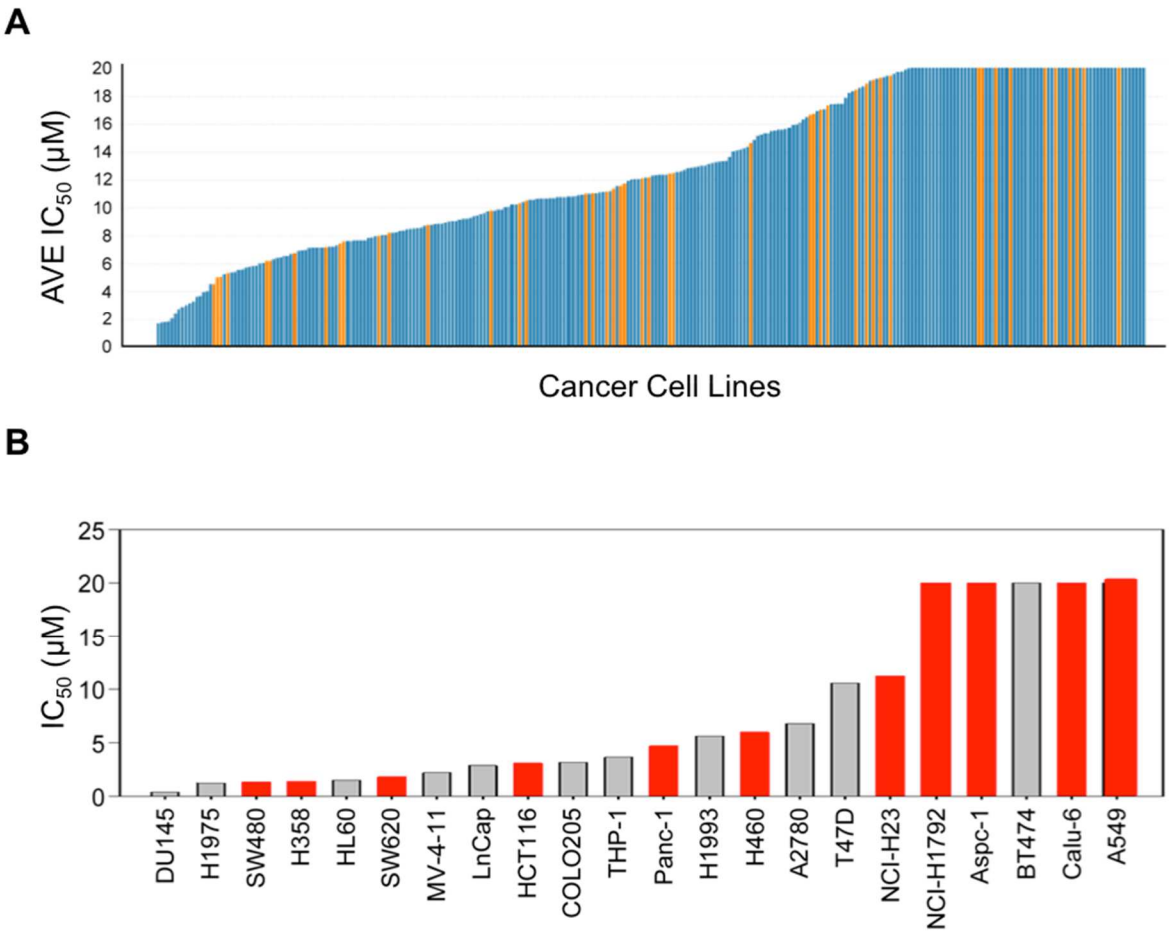



**Figure 3.2: Sensitivity of human tumor cell lines to LSN3090729 treatment does not correlate with RAS mutation status.** A, A panel of cancer cell lines was treated with LSN3090729 and anchorage-dependent proliferation was monitored by using the CellTiter-Glo® Luminescent Cell Viability Assay (orange, RAS-mutant; blue, RAS wild-type). B, The ability of LSN3090729 treatment to inhibit the anchorage-independent growth of a panel of human cancer cell lines was determined by measuring colony formation in soft agar (red, mutant KRAS; grey, wild-type KRAS).

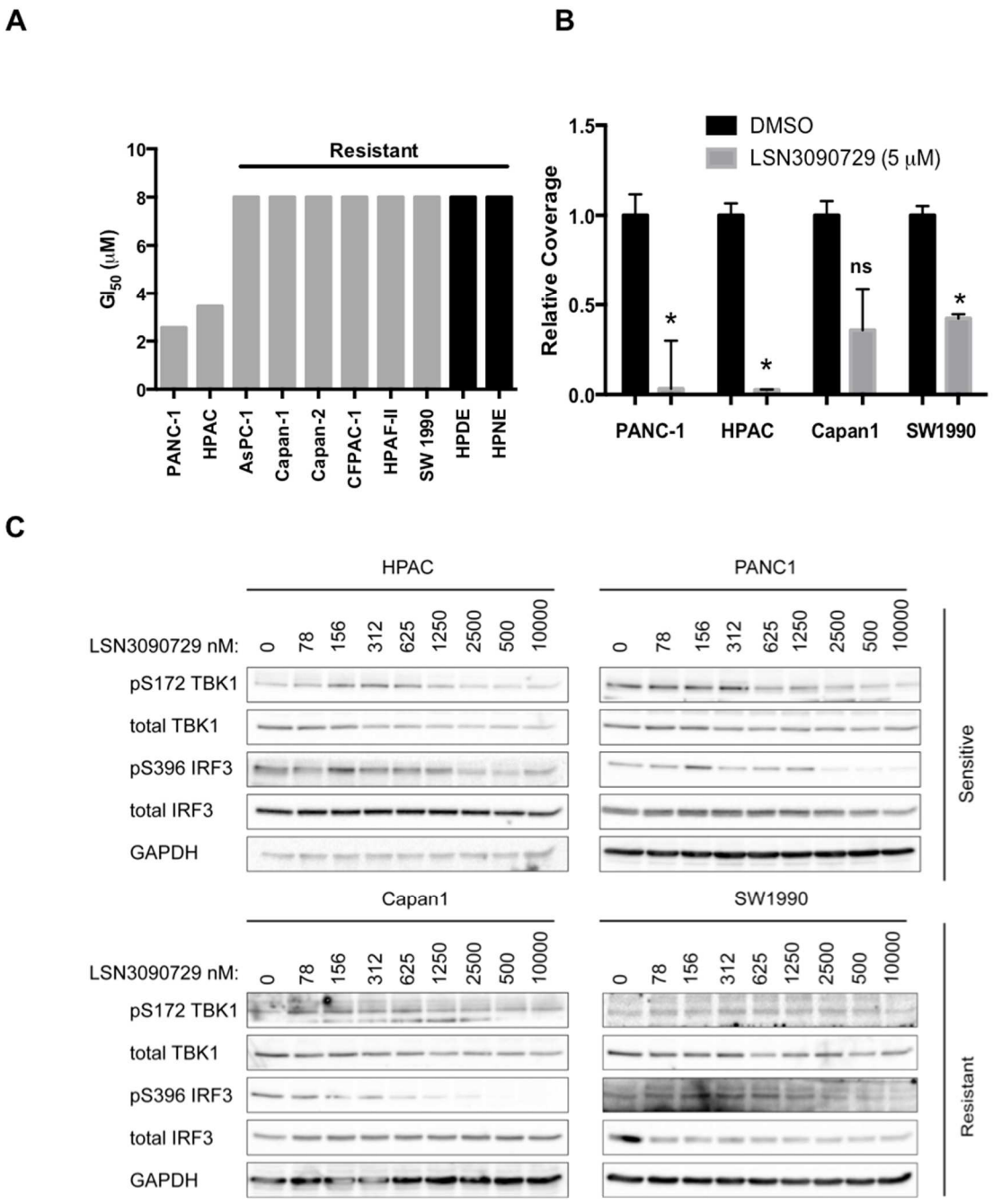



**Figure 3.3: KRAS-mutant PDAC cell lines are differentially sensitive to LSN3090279 treatment in a manner dependend on the growth endpoint.** A, $GI_{50}$ of PDAC cells treated with LSN3090279 for 72 hl anchorage-dependent proliferation was monitored by using an MTT viability assay. B, Sensitive and resistant cell lines were seeded for clonogenic assays on day 0, treated with LSN3090279 at the indicated concentrations on day 1, and colonies were quantified on day 10. C, Sensitive and resistant PDAC cells were probed as indicated at 72 h post-treatment with LSN3090279 to determine the $IC_{50}$ of TBK1 inhibition, using pIRF3 pTBK1 as markers of TBK1 activity.

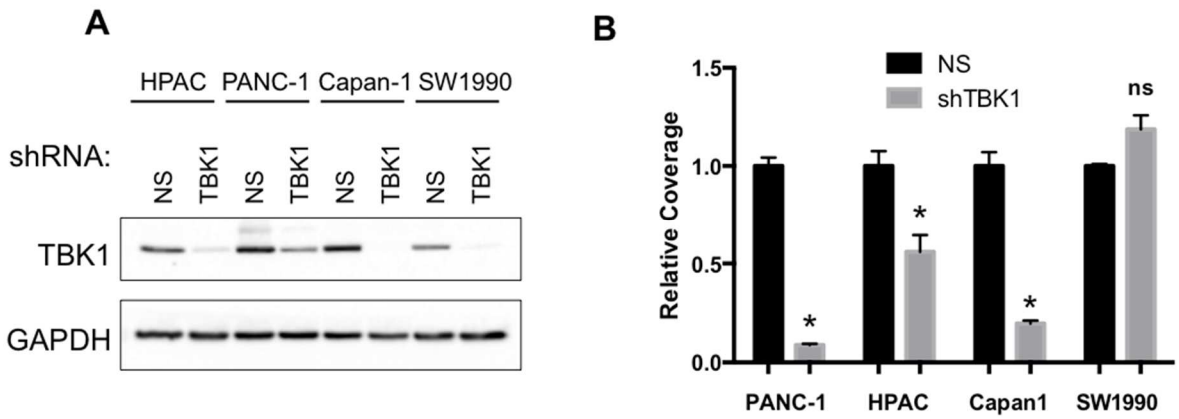



**Figure 3.4: TBK1 depletion reduces clonogenic single cell colony formation.** A, TBK1 was stably knocked down by shRNA and knockdown was verified by western blot analysis. B, Cells were seeded for clonogenic single cell colony formation assays on day 0. Plate coverage was quantified on day 10 by using ImageJ. Results are presented as mean +/- SEM of three replicates.

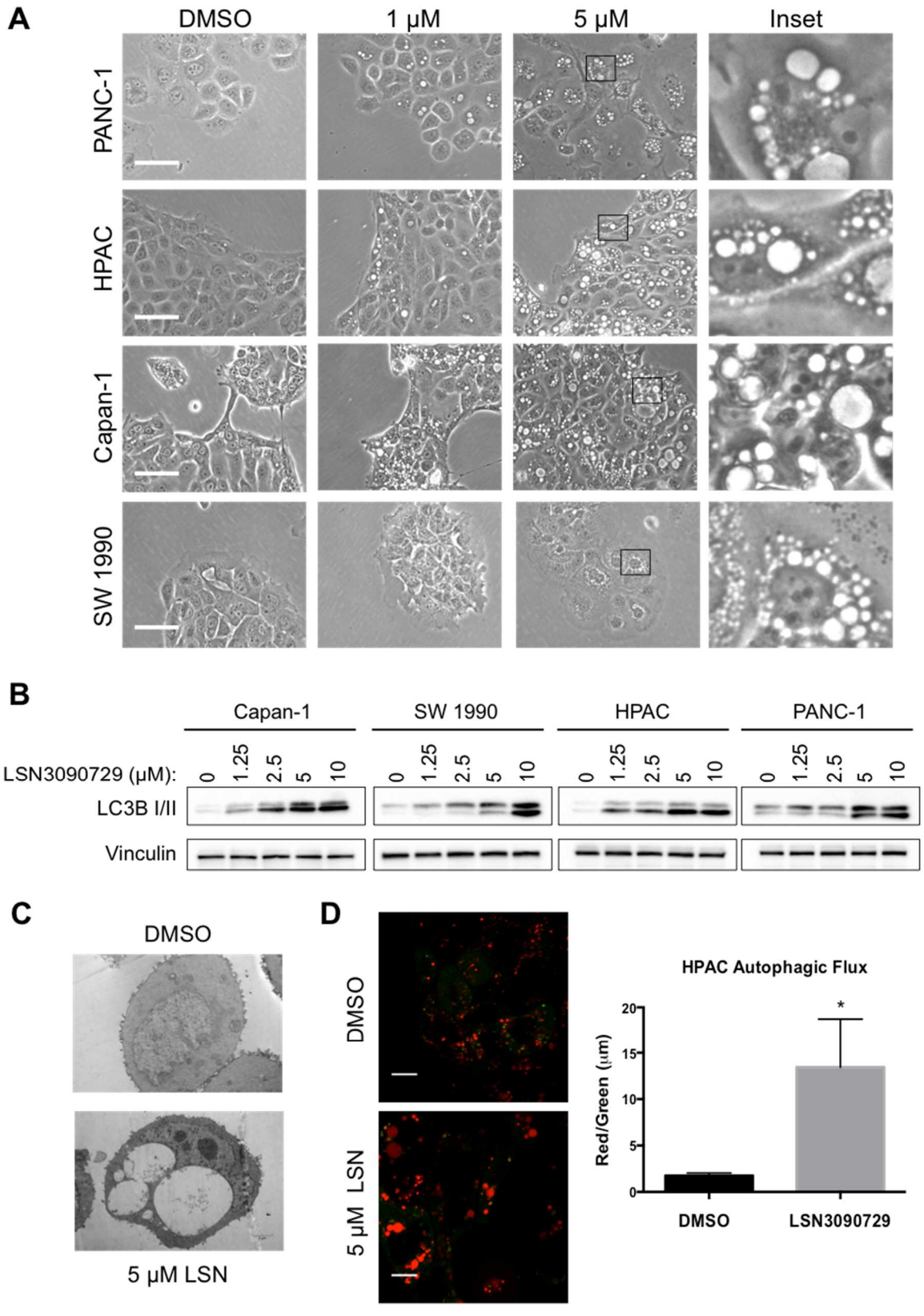

A
DMSO
1 μM
5 μM
Inset
PANC-1
HPAC
Capan-1
SW 1990
B
Capan-1
SW 1990
HPAC
PANC-1
LSN3090729 (μM):
0
1.25
2.5
5
10
LC3B I/II
Vinculin
C
DMSO
5 μM LSN
D
DMSO
5 μM LSN
HPAC Autophagic Flux
Red/Green (μm)
20
15
10
5
0
*
DMSO
LSN3090729

**Figure 3.5: LSN3090729 increases autophagic flux and autolysosome formation in PDAC cells.** A, Indicated PDAC cell lines were treated with increasing doses of LSN3090729. Cells were imaged at 24 h at 20x magnification. Scale bar represents 400 μm. B, Cells were treated with LSN3090279 for 72 h and probed for LC3B I and II. C, HPAC cells were treated for 24 h with DMSO or 5 μM LSN3090729, fixed and imaged via transmission electron microscopy. Scale bar represents 2 μm. D, HPAC cells infected with EGFP-mCherry-LC3 were treated with 5 μM LSN3090729 for 24 h and the ratio of red (autolysosomes) to green (autophagosomes) was quantitated with ImageJ.

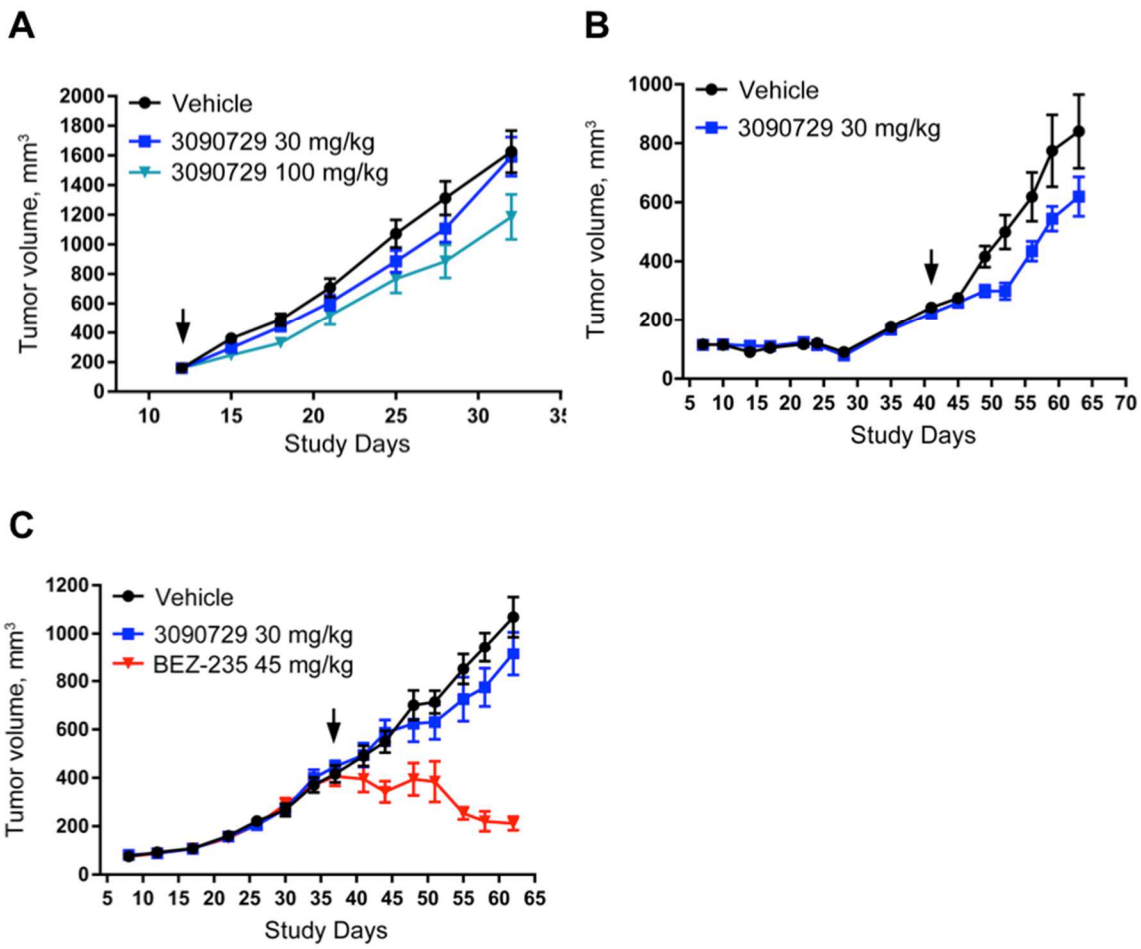



**Figure 3.6: LSN3090729 marginally impairs KRAS-mutant tumor growth in vivo.** A, Mice harboring HCT116-derived subcutaneous tumors in a colon cancer xenograft model were dosed with vehicle or one of 2 doses of LSN3090729. B, Mice bearing subcutaneous tumors derived from PANC-1 PDAC cell lines were dosed with vehicle or a single dose of LSN3090729. C, Mice bearing subcutaneous tumors derived from A549 lung cancer cells were dosed with vehicle, LSN3090729, or the PI3K/mTOR inhibitor BEZ-235 in order to compare the effectiveness of TBK1 and the dual PI3K/mTOR inhibitors at reducing tumor cell viability. For each experiment, n = 10 mice per treatment group.

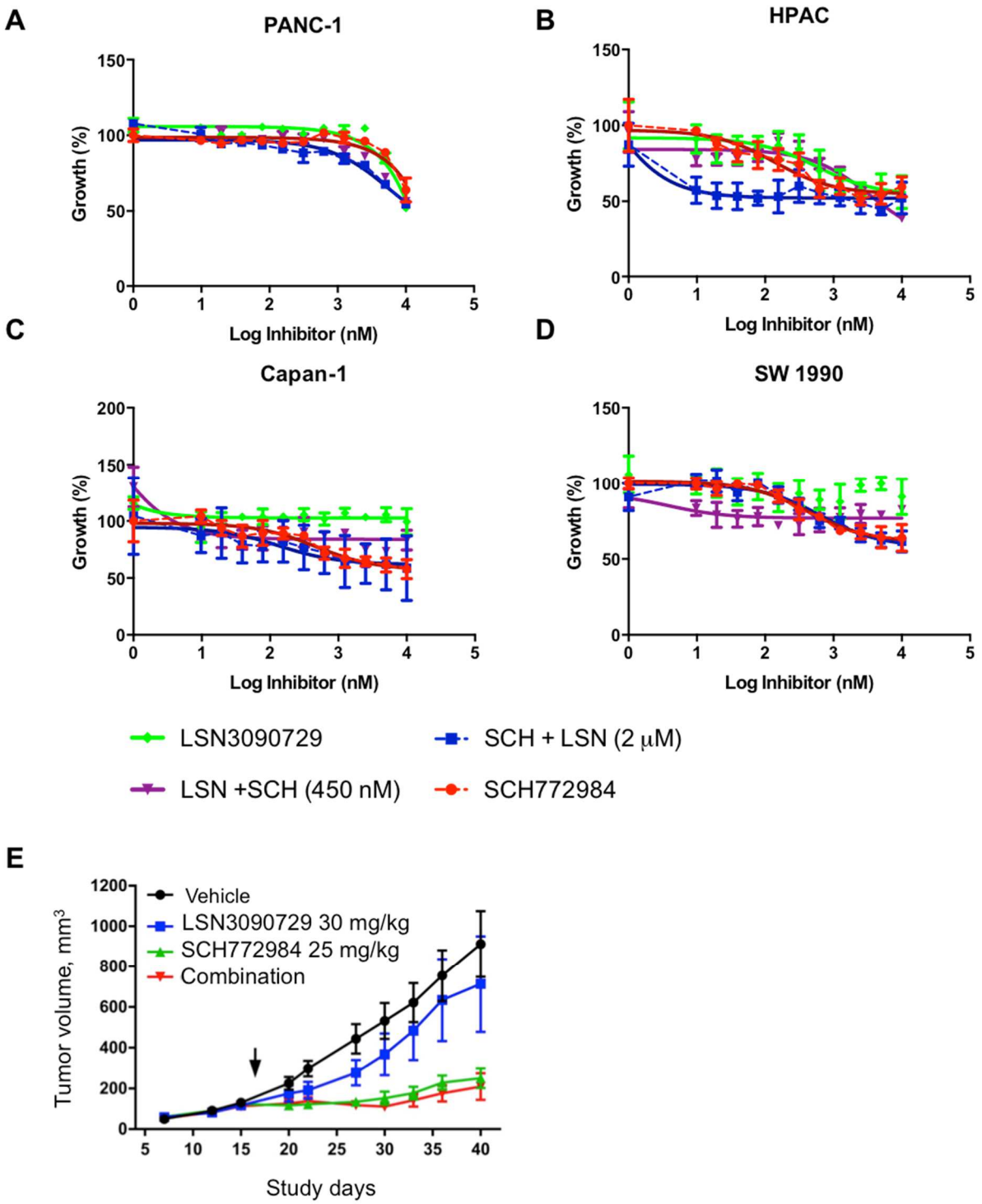
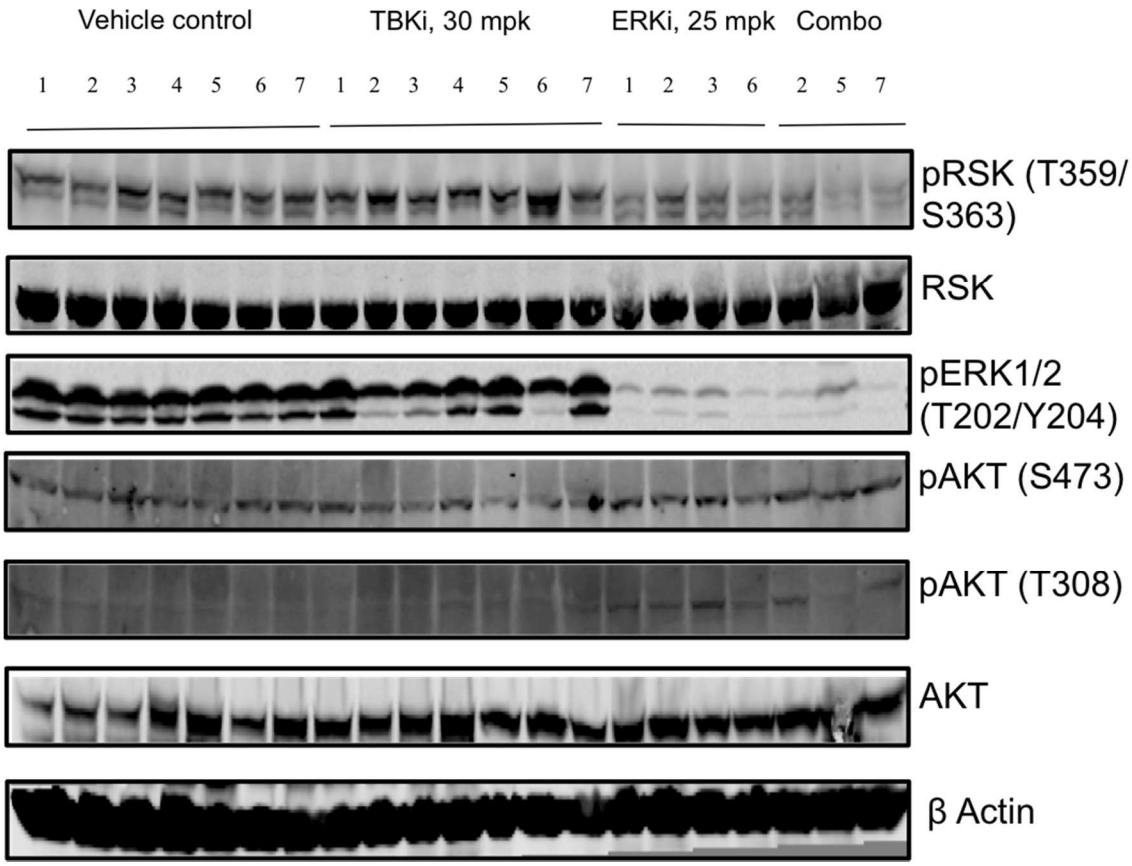



**Figure 3.7: Combined treatment with TBK1 inhibitor LSN3090729 and ERK1/2 inhibitor SCH772984 does not efficiently reduce PDAC growth.** A-D, PDAC cell lines were treated with a range of concentrations of LSN3090729 both with (purple) and without (green) constant SCH772984 (450 nM), and also with a range of SCH772984 concentrations with (blue) or without (red) constant LSN3090729 (2 μM). E, Mice were subcutaneously injected with SW1990 PDAC cells and treated with vehicle or inhibitor starting on day 16 as indicated by the arrow. n = 7 mice per treatment group.




**Supplemental Figure 3.1: Western blot analysis of tumor cell lysates collected from subcutaneous mouse tumors derived from SW1990 PDAC cells.** A reduction in pRSK was used as a marker of ERK1/2 inhibition and pAKT was used to confirm inhibition of TBK1.

# CHAPTER 4: CONCLUDING REMARKS AND FUTURE DIRECTIONS

To date, the effective chemotherapeutic approaches for pancreatic cancer are comprised of the classical cytotoxic drugs (141). Despite our now advanced and detailed genetic understanding of pancreatic cancer, disappointingly, this wealth of information has failed to lead to effective targeted therapies. With death from pancreatic cancer surpassing breast cancer in 2016 (139), and projected to be second only to lung cancer death by 2020 (140), the need for improved therapies is now acute. As KRAS mutations are found in nearly 100% of pancreatic cancers (2), there is much hope that the successful development of anti-KRAS therapies will be the key to accomplishing this feat. Targeting KRAS has become a major effort in cancer research, and there are many ongoing strategies and ideas (43). My studies explore directions where there is promise, but where there has not been sufficient effort. To address this need, my research has centered on two less studied kinase effectors of KRAS in pancreatic cancer, PAK1 and TBK1. My findings reveal both the promise and complexities of targeting effector signaling to develop the elusive anti-KRAS therapy for pancreatic cancer.

Currently, there are at least five major directions for anti-KRAS drug discovery (43). Of these, targeting KRAS effector signaling is arguably considered one of the most promising. This promise is based in large part on the demonstrated requirement for KRAS effector signaling to drive cancer growth, and also in part on the fact that many effector signaling components are protein kinases, which are highly tractable drug targets (201).

Since the 1990s when RAF and PI3K were identified and validated as effectors of RAS, there has been intensive effort by the pharmaceutical industry to develop inhibitors of

these two crucial downstream effector signaling components (43). There has been impressive success in developing potent inhibitors of components of these two canonical effector pathways, with many now under clinical evaluation (ClinicalTrials.gov). However, despite intensive clinical trial analyses, when evaluated as monotherapy, molecules that inhibit RAF, MEK, ERK, PI3K, AKT, or mTOR have demonstrated limited to no clinical efficacy for pancreatic cancer. Do these failures argue that targeting effector signaling is not a useful anti-KRAS strategy? No – instead, I argue that, because of the complexity of effectors that KRAS utilizes to drive cancer growth, concurrent inhibition of multiple effector pathways will be needed.

In my studies, I have addressed the role of two less studied effector pathways, the Tiam1-RAC1 (56, 57, 69) and the RalGEF-RAL (8, 11) small GTPase signaling networks. While there are considerable cell culture and mouse model studies that validate their roles in mutant RAS-initiated and -maintained cancer growth, how these pathways can be targeted therapeutically has been the challenge. My studies explored the PAK1 and TBK1 serine/threonine kinases as mediators of RAC1- and RAL-driven growth.

Previous studies of RAC1 and RAL applied genetic approaches to interrogate their roles in tumor growth. However, each small GTPase can interact with multiple downstream effectors and which effectors account for their driver roles in cancer remains to be determined. Among the multitude of RAC1 and RAL effectors, I focused on PAK1 and TBK1, respectively, for two main reasons. First, these effectors are serine/threonine protein kinases. With the greatest success in targeted anti-cancer drug development seen with protein kinases, PAK1 and TBK1 are highly tractable drug targets. Second, there is evidence that each of these protein kinases can serve driver roles in cancer (54, 86, 151). My studies benefited from collaborative interactions with pharmaceutical companies that shared their unpublished inhibitors. I have employed pharmacologic inhibitors of these molecules to evaluate PAK1 and TBK1 as possible therapeutic targets in the treatment of

PDAC. Though both genetic suppression and pharmacologic inhibition of PAK1 and TBK1 caused reduced cellular proliferation, this reduction was limited and observed only in a subset of PDAC lines. Thus their merit as stand-alone therapeutic targets for KRAS-mutant PDAC may be limited. This is in contrast to published studies reporting the critical importance of these kinases in supporting other cancer types, such as breast and skin cancer for PAK1 and non-small cell cancer for TBK1, where loss or inhibition of these proteins resulted in significant growth inhibition of these tumor lines (54, 86, 151). It is possible that PAK1 and TBK1 may be suitable for monotherapy in these cancer types, though, undoubtedly, combination strategies involving multiple inhibitors will be required to prevent tumor cell resistance to PAK1 or TBK1 inhibition alone. As an additional note for TBK1, in our screen of 300 cell lines, described in Chapter 3, we observed no clustering of tumor cell type and dependency on TBK1. It remains to be determined whether tumor type differences and tumor heterogeneity (or some other factor) account for these disparate dependencies on PAK1 and TBK1.

My PAK1 studies described in Chapter 2 reveal that PAK1 is a moderately effective target for decreasing cellular viability, especially in an anchorage-independent context. Either genetic suppression of PAK1 by RNAi or pharmacologic inhibition of PAK1 by a recently developed, highly selective PAK1 inhibitor, AZ13705339, reduced PDAC tumor cell viability in vitro. While I used the canonical phosphorylation of MEK1 at S298 (163) to demonstrate the efficacy of the PAK1 inhibitor, loss of pMEK1 did not always correlate with a reduction in PDAC cell growth. As PAK1 can also act as a scaffold for PDK1 and AKT (85), inhibition of kinase activity does not abolish all PAK1 functions. Furthermore, since pMEK1 S298 reduction did not correlate with a significant reduction in phosphorylation of ERK1/2 signaling (data not shown), the only well-validated MEK1 substrate and a key mediator of RAS signaling, it is likely that other PAK1 effectors are the key substrates involved in PAK1-dependent cancer growth. To determine which PAK1 effectors are truly

critical for driving PDAC, I would propose to do two experiments: 1) Determine how loss of PAK1 protein and pharmacologic inhibition of PAK1 modulate kinome-wide activity levels in cell lines dependent upon PAK1 for maintaining proliferation and macropinocytosis. To do this I would utilize multiplexed kinase inhibitor beads and mass spectrometry (MIB/MS) as described by Duncan and colleagues (88). 2) I would perform metabolic profiling of PDAC cell lines that exhibit reduced macropinocytosis following loss of PAK1 activity. This might reveal a clue about the metabolic pathways being modulated following loss or inhibition of PAK1, and could point to known proteins that may function downstream of PAK1.

In the absence of a clear signaling mechanism that would indicate why loss of PAK1 activity led to a reduction in PDAC cellular viability, I began to assess other biological phenotypes associated with PAK1. Earlier studies in mouse fibroblast models suggested a role for PAK1 in macropinocytosis (103). However, these observations had not been extended to cancers with bona fide RAS mutations. As pancreatic cancers are especially reliant on the scavenging of protein, carbohydrates, and other nutrients to fuel their increased metabolic needs in their hypoxic, nutrient-scarce tumor/stromal environment (36, 37, 202), I began to assess the role of PAK1 in macropinocytosis as a potential mechanism to support the elevated metabolism of pancreatic cancer.

Previous reports describe the role of PAK1 in promoting macropinocytosis in the context of extracellular protein scavenging and as a means of bacterial/viral entry into cells (103, 104). Additionally, macropinocytosis is a known contributor to pancreatic cancer amino acid pools and necessary to maintain the unremitting metabolic needs of PDAC cell growth, both *in vitro* and *in vivo* (105, 156). My studies revealed that PAK1 does indeed regulate macropinocytosis in PDAC. Both suppression of PAK1 protein expression and inhibition of PAK1 catalytic activity resulted in a reduction of macropinocytosis in the majority of cell lines I tested. This indicated that PAK1 could be partially supporting PDAC tumor cell growth and survival through macropinocytic protein scavenging. Future studies in mouse models of

*Kras*-driven PDAC, such as the widely studied KPC (KrasLSL.G12D/+; p53R172H/+; PdxCretg/+) mouse model (203), will be needed to address the potential relevance of my findings for the pancreatic cancer patient. Due to the large stromal component of PDAC tumors, composed of cancer associated fibroblasts, pancreatic stellate cells, and a large immune inflammatory component (141, 204, 205), it will be critical to determine whether PAK1 modulates the macropinocytic index of tumor cell or stromal cells. A critical question is: Will genetic ablation or pharmacologic inhibition of PAK1 also reduce macropinocytosis activity *in vivo*, where tumor cells are associated with stromal cells that can, and do, influence tumor cell behavior?

In Chapter 3 I detailed my studies of TBK1 in PDAC. I utilized a novel ATP-competitive inhibitor of TBK1, LSN3090729, as well as RNAi targeting TBK1 mRNA, to demonstrate that loss of TBK1 activity marginally reduces pancreatic cancer cell growth in a subset of cell lines. Though most PDAC lines exhibited some degree of resistance to TBK1 inhibition in terms of viability, all cell lines treated with LSN3090729 developed large autolysosomal vacuoles. Since this vacuole formation was also seen with RNAi suppression of TBK1, I believe that this phenotype is not likely to be due to an off-target activity of this inhibitor. This result indicated that loss of TBK1 leads to misregulation of autophagic flux, leading to non-productive autophagy that culminates in swollen lysosomes that do not complete the processing of the cargo to provide the required nutrients to sustain cellular metabolic needs. Though this non-productive autophagy did not appear to result in cell death, proliferation appeared to be marginally impaired. This opens up the potential for combination studies with LSN3090729 and inhibitors of other metabolic pathways, such as chloroquine (112), which inhibits autophagy, or 2-deoxy-D-glucose (206), which inhibits glycolysis, that may together stress PDAC tumor cells sufficiently to lead to significant loss of viability. Studies that could be done to address this possibility are described below.

Overall, my research has described the role of two serine-threonine kinases implicated downstream of mutant KRAS in PDAC. While loss or inhibition of both kinases led to a marginal decline in tumor cell viability, the more interesting implications of my data involve how both PAK1 and TBK1 regulate aspects of cellular metabolism in PDAC. Perhaps the marginal reduction in cell viability following application of these inhibitors reflects the limited potency and selectivity of the inhibitors that I used in my studies. Future development of more potent and selective inhibitors may well find more robust anti-tumor activities than I observed. Also, my studies have been limited to cell culture analyses and subcutaneous nude mouse tumorigenicity studies. Cancer cells are likely to show different dependencies when in a three-dimensional *in vivo* environment, with stromal cell interactions and immune responses also likely to greatly impact drug sensitivities of PDAC tumors. Therefore, using immune-competent, orthotopic mouse models of PDAC to test these inhibitors *in vivo*, alone or in combination with other compounds, will be valuable. The following sections describe potential future directions of these studies and methods for resolving the unanswered questions provoked by these data.

**What regulates PAK1 activation in PDAC if not KRAS-RAC1?**

The original premise of a KRAS-Tiam1-RAC1-PAK1 pathway was based on multiple earlier reports that used overexpression studies to demonstrate that individual components of this proposed pathway interact (57, 207-209). Yet, no study has evaluated endogenous proteins in KRAS-mutant cancer cells to comprehensively link all of these molecules together in a direct signaling cascade. My data indicate that, contrary to this initial hypothesis, neither KRAS nor RAC1 play a large role in regulating phosphorylation of MEK1 by PAK1. My initial hypothesis that PAK1 signals downstream of a KRAS-RAC1 signaling axis in PDAC was revealed to be contextually false. Additionally, only two PDAC cell lines, AsPC-1 and MIA PaCa-2, appeared to require expression of KRAS to drive PAK-1

dependent macropinocytosis. RAC1 appeared largely dispensable for PAK1-dependent macropinocytosis. Furthermore, loss of KRAS expression did not affect RAC1-GTP levels in most cells. These data beg the question of how PAK1 is truly regulated in PDAC cell lines, if not downstream of KRAS and RAC1 as has been both hypothesized and reported in previous studies (79, 86, 92, 165). That PAK1 has been shown to be aberrantly activated in cancer types where KRAS mutations are not common (e.g., breast cancer (151)) may provide clues for these mechanisms.

PAK proteins remain the most validated effectors of RAC1, but they are also activated downstream of both receptor and non-receptor tyrosine kinases, GPCRs, scaffolding molecules such Nck and Grb2, various protein-protein interactions, and WNT-WRCH1 signaling (165).

To determine the mechanism of PAK1 activation in PDAC, I propose to perform co-immunoprecipitation (co-IP) mass spectrometry experiments with exogenously expressed PAK1 across different PDAC cell lines. These co-IP experiments could reveal candidate proteins that PAK1 is in complex with in each cell type. Additionally, I could use mutants of PAK1, such as L83/86H, which does not bind to RAC1 or Cdc42 (113), to officially rule out a contribution of the latter small GTPases to PAK1-dependent macropinocytosis in PDAC.

**What is the contribution of other PAK isoforms to PDAC tumor growth?**

My work has demonstrated a role for PAK1 in supporting PDAC cell growth and macropinocytosis. However, PAK1 is one of six PAK family members. While the Group II PAK family proteins, PAK4, PAK5/7, and PAK6, have some overlapping roles with those of the Group I PAK family proteins, they are regulated in a different manner and have distinct tissue distribution to that of PAK1-3 (79, 165). PAK2 and PAK3 harbor kinase domains that are respectively 93% and 95% identical to PAK1. I was unable to detect expression of PAK3 in PDAC cell lines, but PAK2 is abundantly present in most PDAC lines. It was beyond the

scope of my studies to distinguish the roles of PAK1 and PAK2 in PDAC, though it is possible that PAK2 activity contributes to PDAC growth and survival.

Most of my data would indicate that, of the Group I PAKs, PAK1 is the dominant contributor to PDAC growth due to the substantial growth impairment observed in anchorage-independent soft agar colony formation assays upon loss of PAK1 expression or inhibition of PAK1 activity. However, if PAK2 were able to partially compensate for loss of PAK1, this compensation may be masking some of the effects of PAK1 loss. The AZ13705339 compound primarily inhibits PAK1, and treatment of PDAC cells with AZ13705339 faithfully recapitulated both the growth impairment and defect in macropinocytosis observed with knockdown of only PAK1 in all but two cell lines. It may be that these two cell lines could represent a subset of tumors where PAK2 could potentially compensate for PAK1. Dual genetic ablation of both PAK1 and PAK2, or use of a small molecule inhibitor of all Group I PAK proteins, such as FRAX597 (118), would reveal whether PAK2 was crucial for supporting growth in the absence of PAK1 kinase activity in these two cell lines. So while my data point to PAK1 as having a more important role in PDAC signaling, exploring the role of PAK2 in PDAC might still be informative.

Furthermore, Group II PAKs functionally overlap with Group I PAK substrates to a small degree (79). Though the expression of PAK5/7 and PAK6 tends to be limited to tissues of a neuronal origin (79, 122), PAK4 amplification and overexpression has been observed in pancreatic cancer, and is able to promote increased cellular motility and invasiveness of PDAC cells downstream of Rio Kinase 3 and RAC1 (210, 211). Additionally, PAK4 is known to contribute to pancreatic cancer cell proliferation via AKT and ERK-mediated induction of NF-κB signaling (212). These data support that further investigation into the role of PAK4 in PDAC may prove fruitful for furthering the body of knowledge encompassing PAK regulation in cancer.

To elucidate the contribution of individual PAK isoforms in pancreatic cancer, I could perform PAK2 or PAK4 RNAi knockdown experiments and assess PDAC cell viability, induction of cell death by apoptosis, anchorage-independent growth, and macropinocytic uptake under these conditions. Additionally, knockdown of individual or multiple isoforms in combination with inhibition of PAK1 by AZ13705339, Group I PAKs with FRAX597 (118), Group II PAKs with GNE-2861 (213), or both at once with the pan-PAK inhibitor, PF3758309 (which was originally a Group II inhibitor, but found to be equally effective at inhibiting all PAKs) (122), could reveal distinct cellular functions of PAK isoforms. Such data would provide valuable information about whether different PAK isoforms can compensate for one another in the context of PDAC cell viability and macropinocytosis.

**Is macropinocytosis a primary mechanism by which PAK1 supports PDAC growth?**

Many previous studies implicate PAK1 in proliferative or survival signaling through the phosphorylation of AKT and MEK1 (75, 76, 165). In breast cancer PAK1 is known to promote phosphorylation of β-catenin downstream of ErbB2 to promote survival (151). A model of KRAS-mutant squamous cell skin cancer revealed that PAK1 was necessary to drive signaling to promote tumor proliferation through MEK and ERK signaling in that tumor type (86). Another study revealed that nuclear PAK1, along with protein kinase A, is able to phosphorylate ERα in the absence of hormone signaling to lead to tamoxifen resistance in breast cancer (214). In pancreatic cancer, MET activation leads to PAK1 activation and induces pancreatic cancer cell migration (155). Still another study observed that PAK1 is able to regulate PDAC cell survival through engagement with NF-κB, which then induces the transcription of fibronectin (110).

My studies took a different angle to evaluate the mechanism of PAK1 growth dependence in PDAC. Early observations in NIH 3T3 cells demonstrated a role for PAK1 in driving macropinocytosis (103). Additionally, PAK1-mediated macropinocytosis was shown

to occur in bladder cancer cells downstream of PI3K-AKT signaling in the context of uptake of Bacille Calmette-Guerin (BCG), an attenuated strain of Mycobacterium bovis that is used as a treatment for bladder carcinoma (104). Due to the grossly elevated metabolic rate of pancreatic cancer cells (36, 37), it is likely that macropinocytosis is a necessary and critical means to encourage tumor cell survival. Most of my studies of viability were done in complete culture medium containing serum and ample growth factors, as well as essential and non-essential amino acids. However, the macropinocytosis assays were performed over the course of 16 h where the cells are starved of FBS. The results of my studies suggest that if cells were to be cultured for a longer period of time in media lacking sufficient growth factors while treating with PAK inhibitor in the absence of serum albumin as an amino acid source, perhaps increased loss of cell viability would be seen upon PAK1 loss or inhibition due to insufficient macropinoctyic uptake of extracellular nutrients. A study performed in MEFs expressing KRAS G12D demonstrated that these cells could thrive in media lacking essential amino acids as long as they were supplied with albumin, which was internalized by the cell via macropinocytosis and catabolized in the lysosome to serve as an amino acid source (215). A similar experimental system using constitutively activated PAK1 (L107F or T423E) could reveal the role of PAK1 in supporting macropinocytosis-dependent cell survival.

**How is PAK1-dependent macropinocytosis regulated and what substrate does PAK1 employ to drive this process in PDAC?**

In two of the four PDAC cell lines I surveyed (AsPC-1 and MIA PaCa-2), expression of mutant KRAS was necessary to drive PAK1-dependent macropinocytosis. These results mirror what was reported in a study by Commisso and colleagues (105), in which the authors showed that mutant KRAS drives macropinocytosis in MIA PaCa-2 cells, which harbor a KRAS G12C mutation. This was in contrast to BxPC-3 PDAC cells, which have WT

KRAS, but mutant BRAF, and thus still possess elevated MAPK signaling. However, tumor cells are heterogenous in their preferences for certain signaling events, so it was not altogether surprising that the macropinoctyic index of two other cell lines were not affected by loss of KRAS expression. HPAF-II cells showed no significant change in macropinocytotic index following siRNA-mediated depletion of KRAS, and PANC-1 cells actually displayed increased macropinocytosis upon loss of KRAS expression. Despite the different roles of KRAS in these cells, all of these cell lines exhibited some level of sensitivity to PAK1 loss. Thus, there exist KRAS-dependent and KRAS-independent mechanisms that drive macropinocytosis through PAK-dependent mechanisms.

The downstream effectors that PAK1 utilizes to promote macropinocytosis are largely unknown and much speculated. MEK1 S298 as a marker of PAK1 activity was sufficient for assessing the level of PAK1 inhibition induced by the AZ13705339 inhibitor, but no studies have directly linked MEK1 to macropinocytosis in human cancer, though my data showing inhibition of ERK reduces macropinocytosis may yet implicate the MAPK pathway in this process. To date, PAK1 has over 40 known substrates (77), many of which are involved in cytoskeletal reorganization. As macropinocytosis is a process largely driven by actin reorganization at the plasma membrane that leads to membrane ruffling and macropinocytic cup formation (40, 166, 167, 169), it is likely that cytoskeletal targets of PAK1 activity are involved. Possible candidate PAK1 substrates involved in PAK1-dependent macropinocytosis include LIMK (216), CtBP1/BARS (164), and p41-ARC (97). To more extensively examine PAK1-mediated macropinocytosis, I could perform an siRNA screen to look for proteins that when lost further reduce macropinocytosis beyond that of a PAK1 inhibitor or loss of PAK1 alone. Hits from this initial screen could be further validated as PAK1 interactors via co-IP and western blotting. If these hits are bona fide regulators of macropinocytosis, loss or inhibition of these proteins should lead to a reduction in macropinocytic index of PDAC cells. These data could help us link PAK1 activity to proteins

that affect macropinocytic nutrient scavenging in PDAC, and perhaps identify another therapeutic target.

**Can combined inhibition of macropinocytosis and other metabolic pathways be useful to treat PDAC?**

If inhibition or loss of PAK1 signaling is capable of preventing extracellular protein scavenging and decreasing cell viability in PDAC cell lines, it is a logical next step to attempt to pair PAK inhibitors with inhibitors of other metabolic pathways. For example, combining a PAK inhibitor with an inhibitor of autophagy, such as chloroquine (36), and thus preventing nutrient scavenging from two distinct mechanisms, may decrease the ability of cancer cells to initiate compensatory mechanisms, leading to more efficient cancer cell growth suppression.

Combinatorial approaches with PAK inhibition and other targeted therapies may also be a viable strategy for diminishing cell viability. As we have observed an additive decrease in macropinocytic uptake with combinatorial treatment of PDAC cells with AZ13705339 and either ERK1/2 inhibition with SCH77298 or PI3Kβ/δ inhibition with AZD8186, these combinations may also result in reduced viability of PDAC cells. Based on my studies in both Chapter 2 and Chapter 3, a combination of both a PAK1 inhibitor and a TBK1 inhibitor may prove to be a very fruitful strategy for reducing PDAC nutrient scavenging from both macropinocytosis and autophagy.

Additionally, I could perform an unbiased chemical inhibitor library screen to identify compounds that act synergistically with a PAK1 inhibitor. Such screens have been useful in identifying combinations that enhance the activities of other RAS effector inhibitors, such as MEK inhibitors.

As a cautionary note, some approved chemotherapies, such as nab-paclitaxel, which is paclitaxel tagged to serum albumin, is thought enter cells via macropinocytosis. In such

cases, combining nab-paclitaxel with a PAK1 inhibitor may prove to be therapeutically unproductive, or worse. However, combining PAK1 inhibition with gemcitabine or FOLFIRINOX, which do not depend on macropinocytotic uptake such that delivery would not be affected by inhibition of PAK, may serve to be a promising therapeutic strategy for PDAC.

**Does inhibition of PAK1 prevent macropinocytosis *in vivo*?**

PDAC tumors are notorious for having extensive stromal tissue and a relatively low number of tumor cells (141). Therefore, the dynamics of amino acid scavenging and tumor cell metabolism are likely very different from what is observed in tissue culture. The Commisso et al. study (105) examined the role of macropinocytosis in supporting PDAC tumors *in vivo*. They showed that tumors derived from MIA PaCa-2 cells were able to internalize FITC-dextran delivered intratumorally, and that this macropinocytosis was abrogated in the presence of EIPA, a non-specific inhibitor of the $Na^+/H^+$ antiporter that incidentally interferes with macropinosome formation. I would propose to extend this type of study to PAK1 inhibition *in vivo*. I could generate mice bearing orthotopic tumors induced by implantation of PDAC cell lines derived from human pancreatic tumors or use the KPC mouse model and treat with a PAK inhibitor. PDAC cell lines that have genetically suppressed PAK1 could also be used to complement the inhibitor studies. I could then intratumorally inject FITC-dextran and excise tumors for imaging to determine the level of macropinocytosis occurring in these tumors. Additionally, these *in vivo* studies could indicate the effectiveness of PAK1 loss at reducing PDAC tumor burden in mice. Unfortunately, the PAK1 inhibitor AZ13705339 does not possess favorable pharmacokinetic and pharmacodynamic traits to make it suitable for *in vivo* studies. However, I could utilize other selective PAK1 inhibitors, or Group I PAK inhibitors, as they become available, to complement PAK1 genetic suppression in these studies.

**Which substrate is a reliable marker of TBK1 activity and sensitivity to TBK1 inhibition in PDAC cells?**

Two of the PDAC cell lines from the panel I surveyed in Chapter 3 displayed high sensitivity to TBK1 inhibition by LSN3090729. While even cells that were resistant to LSN3090729 became more sensitized in a single cell clonogenic assay, there was a large discrepancy in the effects between the PANC-1 and HPAC cells, which were significantly sensitive to TBK1 inhibition, and the rest of the PDAC lines. Moreover, studies have shown that many tumor cell lines exhibit differential sensitivity to loss of TBK1, with many cell lines being fairly resistant (55, 137). One study suggested that TBK1 is necessary for KRAS-mutant proliferation in NSCLC (54), but this does not appear to be the case in PDAC (55).

Phosphorylation of specific sites in both IRF3 S386 (55) and AKT T308/S473 (137) have been previously validated as markers of TBK1 activity in tumor cells, including in PDAC. However, my studies revealed that neither pIRF3 nor pAKT were clear indicators of PDAC cell sensitivity to LSN3090729 or genetic ablation of TBK1. PANC-1 cells did not show a consistent, dose-dependent reduction of pAKT S473 in the presence of LSN3090729 despite showing reduced viability (data not shown). On the other hand, all cell lines, whether highly sensitive or fairly resistant, showed moderate loss of pIRF3 S386 levels. To discern the cellular signaling consequences of TBK1 inhibition in hopes of identifying a better biological marker for distinguishing sensitive cell lines from resistant cell lines, I propose to perform reverse phase protein array (RPPA) (217) analysis following TBK1 suppression or inhibition with LSN3090729. RPPA is a method for assessing the phosphorylation status of a large panel of protein kinases via phospho-specific antibodies. It is akin to a large western blot. This type of study would reveal the phosphorylation status of a large array of signaling proteins and thereby provide a clue as to which pathways are modulated following loss of TBK1 activity. If the results of such an experiment do show differences between the phosphorylation profiles of sensitive and resistant cell lines,

validation of these differences and whether these identified kinases actually contribute to promoting tumor growth downstream of TBK1 would then direct future studies aimed at identifying these hits as potential biomarkers that define the patients that may benefit from treatment with LSN3090729.

**What is the mechanism of TBK1-mediated autolysosome accumulation?**

Our studies demonstrated that TBK1 inhibition leads to upregulation of non-productive autophagy that results in the accumulation of large vesicles that are unable to resolve. The accumulation of mCherry-tagged LC3 in these large vesicles indicates that these are unresolved autolysosomes that have acidified sufficiently to cause the degradation of the EGFP signal, but not of the mCherry signal (112). These autolysosomes continuously swell in size rather than undergo normal degradation and export of cargo to other endocytic vesicles. This non-productive autophagy, while not immediately lethal to PDAC cell lines, correlated with slight reduction in cellular proliferation rates following treatment with LSN3090729.

To determine the mechanism of the observed autolysosomal accumulation in PDAC cells, I propose to use RNAi-mediated knockdown or pharmacologic inhibition of individual regulators of autophagy and lysosomal fusion (e.g., Atg-5/7, Ulk1/2, beclin-1, VSP34, etc.) (36, 112) to determine which of these proteins produce a similar phenotype to loss of TBK1 activity. Any positive hit could identify a potential, novel TBK1 binding partner or TBK1-regulated protein associated with autophagy. These data might give mechanistic insight into how these autolysosomes accumulate.

**Can TBK1 inhibitors be paired successfully with other targeted therapies or chemotherapies to drive the cells towards death?**

Though PDAC cells develop enormous autolysomes as a result of non-productive autophagy, this alone is not sufficient to induce apoptotic cell death or significantly reduce

cellular proliferation in all cell lines. I observed no cleavage of PARP or caspase-3 upon TBK1 inhibition (data not shown), and at up to 72 h, cells containing these large autolysosomes were still viable and able to metabolize MTT while remaining adhered to the tissue culture flask. The grotesque morphological phenotypes of these cells would suggest that viability should be compromised and that TBK1 inhibition could be driving these cells, albeit weakly and slowly, towards cell death.

To determine whether TBK1 inhibition may yet be a fruitful strategy for the treatment of PDAC, I would first culture cells for a long period of time (>2 weeks) in TBK1 inhibitor to determine if this non-productive autophagy eventually leads to cell death at time points longer than 72 h. Death by autolysosomal accumulation may very well prove to be a slow, but efficient process. These non-productive autolysosomes would allow substances to accumulate in the cell and interfere with normal cellular physiology, perhaps similar to lysosomal storage diseases, such as Niemann-Pick disease (218). Secondly, it is highly possible that TBK1 inhibition alone will never be enough to drive PDAC cell toward death, and that combined inhibition of multiple cellular processes should be evaluated. Our studies with the ERK1/2 inhibitor, SCH772984 did not lead to a significant loss of viability in PDAC, either *in vitro* or *in vivo*, but this excludes only one of many potential therapeutic targets. No tumor cell is solely reliant on one oncogenic pathway for survival, so I propose pairing LSN3090729 with other targeted therapies, such as a PI3K or PAK1 inhibitor. Previous studies cited above, including my own studies that are the subject of this document, indicate that PI3K and PAK1 are involved in driving macropinocytosis in PDAC. Macropinocytosis and autophagy can be considered as different sides of the same coin with relation to nutrient scavenging in tumor cells. In theory, if one could concurrently block both macropinocytosis (with PI3K and PAK1 inhibition) and autophagy (with a TBK1 inhibitor), pancreatic tumor cells would be less able to meet the demands of their hyperactive metabolism. This could lead to cell death. Additionally, I would like to assay the effect of dual treatment with

LSN3090729 and gemcitabine or nab-paclitaxel, which are currently approved chemotherapeutic regimens used in the treatment of pancreatic cancer (141).

**Conclusion**

In summary, I have found that both PAK1 and TBK1 play a role in mediating metabolic processes in PDAC. While PAK1 appears to be a more promising target for therapeutic intervention in PDAC, TBK1 exhibited more limited potential as a single agent. My studies demonstrate the need for further evaluation of the mechanisms whereby these kinases modulate PDAC metabolic processes. Further, they support the idea that inhibitors of these proteins would be effective in combination with other therapies for the treatment of pancreatic cancer.

## REFERENCES


1. Cox AD, Der CJ. Ras history: The saga continues. Small GTPases 2010;1:2-27.

2. Hobbs GA, Der CJ, Rossman KL. RAS isoforms and mutations in cancer at a glance. J Cell Sci 2016;129:1287-92.

3. Wennerberg K, Rossman KL, Der CJ. The Ras superfamily at a glance. J Cell Sci 2005;118:843-6.

4. Khosravi-Far R, Campbell S, Rossman KL, Der CJ. Increasing complexity of Ras signal transduction: involvement of Rho family proteins. Adv Cancer Res 1998;72:57-107.

5. Wennerberg K, Der CJ. Rho-family GTPases: it's not only Rac and Rho (and I like it). J Cell Sci 2004;117:1301-12.

6. Zohn IM, Campbell SL, Khosravi-Far R, Rossman KL, Der CJ. Rho family proteins and Ras transformation: the RHOad less traveled gets congested. Oncogene 1998;17:1415-38.

7. Bodemann BO, White MA. Ral GTPases and cancer: linchpin support of the tumorigenic platform. Nat Rev Cancer 2008;8:133-40.

8. Gentry LR, Martin TD, Reiner DJ, Der CJ. Ral small GTPase signaling and oncogenesis: More than just 15minutes of fame. Biochim Biophys Acta 2014;1843:2976-88.

9. Jaffe AB, Hall A. Rho GTPases: biochemistry and biology. Annu Rev Cell Dev Biol 2005;21:247-69.

10. Vega FM, Ridley AJ. Rho GTPases in cancer cell biology. FEBS Lett 2008;582:2093-101.

11. Feig LA. Ral-GTPases: approaching their 15 minutes of fame. Trends Cell Biol 2003;13:419-25.

12. Downward J. Targeting RAS signalling pathways in cancer therapy. Nat Rev Cancer 2003;3:11-22.

13. Khosravi-Far R, Solski PA, Clark GJ, Kinch MS, Der CJ. Activation of Rac1, RhoA, and mitogen-activated protein kinases is required for Ras transformation. Mol Cell Biol 1995;15:6443-53.

14. Kunz M. The genetic basis of new treatment modalities in melanoma. Curr Drug Targets 2015;16:233-48.

15. Berndt N, Hamilton AD, Sebti SM. Targeting protein prenylation for cancer therapy. Nat Rev Cancer 2011;11:775-91.

16. Ahearn IM, Haigis K, Bar-Sagi D, Philips MR. Regulating the regulator: post-translational modification of RAS. Nat Rev Mol Cell Biol 2011;13:39-51.

17. Der CJ, Krontiris TG, Cooper GM. Transforming genes of human bladder and lung carcinoma cell lines are homologous to the ras genes of Harvey and Kirsten sarcoma viruses. Proc Natl Acad Sci U S A 1982;79:3637-40.

18. Goldfarb M, Shimizu K, Perucho M, Wigler M. Isolation and preliminary characterization of a human transforming gene from T24 bladder carcinoma cells. Nature 1982;296:404-9.

19. Parada LF, Tabin CJ, Shih C, Weinberg RA. Human EJ bladder carcinoma oncogene is homologue of Harvey sarcoma virus ras gene. Nature 1982;297:474-8.

20. Pulciani S, Santos E, Lauver AV, Long LK, Robbins KC, Barbacid M. Oncogenes in human tumor cell lines: molecular cloning of a transforming gene from human bladder carcinoma cells. Proc Natl Acad Sci U S A 1982;79:2845-9.

21. Santos E, Tronick SR, Aaronson SA, Pulciani S, Barbacid M. T24 human bladder carcinoma oncogene is an activated form of the normal human homologue of BALB- and Harvey-MSV transforming genes. Nature 1982;298:343-7.

22. Shih C, Weinberg RA. Isolation of a transforming sequence from a human bladder carcinoma cell line. Cell 1982;29:161-9.

23. Prior IA, Lewis PD, Mattos C. A comprehensive survey of Ras mutations in cancer. Cancer Res 2012;72:2457-67.

24. Newlaczyl AU, Hood FE, Coulson JM, Prior IA. Decoding RAS isoform and codon-specific signalling. Biochem Soc Trans 2014;42:742-6.

25. Cox AD, Der CJ. Ras family signaling: therapeutic targeting. Cancer Biol Ther 2002;1:599-606.

26. Feuerstein J, Goody RS, Wittinghofer A. Preparation and characterization of nucleotide-free and metal ion-free p21 "apoprotein". J Biol Chem 1987;262:8455-8.

27. Ostrem JM, Peters U, Sos ML, Wells JA, Shokat KM. K-Ras(G12C) inhibitors allosterically control GTP affinity and effector interactions. Nature 2013;503:548-51.

28. James GL, Goldstein JL, Brown MS, Rawson TE, Somers TC, McDowell RS, et al. Benzodiazepine peptidomimetics: potent inhibitors of Ras farnesylation in animal cells. Science 1993;260:1937-42.

29. Kohl NE, Mosser SD, deSolms SJ, Giuliani EA, Pompliano DL, Graham SL, et al. Selective inhibition of ras-dependent transformation by a farnesyltransferase inhibitor. Science 1993;260:1934-7.

30. Whyte DB, Kirschmeier P, Hockenberry TN, Nunez-Oliva I, James L, Catino JJ, et al. K- and N-Ras are geranylgeranylated in cells treated with farnesyl protein transferase inhibitors. J Biol Chem 1997;272:14459-64.

31. Papke B, Murarka S, Vogel HA, Martin-Gago P, Kovacevic M, Truxius DC, et al. Identification of pyrazolopyridazinones as PDEdelta inhibitors. Nat Commun 2016;7:11360.

32. Zimmermann G, Papke B, Ismail S, Vartak N, Chandra A, Hoffmann M, et al. Small molecule inhibition of the KRAS-PDEdelta interaction impairs oncogenic KRAS signalling. Nature 2013;497:638-42.

33. Cox AD, Der CJ, Philips MR. Targeting RAS Membrane Association: Back to the Future for Anti-RAS Drug Discovery? Clin Cancer Res 2015;21:1819-27.

34. White E. Exploiting the bad eating habits of Ras-driven cancers. Genes Dev 2013;27:2065-71.

35. Kimmelman AC. Metabolic Dependencies in RAS-Driven Cancers. Clin Cancer Res 2015;21:1828-34.

36. Bryant KL, Mancias JD, Kimmelman AC, Der CJ. KRAS: feeding pancreatic cancer proliferation. Trends Biochem Sci 2014;39:91-100.

37. Perera RM, Bardeesy N. Pancreatic Cancer Metabolism: Breaking It Down to Build It Back Up. Cancer Discov 2015;5:1247-61.

38. Hjelmeland A, Zhang J. Metabolic, autophagic, and mitophagic activities in cancer initiation and progression. Biomed J 2016;39:98-106.

39. Barton LA, Ren J. Pancreatic Neoplasms and Autophagy. Curr Drug Targets 2016.

40. Ha KD, Bidlingmaier SM, Liu B. Macropinocytosis Exploitation by Cancers and Cancer Therapeutics. Front Physiol 2016;7:381.

41. Vigil D, Cherfils J, Rossman KL, Der CJ. Ras superfamily GEFs and GAPs: validated and tractable targets for cancer therapy? Nature reviews Cancer 2010;10:842-57.

42. Collins MA, Bednar F, Zhang Y, Brisset JC, Galban S, Galban CJ, et al. Oncogenic Kras is required for both the initiation and maintenance of pancreatic cancer in mice. J Clin Invest 2012;122:639-53.

43. Cox AD, Fesik SW, Kimmelman AC, Luo J, Der CJ. Drugging the undruggable RAS: Mission possible? Nat Rev Drug Discov 2014;13:828-51.

44. Baines AT, Xu D, Der CJ. Inhibition of Ras for cancer treatment: the search continues. Future medicinal chemistry 2011;3:1787-808.

45. Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, et al. Mutations of the BRAF gene in human cancer. Nature 2002;417:949-54.

46. Gupta S, Ramjaun AR, Haiko P, Wang Y, Warne PH, Nicke B, et al. Binding of ras to phosphoinositide 3-kinase p110alpha is required for ras-driven tumorigenesis in mice. Cell 2007;129:957-68.

47. Castellano E, Sheridan C, Thin MZ, Nye E, Spencer-Dene B, Diefenbacher ME, et al. Requirement for interaction of PI3-kinase p110alpha with RAS in lung tumor maintenance. Cancer Cell 2013;24:617-30.

48. Fruman DA, Rommel C. PI3K and cancer: lessons, challenges and opportunities. Nat Rev Drug Discov 2014;13:140-56.

49. Samatar AA, Poulikakos PI. Targeting RAS-ERK signalling in cancer: promises and challenges. Nat Rev Drug Discov 2014;13:928-42.

50. Ryan MB, Der CJ, Wang-Gillam A, Cox AD. Targeting RAS-mutant cancers: is ERK the key? Trends Cancer 2015;1:183-98.

51. ClinicalTrials.gov: A service of the U.S. National Institutes of Health. 2014 [cited; Available from: http://clinicaltrials.gov

52. Yu B, Luo J. Synthetic lethal genetic screens in Ras mutant cancers. Enzymes 2013;34 Pt. B:201-19.

53. Downward J. RAS Synthetic Lethal Screens Revisited: Still Seeking the Elusive Prize? Clin Cancer Res 2015;21:1802-9.

54. Barbie DA, Tamayo P, Boehm JS, Kim SY, Moody SE, Dunn IF, et al. Systematic RNA interference reveals that oncogenic KRAS-driven cancers require TBK1. Nature 2009;462:108-12.

55. Muvaffak A, Pan Q, Yan H, Fernandez R, Lim J, Dolinski B, et al. Evaluating TBK1 as a therapeutic target in cancers with activated IRF3. Mol Cancer Res 2014;12:1055-66.

56. Malliri A, van der Kammen RA, Clark K, van der Valk M, Michiels F, Collard JG. Mice deficient in the Rac activator Tiam1 are resistant to Ras-induced skin tumours. Nature 2002;417:867-71.

57. Lambert JM, Lambert QT, Reuther GW, Malliri A, Siderovski DP, Sondek J, et al. Tiam1 mediates Ras activation of Rac by a PI(3)K-independent mechanism. Nat Cell Biol 2002;4:621-5.

58. Welch HC, Coadwell WJ, Ellson CD, Ferguson GJ, Andrews SR, Erdjument-Bromage H, et al. P-Rex1, a PtdIns(3,4,5)P3- and Gbetagamma-regulated guanine-nucleotide exchange factor for Rac. Cell 2002;108:809-21.

59. Han J, Luby-Phelps K, Das B, Shu X, Xia Y, Mosteller RD, et al. Role of substrates and products of PI 3-kinase in regulating activation of Rac-related guanosine triphosphatases by Vav. Science 1998;279:558-60.

60. Archer H, Bar-Sagi D. Ras and Rac as activators of reactive oxygen species (ROS). Methods Mol Biol 2002;189:67-73.

61. Hodis E, Watson IR, Kryukov GV, Arold ST, Imielinski M, Theurillat JP, et al. A landscape of driver mutations in melanoma. Cell 2012;150:251-63.

62. Krauthammer M, Kong Y, Ha BH, Evans P, Bacchiocchi A, McCusker JP, et al. Exome sequencing identifies recurrent somatic RAC1 mutations in melanoma. Nat Genet 2012;44:1006-14.

63. Bustelo XR, Sauzeau V, Berenjeno IM. GTP-binding proteins of the Rho/Rac family: regulation, effectors and functions in vivo. Bioessays 2007;29:356-70.

64. Bar-Sagi D, Feramisco JR. Induction of membrane ruffling and fluid-phase pinocytosis in quiescent fibroblasts by ras proteins. Science 1986;233:1061-8.

65. Walsh AB, Bar-Sagi D. Differential activation of the Rac pathway by Ha-Ras and K-Ras. J Biol Chem 2001;276:15609-15.

66. Qiu RG, Chen J, Kirn D, McCormick F, Symons M. An essential role for Rac in Ras transformation. Nature 1995;374:457-9.

67. Joneson T, White MA, Wigler MH, Bar-Sagi D. Stimulation of membrane ruffling and MAP kinase activation by distinct effectors of RAS. Science 1996;271:810-2.

68. Kissil JL, Walmsley MJ, Hanlon L, Haigis KM, Bender Kim CF, Sweet-Cordero A, et al. Requirement for Rac1 in a K-ras induced lung cancer in the mouse. Cancer Res 2007;67:8089-94.

69. Heid I, Lubeseder-Martellato C, Sipos B, Mazur PK, Lesina M, Schmid RM, et al. Early requirement of Rac1 in a mouse model of pancreatic cancer. Gastroenterology 2011;141:719-30, 30 e1-7.

70. Samuel MS, Lourenco FC, Olson MF. K-Ras mediated murine epidermal tumorigenesis is dependent upon and associated with elevated Rac1 activity. PloS one 2011;6:e17143.

71. Westwick JK, Lambert QT, Clark GJ, Symons M, Van Aelst L, Pestell RG, et al. Rac regulation of transformation, gene expression, and actin organization by multiple, PAK-independent pathways. Molecular and cellular biology 1997;17:1324-35.

72. Hamad NM, Elconin JH, Karnoub AE, Bai W, Rich JN, Abraham RT, et al. Distinct requirements for Ras oncogenesis in human versus mouse cells. Genes Dev 2002;16:2045-57.

73. Rangarajan A, Hong SJ, Gifford A, Weinberg RA. Species- and cell type-specific requirements for cellular transformation. Cancer Cell 2004;6:171-83.

74. Ahmed S, Prigmore E, Govind S, Veryard C, Kozma R, Wientjes FB, et al. Cryptic Rac-binding and p21(Cdc42Hs/Rac)-activated kinase phosphorylation sites of NADPH oxidase component p67(phox). J Biol Chem 1998;273:15693-701.

75. Chan PM, Manser E. PAKs in human disease. Prog Mol Biol Transl Sci 2012;106:171-87.

76. Radu M, Semenova G, Kosoff R, Chernoff J. PAK signalling during the development and progression of cancer. Nat Rev Cancer 2014;14:13-25.

77. Baker NM, Yee Chow H, Chernoff J, Der CJ. Molecular pathways: targeting RAC-p21-activated serine-threonine kinase signaling in RAS-driven cancers. Clin Cancer Res 2014;20:4740-6.

78. Ye DZ, Field J. PAK signaling in cancer. Cell Logist 2012;2:105-16.

79. Arias-Romero LE, Chernoff J. A tale of two Paks. Biol Cell 2008;100:97-108.

80. King H, Nicholas NS, Wells CM. Role of p-21-activated kinases in cancer progression. Int Rev Cell Mol Biol 2014;309:347-87.

81. Chaudhary A, King WG, Mattaliano MD, Frost JA, Diaz B, Morrison DK, et al. Phosphatidylinositol 3-kinase regulates Raf1 through Pak phosphorylation of serine 338. Curr Biol 2000;10:551-4.

82. Coles LC, Shaw PE. PAK1 primes MEK1 for phosphorylation by Raf-1 kinase during cross-cascade activation of the ERK pathway. Oncogene 2002;21:2236-44.

83. Sun H, King AJ, Diaz HB, Marshall MS. Regulation of the protein kinase Raf-1 by oncogenic Ras through phosphatidylinositol 3-kinase, Cdc42/Rac and Pak. Curr Biol 2000;10:281-4.

84. Eblen ST, Slack JK, Weber MJ, Catling AD. Rac-PAK signaling stimulates extracellular signal-regulated kinase (ERK) activation by regulating formation of MEK1-ERK complexes. Mol Cell Biol 2002;22:6023-33.

85. Higuchi M, Onishi K, Kikuchi C, Gotoh Y. Scaffolding function of PAK in the PDK1-Akt pathway. Nat Cell Biol 2008;10:1356-64.

86. Chow HY, Jubb AM, Koch JN, Jaffer ZM, Stepanova D, Campbell DA, et al. p21-Activated kinase 1 is required for efficient tumor formation and progression in a Ras-mediated skin cancer model. Cancer Res 2012;72:5966-75.

87. Little AS, Smith PD, Cook SJ. Mechanisms of acquired resistance to ERK1/2 pathway inhibitors. Oncogene 2013;32:1207-15.

88. Duncan JS, Whittle MC, Nakamura K, Abell AN, Midland AA, Zawistowski JS, et al. Dynamic reprogramming of the kinome in response to targeted MEK inhibition in triple-negative breast cancer. Cell 2012;149:307-21.

89. Graves LM, Duncan JS, Whittle MC, Johnson GL. The dynamic nature of the kinome. Biochem J 2013;450:1-8.

90. Burris HA, 3rd. Overcoming acquired resistance to anticancer therapy: focus on the PI3K/AKT/mTOR pathway. Cancer Chemother Pharmacol 2013;71:829-42.

91. Tabusa H, Brooks T, Massey AJ. Knockdown of PAK4 or PAK1 inhibits the proliferation of mutant KRAS colon cancer cells independently of RAF/MEK/ERK and PI3K/AKT signaling. Mol Cancer Res 2013;11:109-21.

92. Arias-Romero LE, Villamar-Cruz O, Huang M, Hoeflich KP, Chernoff J. Pak1 kinase links ErbB2 to beta-catenin in transformation of breast epithelial cells. Cancer Res 2013;73:3671-82.

93. Zhu G, Wang Y, Huang B, Liang J, Ding Y, Xu A, et al. A Rac1/PAK1 cascade controls beta-catenin activation in colon cancer cells. Oncogene 2012;31:1001-12.

94. Ye DZ, Jin S, Zhuo Y, Field J. p21-Activated kinase 1 (Pak1) phosphorylates BAD directly at serine 111 in vitro and indirectly through Raf-1 at serine 112. PLoS One 2011;6:e27637.

95. Misra UK, Deedwania R, Pizzo SV. Binding of activated alpha2-macroglobulin to its cell surface receptor GRP78 in 1-LN prostate cancer cells regulates PAK-2-dependent activation of LIMK. J Biol Chem 2005;280:26278-86.

96. Bagheri-Yarmand R, Mazumdar A, Sahin AA, Kumar R. LIM kinase 1 increases tumor metastasis of human breast cancer cells via regulation of the urokinase-type plasminogen activator system. Int J Cancer 2006;118:2703-10.

97. Vadlamudi RK, Li F, Barnes CJ, Bagheri-Yarmand R, Kumar R. p41-Arc subunit of human Arp2/3 complex is a p21-activated kinase-1-interacting substrate. EMBO Rep 2004;5:154-60.

98. Webb BA, Eves R, Crawley SW, Zhou S, Cote GP, Mak AS. PAK1 induces podosome formation in A7r5 vascular smooth muscle cells in a PAK-interacting exchange factor-dependent manner. Am J Physiol Cell Physiol 2005;289:C898-907.

99. Vadlamudi RK, Barnes CJ, Rayala S, Li F, Balasenthil S, Marcus S, et al. p21-activated kinase 1 regulates microtubule dynamics by phosphorylating tubulin cofactor B. Mol Cell Biol 2005;25:3726-36.

100. Banerjee M, Worth D, Prowse DM, Nikolic M. Pak1 phosphorylation on t212 affects microtubules in cells undergoing mitosis. Curr Biol 2002;12:1233-9.

101. Takahashi K, Suzuki K. Membrane transport of WAVE2 and lamellipodia formation require Pak1 that mediates phosphorylation and recruitment of stathmin/Op18 to Pak1-WAVE2-kinesin complex. Cell Signal 2009;21:695-703.

102. Morimura S, Takahashi K. Rac1 and Stathmin but Not EB1 Are Required for Invasion of Breast Cancer Cells in Response to IGF-I. Int J Cell Biol 2011;2011:615912.

103. Dharmawardhane S, Schurmann A, Sells MA, Chernoff J, Schmid SL, Bokoch GM. Regulation of macropinocytosis by p21-activated kinase-1. Mol Biol Cell 2000;11:3341-52.

104. Redelman-Sidi G, Iyer G, Solit DB, Glickman MS. Oncogenic activation of Pak1-dependent pathway of macropinocytosis determines BCG entry into bladder cancer cells. Cancer Res 2013;73:1156-67.

105. Commisso C, Davidson SM, Soydaner-Azeloglu RG, Parker SJ, Kamphorst JJ, Hackett S, et al. Macropinocytosis of protein is an amino acid supply route in Ras-transformed cells. Nature 2013;497:633-7.

106. Lightcap CM, Kari G, Arias-Romero LE, Chernoff J, Rodeck U, Williams JC. Interaction with LC8 is required for Pak1 nuclear import and is indispensable for zebrafish development. PloS one 2009;4:e6025.

107. Qing H, Gong W, Che Y, Wang X, Peng L, Liang Y, et al. PAK1-dependent MAPK pathway activation is required for colorectal cancer cell proliferation. Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine 2012.

108. Rayala SK, Molli PR, Kumar R. Nuclear p21-activated kinase 1 in breast cancer packs off tamoxifen sensitivity. Cancer Res 2006;66:5985-8.

109. Holm C, Rayala S, Jirstrom K, Stal O, Kumar R, Landberg G. Association between Pak1 expression and subcellular localization and tamoxifen resistance in breast cancer patients. J Natl Cancer Inst 2006;98:671-80.

110. Jagadeeshan S, Krishnamoorthy YR, Singhal M, Subramanian A, Mavuluri J, Lakshmi A, et al. Transcriptional regulation of fibronectin by p21-activated kinase-1 modulates pancreatic tumorigenesis. Oncogene 2015;34:455-64.

111. Osada S, Izawa M, Koyama T, Hirai S, Ohno S. A domain containing the Cdc42/Rac interactive binding (CRIB) region of p65PAK inhibits transcriptional activation and cell transformation mediated by the Ras-Rac pathway. FEBS letters 1997;404:227-33.

112. Klionsky DJ, Abdelmohsen K, Abe A, Abedin MJ, Abeliovich H, Acevedo Arozena A, et al. Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). Autophagy 2016;12:1-222.

113. Tang Y, Chen Z, Ambrose D, Liu J, Gibbs JB, Chernoff J, et al. Kinase-deficient Pak1 mutants inhibit Ras transformation of Rat-1 fibroblasts. Mol Cell Biol 1997;17:4454-64.

114. Tang Y, Marwaha S, Rutkowski JL, Tennekoon GI, Phillips PC, Field J. A role for Pak protein kinases in Schwann cell transformation. Proceedings of the National Academy of Sciences of the United States of America 1998;95:5139-44.

115. Rudolph J, Crawford, J.J., Hoeflich, K.P. and Chernoff, J. . Inhibitors of the Ras Superfamily G-proteins, Part B. p21-activated kinase inhibitors. The Enzymes; 2013. p. 157-80.

116. Murray BW, Guo C, Piraino J, Westwick JK, Zhang C, Lamerdin J, et al. Small-molecule p21-activated kinase inhibitor PF-3758309 is a potent inhibitor of oncogenic signaling and tumor growth. Proceedings of the National Academy of Sciences of the United States of America 2010;107:9446-51.

117. Guo C, McAlpine I, Zhang J, Knighton DD, Kephart S, Johnson MC, et al. Discovery of pyrroloaminopyrazoles as novel PAK inhibitors. J Med Chem 2012;55:4728-39.

118. Licciulli S, Maksimoska J, Zhou C, Troutman S, Kota S, Liu Q, et al. FRAX597, a small molecule inhibitor of the p21-activated kinases, inhibits tumorigenesis of neurofibromatosis type 2 (NF2)-associated Schwannomas. J Biol Chem 2013;288:29105-14.

119. Deacon SW, Beeser A, Fukui JA, Rennefahrt UE, Myers C, Chernoff J, et al. An isoform-selective, small-molecule inhibitor targets the autoregulatory mechanism of p21-activated kinase. Chemistry & biology 2008;15:322-31.

120. Viaud J, Peterson JR. An allosteric kinase inhibitor binds the p21-activated kinase autoregulatory domain covalently. Molecular cancer therapeutics 2009;8:2559-65.

121. Crawford JJ, Hoeflich KP, Rudolph J. p21-Activated kinase inhibitors: a patent review. Expert opinion on therapeutic patents 2012;22:293-310.

122. Rudolph J, Crawford JJ, Hoeflich KP, Wang W. Inhibitors of p21-activated kinases (PAKs). J Med Chem 2015;58:111-29.

123. Albright CF, Giddings BW, Liu J, Vito M, Weinberg RA. Characterization of a guanine nucleotide dissociation stimulator for a ras-related GTPase. EMBO J 1993;12:339-47.

124. Hofer F, Fields S, Schneider C, Martin GS. Activated Ras interacts with the Ral guanine nucleotide dissociation stimulator. Proc Natl Acad Sci U S A 1994;91:11089-93.

125. Spaargaren M, Bischoff JR. Identification of the guanine nucleotide dissociation stimulator for Ral as a putative effector molecule of R-ras, H-ras, K-ras, and Rap. Proc Natl Acad Sci U S A 1994;91:12609-13.

126. Shao H, Andres DA. A novel RalGEF-like protein, RGL3, as a candidate effector for rit and Ras. J Biol Chem 2000;275:26914-24.

127. Wolthuis RM, Bauer B, van 't Veer LJ, de Vries-Smits AM, Cool RH, Spaargaren M, et al. RalGDS-like factor (Rlf) is a novel Ras and Rap 1A-associating protein. Oncogene 1996;13:353-62.

128. Chien Y, White MA. RAL GTPases are linchpin modulators of human tumour-cell proliferation and survival. EMBO Rep 2003;4:800-6.

129. Lim KH, O'Hayer K, Adam SJ, Kendall SD, Campbell PM, Der CJ, et al. Divergent roles for RalA and RalB in malignant growth of human pancreatic carcinoma cells. Curr Biol 2006;16:2385-94.

130. Gentry LR, Nishimura A, Cox AD, Martin TD, Tsygankov D, Nishida M, et al. Divergent roles of CAAX motif-signaled posttranslational modifications in the regulation and subcellular localization of Ral GTPases. J Biol Chem 2015;290:22851-61.

131. Moskalenko S, Henry DO, Rosse C, Mirey G, Camonis JH, White MA. The exocyst is a Ral effector complex. Nat Cell Biol 2002;4:66-72.

132. Chien Y, Kim S, Bumeister R, Loo YM, Kwon SW, Johnson CL, et al. RalB GTPase-mediated activation of the IkappaB family kinase TBK1 couples innate immune signaling to tumor cell survival. Cell 2006;127:157-70.

133. Goetz CA, Baldwin AS. NF-kappaB pathways in the immune system: control of the germinal center reaction. Immunol Res 2008;41:233-47.

134. Beg AA, Baldwin AS, Jr. The I kappa B proteins: multifunctional regulators of Rel/NF-kappa B transcription factors. Genes Dev 1993;7:2064-70.

135. Shen RR, Hahn WC. Emerging roles for the non-canonical IKKs in cancer. Oncogene 2011;30:631-41.

136. Clark K, Peggie M, Plater L, Sorcek RJ, Young ER, Madwed JB, et al. Novel cross-talk within the IKK family controls innate immunity. Biochem J 2011;434:93-104.

137. Ou YH, Torres M, Ram R, Formstecher E, Roland C, Cheng T, et al. TBK1 directly engages Akt/PKB survival signaling to support oncogenic transformation. Mol Cell 2011;41:458-70.

138. Kamisawa T, Wood LD, Itoi T, Takaori K. Pancreatic cancer. Lancet 2016;388:73-85.

139. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. CA Cancer J Clin 2016;66:7-30.

140. Rahib L, Smith BD, Aizenberg R, Rosenzweig AB, Fleshman JM, Matrisian LM. Projecting cancer incidence and deaths to 2030: the unexpected burden of thyroid, liver, and pancreas cancers in the United States. Cancer Res 2014;74:2913-21.

141. Wolfgang CL, Herman JM, Laheru DA, Klein AP, Erdek MA, Fishman EK, et al. Recent progress in pancreatic cancer. CA Cancer J Clin 2013;63:318-48.

142. Ryan DP, Hong TS, Bardeesy N. Pancreatic adenocarcinoma. N Engl J Med 2014;371:2140-1.

143. Biankin AV, Waddell N, Kassahn KS, Gingras MC, Muthuswamy LB, Johns AL, et al. Pancreatic cancer genomes reveal aberrations in axon guidance pathway genes. Nature 2012;491:399-405.

144. Jones S, Zhang X, Parsons DW, Lin JC, Leary RJ, Angenendt P, et al. Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. Science 2008;321:1801-6.

145. Waddell N, Pajic M, Patch AM, Chang DK, Kassahn KS, Bailey P, et al. Whole genomes redefine the mutational landscape of pancreatic cancer. Nature 2015;518:495-501.

146. Witkiewicz AK, McMillan EA, Balaji U, Baek G, Lin WC, Mansour J, et al. Whole-exome sequencing of pancreatic cancer defines genetic diversity and therapeutic targets. Nat Commun 2015;6:6744.

147. Bailey P, Chang DK, Nones K, Johns AL, Patch AM, Gingras MC, et al. Genomic analyses identify molecular subtypes of pancreatic cancer. Nature 2016;531:47-52.

148. Ma WW, Herman JM, Jimeno A, Laheru D, Messersmith WA, Wolfgang CL, et al. A tolerability and pharmacokinetic study of adjuvant erlotinib and capecitabine with concurrent radiation in resected pancreatic cancer. Transl Oncol 2010;3:373-9.

149. Moore MJ, Goldstein D, Hamm J, Figer A, Hecht JR, Gallinger S, et al. Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. J Clin Oncol 2007;25:1960-6.

150. Ying H, Kimmelman AC, Lyssiotis CA, Hua S, Chu GC, Fletcher-Sananikone E, et al. Oncogenic Kras maintains pancreatic tumors through regulation of anabolic glucose metabolism. Cell 2012;149:656-70.

151. Arias-Romero LE, Villamar-Cruz O, Pacheco A, Kosoff R, Huang M, Muthuswamy SK, et al. A Rac-Pak signaling pathway is essential for ErbB2-mediated transformation of human breast epithelial cancer cells. Oncogene 2010;29:5839-49.

152. Shrestha Y, Schafer EJ, Boehm JS, Thomas SR, He F, Du J, et al. PAK1 is a breast cancer oncogene that coordinately activates MAPK and MET signaling. Oncogene 2012;31:3397-408.

153. Chen MJ, Wu DW, Wang YC, Chen CY, Lee H. PAK1 confers chemoresistance and poor outcome in non-small cell lung cancer via beta-catenin-mediated stemness. Sci Rep 2016;6:34933.

154. Mortazavi F, Lu J, Phan R, Lewis M, Trinidad K, Aljilani A, et al. Significance of KRAS/PAK1/Crk pathway in non-small cell lung cancer oncogenesis. BMC Cancer 2015;15:381.

155. Zhou W, Jubb AM, Lyle K, Xiao Q, Ong CC, Desai R, et al. PAK1 mediates pancreatic cancer cell migration and resistance to MET inhibition. J Pathol 2014;234:502-13.

156. Kamphorst JJ, Nofal M, Commisso C, Hackett SR, Lu W, Grabocka E, et al. Human pancreatic cancer tumors are nutrient poor and tumor cells actively scavenge extracellular protein. Cancer Res 2015;75:544-53.

157. Cox AD, Der CJ. Biological assays for cellular transformation. Methods Enzymol 1994;238:277-94.

158. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nat Methods 2012;9:671-5.

159. Marques RB, Aghai A, de Ridder CM, Stuurman D, Hoeben S, Boer A, et al. High Efficacy of Combination Therapy Using PI3K/AKT Inhibitors with Androgen Deprivation in Prostate Cancer Preclinical Models. Eur Urol 2015;67:1177-85.

160. Morris EJ, Jha S, Restaino CR, Dayananth P, Zhu H, Cooper A, et al. Discovery of a novel ERK inhibitor with activity in models of acquired resistance to BRAF and MEK inhibitors. Cancer Discov 2013;3:742-50.

161. Commisso C, Flinn RJ, Bar-Sagi D. Determining the macropinocytic index of cells through a quantitative image-based assay. Nat Protoc 2014;9:182-92.

162. Fiordalisi JJ, Keller PJ, Cox AD. PRL tyrosine phosphatases regulate rho family GTPases to promote invasion and motility. Cancer Res 2006;66:3153-61.

163. Frost JA, Steen H, Shapiro P, Lewis T, Ahn N, Shaw PE, et al. Cross-cascade activation of ERKs and ternary complex factors by Rho family proteins. EMBO J 1997;16:6426-38.

164. Liberali P, Kakkonen E, Turacchio G, Valente C, Spaar A, Perinetti G, et al. The closure of Pak1-dependent macropinosomes requires the phosphorylation of CtBP1/BARS. EMBO J 2008;27:970-81.

165. Molli PR, Li DQ, Murray BW, Rayala SK, Kumar R. PAK signaling in oncogenesis. Oncogene 2009;28:2545-55.

166. Koivusalo M, Welch C, Hayashi H, Scott CC, Kim M, Alexander T, et al. Amiloride inhibits macropinocytosis by lowering submembranous pH and preventing Rac1 and Cdc42 signaling. J Cell Biol 2010;188:547-63.

167. Swanson JA. Shaping cups into phagosomes and macropinosomes. Nat Rev Mol Cell Biol 2008;9:639-49.

168. Hancox U, Cosulich S, Hanson L, Trigwell C, Lenaghan C, Ellston R, et al. Inhibition of PI3Kbeta signaling with AZD8186 inhibits growth of PTEN-deficient breast and prostate tumors alone and in combination with docetaxel. Mol Cancer Ther 2015;14:48-58.

169. Lim JP, Gleeson PA. Macropinocytosis: an endocytic pathway for internalising large gulps. Immunol Cell Biol 2011;89:836-43.

170. Hayes TK, Neel NF, Hu C, Gautam P, Chenard M, Long B, et al. Long-Term ERK Inhibition in KRAS-Mutant Pancreatic Cancer Is Associated with MYC Degradation and Senescence-like Growth Suppression. Cancer Cell 2016;29:75-89.

171. Chen SH, Zhang Y, Van Horn RD, Yin T, Buchanan S, Yadav V, et al. Oncogenic BRAF Deletions That Function as Homodimers and Are Sensitive to Inhibition by RAF Dimer Inhibitor LY3009120. Cancer Discov 2016;6:300-15.

172. Moffitt RA, Marayati R, Flate EL, Volmar KE, Loeza SG, Hoadley KA, et al. Virtual microdissection identifies distinct tumor- and stroma-specific subtypes of pancreatic ductal adenocarcinoma. Nat Genet 2015;47:1168-78.

173. Lito P, Pratilas CA, Joseph EW, Tadi M, Halilovic E, Zubrowski M, et al. Relief of profound feedback inhibition of mitogenic signaling by RAF inhibitors attenuates their activity in BRAFV600E melanomas. Cancer Cell 2012;22:668-82.

174. Thillai K, Lam H, Sarker D, Wells CM. Deciphering the link between PI3K and PAK: An opportunity to target key pathways in pancreatic cancer? Oncotarget 2016.

175. Tan L, Zhang Y, Zhan Y, Yuan Y, Sun Y, Qiu X, et al. Newcastle disease virus employs macropinocytosis and Rab5a-dependent intracellular trafficking to infect DF-1 cells. Oncotarget 2016.

176. Carter GC, Bernstone L, Baskaran D, James W. HIV-1 infects macrophages by exploiting an endocytic route dependent on dynamin, Rac1 and Pak1. Virology 2011;409:234-50.

177. Saeed MF, Kolokoltsov AA, Albrecht T, Davey RA. Cellular entry of ebola virus involves uptake by a macropinocytosis-like mechanism and subsequent trafficking through early and late endosomes. PLoS Pathog 2010;6:e1001110.

178. Camonis JH, White MA. Ral GTPases: corrupting the exocyst in cancer cells. Trends Cell Biol 2005;15:327-32.

179. Kim JY, Beg AA, Haura EB. Non-canonical IKKs, IKK and TBK1, as novel therapeutic targets in the treatment of non-small cell lung cancer. Expert Opin Ther Targets 2013;17:1109-12.

180. Kim JY, Welsh EA, Oguz U, Fang B, Bai Y, Kinose F, et al. Dissection of TBK1 signaling via phosphoproteomics in lung cancer cells. Proc Natl Acad Sci U S A 2013;110:12414-9.

181. Junttila MR, Devasthali V, Cheng JH, Castillo J, Metcalfe C, Clermont AC, et al. Modeling targeted inhibition of MEK and PI3 kinase in human pancreatic cancer. Mol Cancer Ther 2015;14:40-7.

182. McIver EG, Bryans J, Birchall K, Chugh J, Drake T, Lewis SJ, et al. Synthesis and structure-activity relationships of a novel series of pyrimidines as potent inhibitors of TBK1/IKKepsilon kinases. Bioorg Med Chem Lett 2012;22:7169-73.

183. Feldman RI, Wu JM, Polokoff MA, Kochanny MJ, Dinter H, Zhu D, et al. Novel small molecule inhibitors of 3-phosphoinositide-dependent kinase-1. J Biol Chem 2005;280:19867-74.

184. Clark K, Plater L, Peggie M, Cohen P. Use of the pharmacological inhibitor BX795 to study the regulation and physiological roles of TBK1 and IkappaB kinase epsilon: a distinct upstream kinase mediates Ser-172 phosphorylation and activation. J Biol Chem 2009;284:14136-46.

185. Vu HL, Aplin AE. Targeting TBK1 inhibits migration and resistance to MEK inhibitors in mutant NRAS melanoma. Mol Cancer Res 2014;12:1509-19.

186. Tyner JW, Bumm TG, Deininger J, Wood L, Aichberger KJ, Loriaux MM, et al. CYT387, a novel JAK2 inhibitor, induces hematologic responses and normalizes inflammatory cytokines in murine myeloproliferative neoplasms. Blood 2010;115:5232-40.

187. Helgason E, Phung QT, Dueber EC. Recent insights into the complexity of Tank-binding kinase 1 signaling networks: the emerging role of cellular localization in the activation and substrate specificity of TBK1. FEBS Lett 2013;587:1230-7.

188. Newman AC, Scholefield CL, Kemp AJ, Newman M, McIver EG, Kamal A, et al. TBK1 kinase addiction in lung cancer cells is mediated via autophagy of Tax1bp1/Ndp52 and non-canonical NF-kappaB signalling. PLoS One 2012;7:e50672.

189. Yang S, Imamura Y, Jenkins RW, Canadas I, Kitajima S, Aref A, et al. Autophagy Inhibition Dysregulates TBK1 Signaling and Promotes Pancreatic Inflammation. Cancer Immunol Res 2016;4:520-30.

190. Yasui LS, Duran M, Andorf C, Kroc T, Owens K, Allen-Durdan K, et al. Autophagic flux in glioblastoma cells. Int J Radiat Biol 2016:1-14.

191. Matrone A, Grossi V, Chiacchiera F, Fina E, Cappellari M, Caringella AM, et al. p38alpha is required for ovarian cancer cell metabolism and survival. Int J Gynecol Cancer 2010;20:203-11.

192. Menon MB, Kotlyarov A, Gaestel M. SB202190-induced cell type-specific vacuole formation and defective autophagy do not depend on p38 MAP kinase inhibition. PLoS One 2011;6:e23054.

193. Majumder B, Baraneedharan U, Thiyagarajan S, Radhakrishnan P, Narasimhan H, Dhandapani M, et al. Predicting clinical response to anticancer drugs using an ex vivo platform that captures tumour heterogeneity. Nat Commun 2015;6:6169.

194. Kreso A, O'Brien CA, van Galen P, Gan OI, Notta F, Brown AM, et al. Variable clonal repopulation dynamics influence chemotherapy response in colorectal cancer. Science 2013;339:543-8.

195. Straussman R, Morikawa T, Shee K, Barzily-Rokni M, Qian ZR, Du J, et al. Tumour micro-environment elicits innate resistance to RAF inhibitors through HGF secretion. Nature 2012;487:500-4.

196. Hensing T, Chawla A, Batra R, Salgia R. A personalized treatment for lung cancer: molecular pathways, targeted therapies, and genomic characterization. Adv Exp Med Biol 2014;799:85-117.

197. Holderfield M, Deuker MM, McCormick F, McMahon M. Targeting RAF kinases for cancer therapy: BRAF-mutated melanoma and beyond. Nat Rev Cancer 2014;14:455-67.

198. Brown-Glaberman U, Dayao Z, Royce M. HER2-targeted therapy for early-stage breast cancer: a comprehensive review. Oncology (Williston Park) 2014;28:281-9.

199. Chien Y, White MA. Characterization of RalB-Sec5-TBK1 function in human oncogenesis. Methods Enzymol 2008;438:321-9.

200. Zhu Z, Aref AR, Cohoon TJ, Barbie TU, Imamura Y, Yang S, et al. Inhibition of KRAS-driven tumorigenicity by interruption of an autocrine cytokine circuit. Cancer Discov 2014;4:452-65.

201. Gross S, Rahal R, Stransky N, Lengauer C, Hoeflich KP. Targeting cancer with kinase inhibitors. J Clin Invest 2015;125:1780-9.

202. Sousa CM, Kimmelman AC. The complex landscape of pancreatic cancer metabolism. Carcinogenesis 2014;35:1441-50.

203. Hingorani SR, Wang L, Multani AS, Combs C, Deramaudt TB, Hruban RH, et al. Trp53R172H and KrasG12D cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice. Cancer Cell 2005;7:469-83.

204. Chu GC, Kimmelman AC, Hezel AF, DePinho RA. Stromal biology of pancreatic cancer. J Cell Biochem 2007;101:887-907.

205. Erkan M, Hausmann S, Michalski CW, Fingerle AA, Dobritz M, Kleeff J, et al. The role of stroma in pancreatic cancer: diagnostic and therapeutic implications. Nat Rev Gastroenterol Hepatol 2012;9:454-67.

206. Zhang D, Li J, Wang F, Hu J, Wang S, Sun Y. 2-Deoxy-D-glucose targeting of glucose metabolism in cancer cells as a potential therapy. Cancer Lett 2014;355:176-83.

207. Michiels F, Habets GG, Stam JC, van der Kammen RA, Collard JG. A role for Rac in Tiam1-induced membrane ruffling and invasion. Nature 1995;375:338-40.

208. Mertens AE, Roovers RC, Collard JG. Regulation of Tiam1-Rac signalling. FEBS Lett 2003;546:11-6.

209. Campbell PM, Singh A, Williams FJ, Frantz K, Ulku AS, Kelley GG, et al. Genetic and pharmacologic dissection of Ras effector utilization in oncogenesis. Methods Enzymol 2006;407:195-217.

210. Kimmelman AC, Hezel AF, Aguirre AJ, Zheng H, Paik JH, Ying H, et al. Genomic alterations link Rho family of GTPases to the highly invasive phenotype of pancreas cancer. Proc Natl Acad Sci U S A 2008;105:19372-7.

211. Chen S, Auletta T, Dovirak O, Hutter C, Kuntz K, El-ftesi S, et al. Copy number alterations in pancreatic cancer identify recurrent PAK4 amplification. Cancer Biol Ther 2008;7:1793-802.

212. Tyagi N, Bhardwaj A, Singh AP, McClellan S, Carter JE, Singh S. p-21 activated kinase 4 promotes proliferation and survival of pancreatic cancer cells through AKT- and ERK-dependent activation of NF-kappaB pathway. Oncotarget 2014;5:8778-89.

213. Zhuang T, Zhu J, Li Z, Lorent J, Zhao C, Dahlman-Wright K, et al. p21-activated kinase group II small compound inhibitor GNE-2861 perturbs estrogen receptor alpha

signaling and restores tamoxifen-sensitivity in breast cancer cells. Oncotarget 2015;6:43853-68.

214. Kok M, Zwart W, Holm C, Fles R, Hauptmann M, Van't Veer LJ, et al. PKA-induced phosphorylation of ERalpha at serine 305 and high PAK1 levels is associated with sensitivity to tamoxifen in ER-positive breast cancer. Breast Cancer Res Treat 2011;125:1-12.

215. Palm W, Park Y, Wright K, Pavlova NN, Tuveson DA, Thompson CB. The Utilization of Extracellular Proteins as Nutrients Is Suppressed by mTORC1. Cell 2015;162:259-70.

216. Edwards DC, Sanders LC, Bokoch GM, Gill GN. Activation of LIM-kinase by Pak1 couples Rac/Cdc42 GTPase signalling to actin cytoskeletal dynamics. Nat Cell Biol 1999;1:253-9.

217. Federici G, Gao X, Slawek J, Arodz T, Shitaye A, Wulfkuhle JD, et al. Systems analysis of the NCI-60 cancer cell lines by alignment of protein pathway activation modules with "-OMIC" data fields and therapeutic response signatures. Mol Cancer Res 2013;11:676-85.

218. Kuech EM, Brogden G, Naim HY. Alterations in membrane trafficking and pathophysiological implications in lysosomal storage disorders. Biochimie 2016;130:152-62.