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PAK4 AS A DRUG TARGET IN BREAST CANCER THERAPY

By

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ABSTRACT OF THE DISSERTATION

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PAK4, belonging to Group II PAKs, is an important signaling protein with key roles in regulating cell growth, cell survival and cell morphology. PAK4 protein levels are higher in several subtypes of breast cancer cells and PAK4 gene is amplified in basal like breast cancer. PAK4 overexpression in a mouse mammary epithelial cell line (iMMEC) results in oncogenic transformation of these cells, while siRNA mediated PAK4 down regulation in a human triple negative breast cancer cell line, MDA-MB-231, resulted in significant inhibition of its tumorigenic potential. These results point to a central role for PAK4 in mammary tumorigenesis. PAK4 expression pattern makes it an attractive drug target, however, it is important to note that PAK4 has both kinase-dependent and –independent functions in regulating tumor formation. Hence, blocking its kinase activity alone is insufficient in blocking its tumorigenic potential. Our lab collaborated with Karyopharm Therapeutics to investigate a novel PAK4 inhibitor, KPT-9274. I observed that treatment with KPT-9274 and KPT-8752 (isoform of KPT-9274) inhibited the cell growth, cell survival and cell motility of breast cancer cell lines, with this effect most

significantly observed in triple negative breast cancer cell lines. I observed that treatment with KPT inhibitors inhibited PAK4 protein levels along with PAK4 associated downstream signaling pathways in triple negative breast cancer cell lines. Most importantly, I observed that KPT-9274 significantly inhibited tumor growth in mouse xenograft models of 3 human triple negative breast cancer cell lines. KPT-9274 was capable of reducing steady state PAK4 protein levels *in vivo*, without significantly affecting PAK1 protein levels, indicating that KPT-9274 exhibits *in vivo* PAK4 specificity. This study shows that PAK4 can serve as a novel drug target in triple negative breast cancer therapy and KPT-9274 can have clinical benefits for the triple negative breast cancer population. Next, I analyzed RNA-seq data of samples collected from non-transformed WT iMMECs and iMMECs overexpressing PAK4, that form tumors in mice. Sequencing analysis identified several genes previously unknown to be regulated by PAK4. Using q-PCR, I was able to validate the RNA-seq data, further suggesting that RNA-seq is a promising and reproducible tool to study PAK4 induced transcriptional changes. This study reveals the PAK4 transcriptome profile in mammary tumor forming cells, and can provide translational utility in other types of cancers as well. Delineating the varied effectors of PAK4 signaling cascade will help uncover novel biomarkers for cancer, with some serving as potential therapeutic targets.

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DEDICATION

I would like to dedicate this dissertation

To my parents,

Krishnanand Rane

&

Chandrakala Rane

To my sister,

Dr. Vrushali Rane

To my niece,

Myra

And to my wife,

Bhargavi Pandit

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Chapter I

General Introduction

1. Introduction to The p21-Activated Kinase (PAK) family:

p21-Activated Kinases (PAKs) belong to a family of serine threonine kinases that were originally identified as downstream effectors of Rho GTPases Cdc42 and Rac. They bind to Cdc42 and Rac through a GTPase binding domain (GBD), also known as Cdc42/Rac binding domain (CRIB). PAKs are broadly classified into two categories based on their sequences and functions, Group A/Group I PAKs consisting of PAK 1,2 and 3; and Group B/Group II PAKs consisting of PAK 4,5 and 6 [1-3]. Mammalian PAKs are related to the yeast serine/threonine kinase, Ste20. PAK homologs are also found in other organisms including *C.elegans*, *Drosophila* and *Xenopus*.

2. PAK Structure and Activation

2.1 Structure of Group I PAKs

Each of the Group I PAKs has a N-terminal regulatory domain and a C-terminal kinase domain. Within the regulatory domain is the GBD, through which PAKs bind to activated Cdc42 and Rac (Figure 1). Binding to activated Cdc42 and Rac relieves an intramolecular interaction between their kinase domain and an autoinhibitory domain (AID), stimulating PAK's kinase activities. Group I PAK members share a high level of sequence homology but have different tissue specific expression patterns [4, 5]. PAK2 is found in all tissues, while PAK1 and PAK3 have more restricted expression patterns. All of the Group I PAKs are highly expressed in the nervous system.

2.2 Structure of Group II PAKs

Similar to Group I PAKs, Group II PAKs have a N-terminal GBD and a C-terminal kinase domain, and a sequence similar to AID found in Group I PAKs. However, the GBD and kinase domains of Group II PAKs share approximate only 50% sequence homology with the Group I PAKs, and they do not contain any other conserved domains found in Group I PAKs (Figure 1). Group II PAKs share some substrates in common with Group I PAKs, but also have some unique substrates of their own [6, 7]. PAK4 is considered to be the founding member of Group II PAKs [8], with ubiquitous expression in many adult tissues, but at low levels. However, PAK4 expression is very high during embryogenesis. PAK4 binds most efficiently to Cdc42 and less so with Rac. PAK5 and PAK6 have more of a tissue restricted expression pattern and they are especially high in the adult brain [9-12]. PAK6 is also found in testes and prostate and plays an important role in androgen receptor signaling [11, 13, 14], indicating that in addition to functioning as Rho GTPase targets, Group II PAKs also have Rho GTPase independent functions.

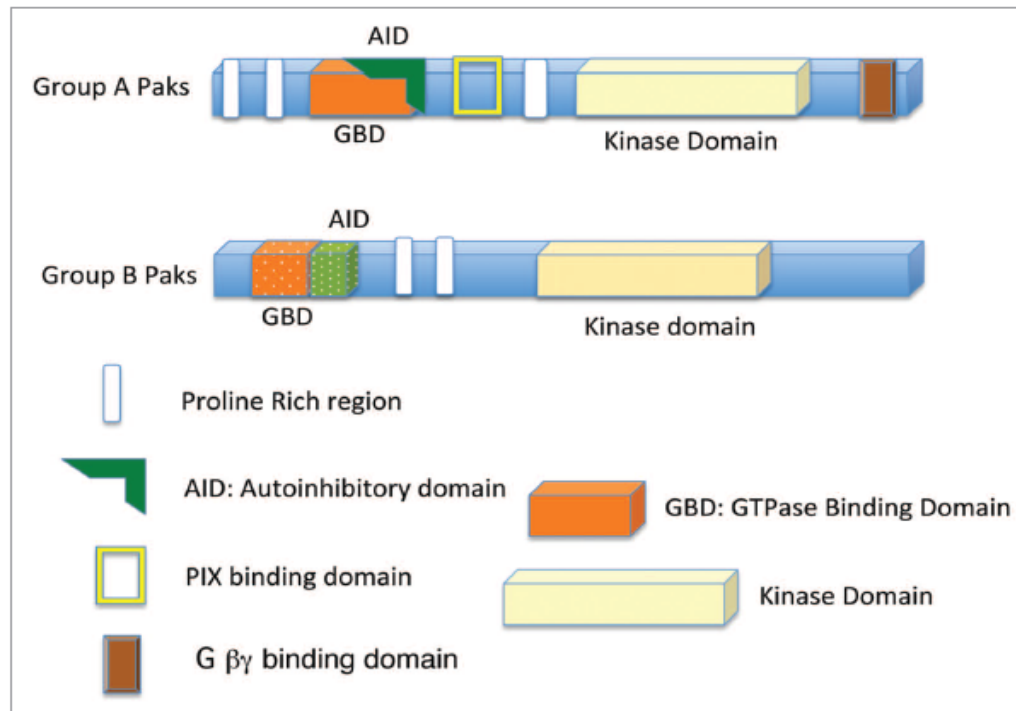


Figure 1: Basic structures of the Group I PAKs (PAKs 1, 2, and 3), and Group II PAKs (PAKs 4, 5, and 6). Group I PAKs have an Autoinhibitory Domain (AID) overlapping the GBD (GTPase Binding Domain), while the Group II PAKs have a related sequence adjacent to the GBD [15].

2.3 Activation of Group I PAKs

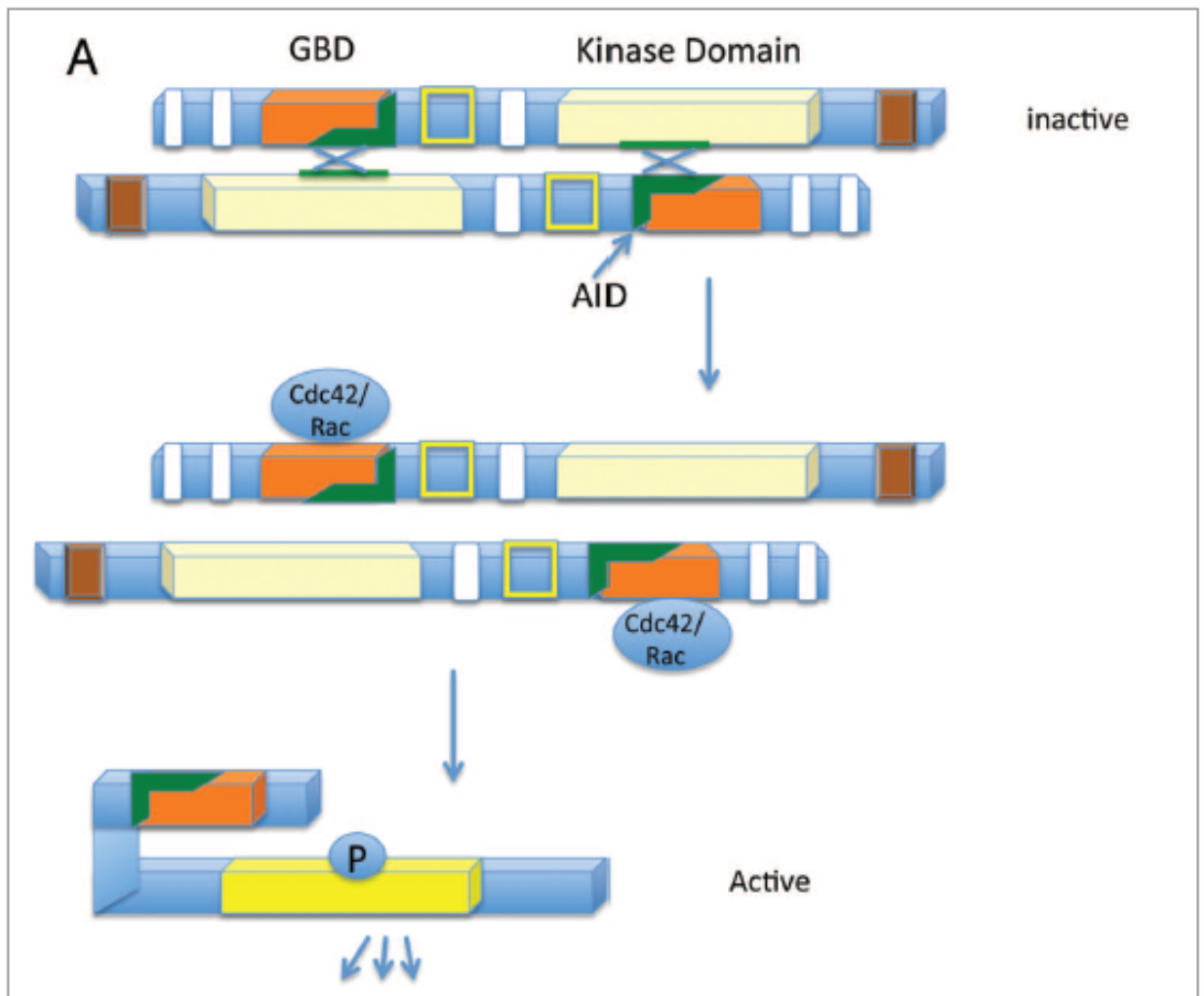
Group I and Group II PAKs are activated by different mechanisms [16, 17]. Group I PAKs function as dimers, where the AID binds in *trans* to the PAK catalytic domain on the dimerizing PAK. This interaction prevents auto phosphorylation at the activation loop (A-loop), and subsequent activation of PAK's kinase activity. Cdc42/Rac binding to the PAK GBD disrupts this interaction between the AID and the dimerizing PAK, leading to a conformational

change and forming a PAK monomer (Figure 2A) [15]. This monomer can get auto phosphorylated on its A-loop and consequently, gets activated [17-23].

2.4 Activation of Group II PAKs

Although Group II PAKs possess AID like domains, they get activated by different mechanisms [17, 21-25]. Group II PAKs function as monomers instead of dimers and are constitutively phosphorylated at the A-loop, even in quiescent cells [16]. Instead of regulating A-loop auto phosphorylation, the AID of Group II PAK is thought to allosterically modify the constitutively phosphorylated kinase, so that it becomes active. A prevailing model of Group II activation is that the AID like domain binds in *cis* to the PAK kinase domain, which keeps it in an inactive conformation, although constitutively phosphorylated (Figure 2B) [15]. When the PAK GBD binds to activated Cdc42, this results in a conformational change, relieving this inhibition and consequently activating its kinase activity [16]. Recently, a second model of Group II PAK activation has been proposed for PAK4 [26], though PAK5 and PAK6 might operate through a similar mechanism. This model involves an auto inhibitory pseudosubstrate (PS), which like the AID, is adjacent to the GBD. The PS is a proline rich region and thus can interact with proteins that have SH3 domains. According to this model, the PAK kinase domain interacts with the PS, which keeps it in an inactive conformation. This interaction is disrupted when the PS binds to proteins that contain the SH3 domains, relieving this auto inhibition. This model relies on a 2 step activation process; the first step involves binding of Cdc42 or Rac to PAK4 GBD, this

binding presumably localizes PAK4 to cellular regions containing other activating proteins and substrates. The second signal involves binding to SH3 domain containing proteins, resulting in relief of auto inhibition and activation of kinase activity (Figure 2B). It is interesting to note that mutations in the auto inhibitory PS region were found in both PAK5 and PAK6 in human cancer cells, including lung cancer and melanoma [26] suggesting that disruption of this regulatory mechanism may be associated with cancer.



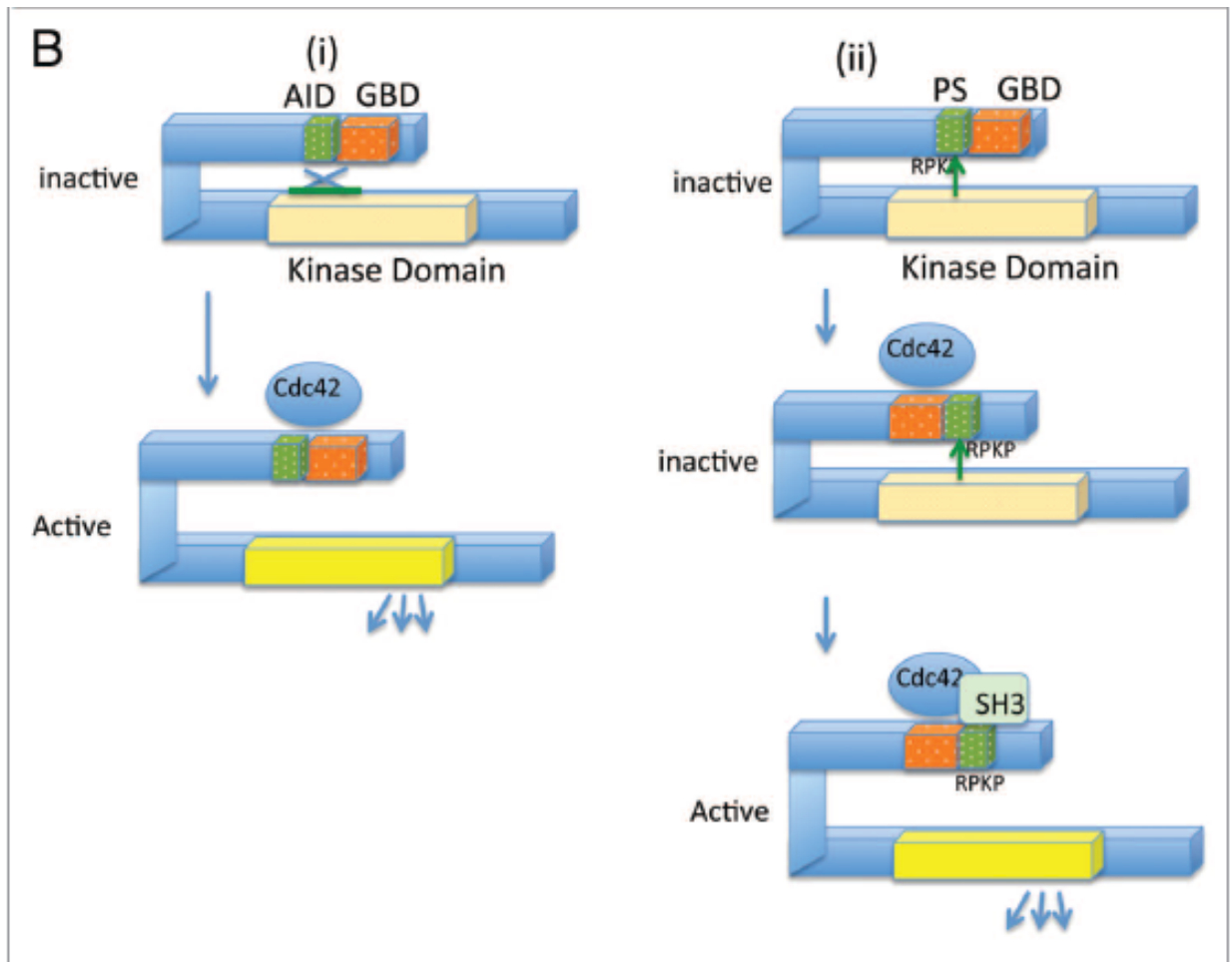


Figure 2: Models representing the activation mechanism of the Group I and Group II PAKs. (A) Activation of the Group I PAKs: Inactivated Group I PAKs form dimers, where the AID of one PAK binds the kinase domain of the dimerizing PAK, and inactivates it. Binding to Cdc42 or Rac can relieve this inhibition, resulting in auto phosphorylation and kinase activation. (B) Two different models for regulation of the Group II PAKs: In the first model (i), the AID binds to the kinase domain of the monomeric PAK, in *cis*, resulting in an inactive conformation. Binding of Cdc42 relieves the inhibition and leads to PAK activation. Unlike the Group I PAKs, the Group II PAKs are constitutively phosphorylated, but the kinase takes on an active conformation upon Cdc42

binding. In the second model (ii), the auto inhibitory pseudosubstrate (PS) containing the sequence RPKP is recognized by the kinase domain. This interaction inhibits PAK kinase activity. Cdc42 binding relocalizes PAK within the cell, and proteins containing SH3 domains, such as Src, subsequently activate PAK by competing with the PAK kinase domain, for interacting with the pseudosubstrate domain [15].

3. PAK kinases and Cytoskeletal Organization:

The PAKs are effectors of Cdc42 and Rac, which are key cytoskeletal regulatory proteins; consequently, they play an important role in regulating the cytoskeleton by affecting cell shape, motility, and adhesion. The PAKs regulate cytoskeletal organization primarily through the regulation of polymerized actin structures, particularly the formation of filopodia and lamellipodia, but they can also act upon microtubule organization. Group II PAKs play an important role in the formation of filopodia in response to Cdc42 activation [8]. Activated PAK5 was shown to lead to cytoskeletal changes that are associated with neuronal structure. These include induction of filopodia and the formation of neurite like extensions in neuroblastoma cells [9]. Not surprisingly, many PAK substrates are known for their roles in cytoskeletal organization. Group I PAKs, for example, phosphorylate a serine residue on the regulatory myosin light chain (MLC) in neural cells [27-30]. Myosins, found in muscle cells, smooth muscle cells, and non-muscle cells, are important cytoskeletal regulatory proteins that interact with actin. Phosphorylation of MLC by PAK stabilizes polymerized actin, and in

neuronal cells it contributes to the regulation of dendritic spine formation [31]. Group A PAKs also phosphorylate the regulatory MLC of myosin VI, a nonconventional myosin involved in membrane trafficking and cell migration [32].

In other cell types, PAKs can in fact have an opposite effect and lead to decreased MLC phosphorylation, which is associated with stress fiber dissolution. PAK proteins lead to formation of polymerized actin structures such as lamellipodia and filopodia, but they also lead to the dissolution of stress fibers [7]. Stress fibers are polymerized actin structures that exert tension on the cell and are directly linked to focal adhesions. Dissolution of stress fibers thus leads to loss of focal adhesions [7]. In the case of PAK1 and PAK2, this was shown to be mediated by phosphorylation of Myosin Light Chain Kinase (MLCK) in fibroblasts, epithelial cells, and endothelial cells [33, 34]. Phosphorylation of MLCK decreases MLCK's kinase activity, leading to decreased MLC phosphorylation and subsequent stress fiber dissolution [33]. MLCK is not a direct substrate for PAK4, and yet PAK4 also causes stress fiber dissolution. In this case, PAK4 phosphorylates GefH1, which in turn inhibits Rho activation. Since Rho leads to stress fiber formation, its inhibition leads to stress fiber dissolution [35].

Another substrate for both Group I and Group II PAKs is LIM Kinase 1 (LIMK1) [36]. When it is phosphorylated, LIMK phosphorylates the actin depolymerization protein cofilin, thereby inhibiting actin depolymerization [37, 38]. Thus, phosphorylation of LIMK1 by PAK kinases represents another mechanism by which the PAKs can regulate the formation or stability of polymerized actin

structures such as filopodia and lamellipodia. In addition to direct phosphorylation of LIMK1, PAK4 also activates LIMK1 indirectly, via inhibition of SlingShot Phosphatase (SSH1), a LIM Kinase phosphatase [39].

Filamin A (FLNa) is another cytoskeletal regulatory protein that is targeted by the PAKs [40]. FLNa is an actin binding protein which connects actin filaments to the cell membrane. Phosphorylation of FLNa by PAK1 modulates actin stability. Interestingly, FLNa can also bind to the PAK1 GBD and stimulate PAK1 kinase activity, indicating the presence of a positive feedback loop [29, 40]. Another mechanism by which PAKs control actin-cytoskeletal organization is via its actions on the ARP 2/3 complex. The ARP 2/3 complex controls actin nucleation and branching. Phosphorylation of the p41-ARC subunit of ARP 2/3 by PAK1 stimulates the assembly of the complex at the cellular cortex of migrating cells. This plays an important role in constitutive and growth-factor induced cell motility [41].

In addition to their effects on the actin cytoskeleton, the PAKs can also affect microtubule organization. One outcome of this is the regulation of mitotic spindles. PAK1 regulates mitotic spindle function and organization by interacting with Tubulin Cofactor B (TCoB), a cofactor in the assembly of α/β -tubulin. It phosphorylates TCoB on Ser65 and Ser128 and co-localizes with TCoB on newly polymerized microtubules and centrosomes [41]. Phosphorylation of TCoB by PAK1 is essential for microtubule polymerization. Coordinated dysregulation of PAK1 and TCoB can promote multiple spindle formation, as seen in human breast tumors. PAK1 thus plays an important role in maintaining microtubule

dynamics, which is crucial for mitotic spindle function. Another mechanism by which PAK1 regulates mitotic spindle function during mitosis is by phosphorylating centrosome-located Polo-like kinase1 (PLK1) and Aurora-A kinase, two important mitotic regulators. The GIT-PIX complex activates PAK1 by recruiting it to the centrosome, which then phosphorylates PLK1 and Aurora-A kinase. PAK1 phosphorylates PLK1 at Ser49 and Aurora-A Kinase at Thr288 and Ser342 [42], thereby activating them. This is followed by colocalization of both PLK1 and Aurora-A Kinase on spindle poles, the central spindle, and the midbody. This is important for establishing a functional bipolar spindle [43]. Enhanced Aurora-A activity can result in abnormal mitotic spindle organization and anchorage- independent growth in human breast epithelial cells [44]. PAKs also play important roles in cell motility by regulating leading edge microtubule dynamics. Microtubules in the protruding edge of migrating cells exhibit decreased catastrophic frequency and increased net growth. PAK1 has been shown to phosphorylate stathmin at Ser16 *in vitro* in Hep-2 cells, resulting in its downregulation. Stathmin inhibits tubulin polymerization, consequently its downregulation results in enhanced cell motility [45]. PAK1 also phosphorylates GIT1, a GAP protein for the Arf GTPase, and this increases GIT1 binding to paxillin, a focal adhesion adaptor protein. This entire pathway is important for regulating focal adhesion turnover [42, 46].

Xenopus and mammalian PAK4 were shown to phosphorylate the GTPase Ran, which in turn regulates the assembly of Ran-dependent complexes on the mitotic spindle, pointing to a role for this complex in mitosis [47]. A

constitutively active PAK4 mutant leads to phosphorylation of p120 [48], a protein that plays an important role in regulating cell shape and adhesion and also in anchorage independent cell growth [49], an important hallmark of cancer. PAK4 also phosphorylates the cytoplasmic tail of β -5 integrin and this has important implications in cell adhesion and migration [50].

4. PAK signaling in cancer

PAK kinases are important signaling proteins involved in key cellular functions including cell proliferation, cell migration and cytoskeletal organization. Neither the Group I or Group II PAKs are frequently mutated in human cancers, however their dysregulated expression is frequently associated with cancer. PAK1 and PAK4 are the PAKs most strongly associated with cancer. Both PAK1 and PAK4 genes are found on chromosomal regions that are frequently amplified in cancer [51].

4.1 Wnt/ β -catenin signaling pathway:

Aberrant activation of the canonical Wnt/ β -catenin signaling pathway is associated with several cancers including colorectal tumors and hepatocellular carcinomas [52, 53]. The Wnt/ β -catenin pathways is activated upon binding of Wnt ligands to Frizzled Receptor (Fz or Fzd) and its co-receptor, low density lipoprotein receptor-related proteins (LRP5 and LRP6). In the absence of ligand binding, β -catenin is phosphorylated and targeted for proteosomal degradation [54] by a multi-protein complex, called as the “destruction complex” that includes

the scaffolding protein Axin, the tumor suppressor adenomatous polyposis coli gene product (APC), casein kinase 1 (CK1), and glycogen synthase (GSK3). Activation of Wnt signaling pathway prevents β -catenin phosphorylation and degradation, allowing it to accumulate and translocate to the nucleus [55], where it can interact with transcription factors of the T-cell factor (TCF) and lymphoid enhancer factor (LEF) families. This leads to activation of Wnt target genes such as c-myc and cyclin D1 [56], genes that are associated with increased cell proliferation and frequently linked to cancer. Thus, stabilization and activation of β -catenin pathway plays an important role in driving cellular processes that mediate tumorigenesis. In the cytoplasm, PAK4 phosphorylates β -catenin, which possibly prevents β -catenin from getting ubiquitinated and consequently targeted for proteosomal degradation. In the nucleus, PAK4 upregulates β -catenin protein expression and is associated with increased TCF/LEF transcriptional activity. This nucleo-plasmic shuttling of PAK4 is important in mediating β -catenin stability and activity, and hence PAK4 plays an important role in activating Wnt/ β -catenin signaling pathway. It is important to note that PAK4 phosphorylates the β -catenin C1 domain, at Ser675 [57], while CK1 and GSK3 mediated phosphorylation of β -catenin occurs on the N terminal region [54], suggesting that the site of phosphorylation is important in mediating β -catenin stability.

SETD6, belongs to a family of protein lysine methyltransferases (PKMTs) and plays an important role in epigenetic regulation as well as cellular signaling pathways through methylation of non-histone proteins [58, 59]. Recent studies have shown that SETD6 binds to and methylates PAK4 both *in vitro* and in cells

at chromatin, and this cross-talk is associated with the regulation of Wnt/ β -catenin pathway [60]. SETD6 binding to PAK4 at chromatin enhances the physical interaction between PAK4 and β -catenin and is responsible for increasing Wnt/ β -catenin transcriptional activity. PAK4 is also important for SETD6- β -catenin association, forming the three protein SETD6-PAK4- β -catenin complex at chromatin and this complex can enhance the transcription of several Wnt/ β -catenin genes.

4.2 Regulation of cell proliferation:

PAK kinases play an important role in regulating cell growth and oncogenic transformation. Anchorage independent growth is an important hallmark of oncogenic transformation. While normal adherent cells stop growing or die when not attached to a surface, cancer cells can survive and proliferate, resulting in anchorage independent growth, which often leads to metastasis.

PAK1 can promote oncogenic transformation by acting as a downstream effector of Ras signaling. This was mediated through activation of MAPK (mitogen-activated protein kinase) signaling pathways. It has also been reported that the observed synergy between Rho GTPase, Rac and Ras to activate ERK signaling requires activated PAK1 [61]. PAK1 was shown to be implicated in anchorage independent growth, by culturing cells in soft agar. PAK1 phosphorylates dynein light-chain 1 (DLC1) on Ser88, resulting in anchorage-independent growth of ZR75 breast cancer cells and making them highly tumorigenic [62].

PAK4 is a downstream effector of HGF receptor [63] and integrin [64] mediated signaling pathways that are involved in oncogenic transformation. PAK4 phosphorylates Raf1, activating the ERK signaling pathway and resulting in increased cell growth [65]. Our lab has also shown that PAK4 mediates TNF α signaling by regulating ERK and NF- κ B signaling pathways [66]. Our lab has found that activated PAK4 promotes anchorage independent growth in immortalized fibroblasts, as efficiently as Ras, a very strong oncogene. Consistent with this, dominant negative PAK4 partially inhibits focus formation in response to oncogenic Dbl in fibroblasts [7], and it also inhibits transformation by oncogenic Ras in some cells [12]. PAK4 null cells also have a decreased capacity to be transformed by Dbl [7] and oncogenic Ras [12].

4.3 Regulation of cell survival and apoptosis:

In response to a cell survival signal, PAK1 directly phosphorylates pro-apoptotic protein Bad at Ser112 and Ser136 [67]. This disrupts Bad interactions with Bcl-2 and Bcl-xL on the mitochondria, allowing them to suppress cell death by blocking the release of mitochondrial cytochrome c. In addition, PAK1 interacts with DLC1 [68], which sequesters the pro-apoptotic Bcl-2 family protein BimL. Unphosphorylated DLC1 interacts with BimL to form DLC1-BimL dimers which then interact with Bcl-2, thereby inhibiting cell survival functions of Bcl-2. However, in the presence of growth factors, PAK1 phosphorylates both DLC1 and BimL, thereby dissociating the DLC1-BimL dimers. Activated PAK1 also reduces BimL1 expression, consequently blocking the pro-apoptotic signal of

BimL [62]. In response to estrogen treatment, PAK1 can also inhibit apoptosis by phosphorylating and inactivating forkhead transcription factor (FKHR) [69].

PAK2, on the other hand, has dual functions and can regulate both cell survival and cell death pathways. Under cellular stress and serum starvation conditions, PAK2 is activated by cleaved caspase 3 to generate a proteolytic fragment, PAK2-p34, thereby promoting cell morphology changes characteristic of apoptosis [70], and promoting a cell death response. On the other hand, activation of full-length PAK2 can also promote cell survival by phosphorylating Bad and reducing the interaction between Bad and Bcl-2; resulting in cell survival [71].

PAK4, like PAK1, can protect cells from apoptosis by phosphorylating Bad, specifically at Ser112. When overexpressed, PAK4 is associated with protecting cells from apoptosis in response to TNF- α treatment, UV irradiation and serum withdrawal [72]. Along with activating anti-apoptotic signals, PAK4 can promote cell survival by inhibiting pro-apoptotic signals. Overexpressed PAK4 prevents apoptosis induced by a fusion of TNFR1 and the Fas receptor, by inhibiting the recruitment of caspase 8 to the complex II or DISC and its activation. It is important to note that PAK4 mediates this function through a kinase independent mechanism. Thus, PAK4 can block the caspase cascade acting upstream of the mitochondrial pathway and effector cascades, instead of preventing phosphorylation of Bad and cytochrome c release [73].

PAK5 and PAK1 can also prevent cell apoptosis via a pathway involving Raf and Bad [74, 75]. PAK1 and PAK5 both phosphorylate Raf on Ser338,

stimulating translocation of Raf1 to mitochondria. Phosphorylated Raf1 forms a complex with Bcl-2 proto-oncogene. This complex phosphorylates the pro-apoptotic protein Bad at Ser112, which prevents its binding to Bcl-2. This prevents the release of pro-apoptotic factors from the mitochondria, thereby preventing apoptosis [74, 76]. Another mechanism by which PAK1 prevents cell apoptosis is by stimulation of transcription factor NFkappaB, which can promote cell survival, cell proliferation and angiogenesis [77, 78] and which inhibits the pro-apoptotic factor FKHR [69]. PAK5 also inhibits camptothecin induced apoptosis in colorectal cancer cells, by inhibition of the caspase-8 signaling pathway [75].

4.4 Regulation of cell motility and invasiveness

Activated PAK4 promotes anchorage independent growth in immortalized fibroblasts [7, 12], an important hallmark of oncogenic transformation. Activated PAK4, in fact, has been shown to be as efficient as oncogenic Ras, an important oncogene, in promoting foci in soft agar [7]. Consistent with this effect, dominant negative PAK4 partially inhibits foci formation in response to Db1 in fibroblasts [7] and also inhibits oncogenic transformation by Ras in some cells [12]. As mentioned above, PAK4 can regulate cytoskeletal organization through phosphorylation of LIMK1. LIMK1 phosphorylation by PAK4 can be regulated by DGCR6L. DGCR6L binds to PAK4, and this interaction enhances LIMK phosphorylation, leading to increased migration of gastric cells [79].

Metastasis, one of the most challenging aspects of cancer treatment, is tightly linked with cell migration and invasiveness, and PAK4 has been shown to regulate metastasis. Overexpression of activated PAK4 in pancreatic ductal cells leads to increased migration and increased invasiveness *in vitro*. Conversely, blocking PAK4 reduces invasiveness in a pancreatic tumor cell line [80]. Similarly, PAK4 overexpression was shown to promote migration and invasiveness of choriocarcinoma cells, while inhibiting PAK4 had the opposite effect [81]. Reducing PAK4 protein levels in prostate cancer cells decreases cell migration and leads to reduced cell-adhesion turnover rates, indicating that PAK4 has a role in prostate cancer cell migration and adhesion.

4.4A Kinase-Independent regulation of cell adhesion:

Previous studies have shown that PAK4 regulation of cell adhesion and migration is dependent on its kinase activity, while there is limited information available on PAK4 kinase-independent functions in cell adhesion. Recent studies have shown that PAK4 can modulate cell adhesion by stabilizing RhoU, a Rho GTPase, and this function was independent of its kinase activity [82]. PAK4 binding to activated Cdc42 is known to be required for activation of its kinase activity. However, it was reported that a Cdc42 binding-deficient mutant, PAK4 (H19, 22L), was able to rescue adhesion of PAK4 knockdown MDA-MB-231 cells, suggesting that an interaction between Cdc42 and PAK4 is not required for regulating cell adhesion. The PAK4 (H19, 22L) mutant was capable of binding RhoU, an atypical Rho GTPase. However, this binding was not responsible for

activation of PAK4 kinase activity, as indicated by unchanged levels of auto phosphorylated PAK4 (Ser474). Furthermore, RhoU was not identified to be a substrate of PAK4 kinase activity. Interestingly, PAK4 depletion in MDA-MB-231 cells, resulted in reduced RhoU expression, while PAK4 overexpression was associated with upregulated RhoU protein expression, suggesting that PAK4 regulation of RhoU expression is independent of its kinase activity. Further studies identified that PAK4 regulates RhoU expression by preventing its ubiquitination and consequently from proteosomal degradation. More importantly, both the kinase dead, PAK4 (K350, 351M), and Cdc42 binding-deficient mutants PAK4 (H19, 22L), were equally competent as full-length PAK4 in preventing RhoU ubiquitination, confirming that PAK4-RhoU interaction is both crucial and sufficient for protecting RhoU from ubiquitination, but does not require PAK4 kinase activity. In addition, PAK4 (H19, 22L), PAK4 (K350, 351M) and RhoU were able to rescue the effects of PAK4 knockdown on cell motility in MDA-MB-231 cells, clearly suggesting that PAK4 can regulate cell adhesion and migration, upstream of RhoU, through its kinase-independent activity [82].

5. PAK expression in cancer

PAK1 is overexpressed in different types of tumors including breast, kidney, colon [41, 83]. Although, PAK1 kinase activity is found to be high in some tumors, most often, PAK1 is present in its wild-type form, without activating mutations. High PAK1 levels were seen in invasive prostate cancer cells as compared to the non-invasive ones. PAK1 was shown to play an important role in micro invasion

of the cells and is necessary for prostate tumor growth and micro metastasis. PAK1 stimulates invasiveness of prostate cancer cells through its actions on cytoskeletal network that enhance the directional migration of these cells. It also stimulates prostate tumor growth through enhanced expression of various tumor-promoting factors such as MMP9 and reduced expression of TGF β , a factor that inhibits tumor growth [84].

PAK1 DNA copy number, mRNA and protein levels are up regulated in human melanoma. However, dysregulated PAK1 expression had a negative correlation with BRAF mutation. While BRAF mutation is linked to a subset of melanoma, PAK1 was upregulated in a subset of melanoma that lacked BRAF mutation. This is significant because wild-type BRAF melanoma has no targeted therapy. Thus, targeted PAK1 inhibition can serve as a pharmacologically effective means of treating wild-type BRAF melanomas [85]. PAK1 is also associated with colon cancer. PAK1 mediates cell proliferation, cell survival and cell migration of colon cancer cells by regulating the Wnt, Erk and Akt pathways [86].

PAK4 is associated with different types of cancers [12, 87-90]. Occasionally, point mutations have been found in PAK4, for example in colorectal cancers [91], however, in most cases, overexpression of wild-type PAK4 is sufficient for oncogenesis. There are different mechanisms of PAK4 overexpression that can be linked to cancer. One such mechanism is PAK4 gene amplification. The PAK4 gene is located on a chromosomal region (19q13.2) that is often found to be amplified in cancer [92]. The PAK4 gene has been shown to be amplified in a

series of pancreatic cancer samples, including pancreatic ductal carcinomas [80, 93, 94] and squamous cell carcinomas [95]. PAK4 gene is also amplified in aggressive breast cancers with basal like features [92].

PAK4 mRNA and protein levels were shown to be elevated in a panel of 60 tumor cell lines, representing different types of cancers [12], while PAK4 protein levels are low in normal tissues. PAK4 is overexpressed in a subset of gastric tumors, and overexpression of PAK4 is associated with poor survival in patients with these types of tumors [89]. High PAK4 expression is also linked to liver cancer. PAK4 has been shown to be overexpressed and activated in hepatocellular cancer carcinomas (HCC) [89]. Studying microRNAs provided evidence for the role of PAK4 in liver cancer. The microRNA miR-199a/b-3p is highly expressed in liver, but consistently decreased in HCC. This microRNA, which has an anti-tumor effect in cells, inhibits PAK4 expression as well as downstream ERK activation [96], suggesting a strong link between PAK4 and liver cancer.

High PAK4 protein levels, with increase in phosphorylated PAK4 levels, are frequently associated with ovarian cancer. High PAK4 levels in ovarian tumors are frequently associated with metastasis, poor survival and reduced chemosensitivity. Reducing PAK4 levels in ovarian cancer cells inhibits cell proliferation, migration and invasion, and abrogates a number of cell growth signaling pathways and also blocks their ability to form tumors in mice. Conversely, PAK4 overexpression in ovarian cancer cells increases cell migration and cell invasion [90].

Recent studies have demonstrated that high PAK4 expression is associated with increased cell growth and survival of pancreatic cancer (PC) cells [97]. PAK4 plays an important role in promoting cell cycle progression and increased apoptosis resistance in PC cells, and this is mediated through PAK4 dependent activation of NF- κ B signaling. PAK4 phosphorylates and activates AKT/ERK pathways, which results in the increased nuclear accumulation and transcriptional activity of NF- κ B. On the other hand, PAK4 silencing in PC cells was associated with stabilization of I κ B α , which sequesters NF- κ B in to the cytoplasm and inhibits its activity [97]. Pancreatic cancer initiating cells/cancer stem cells (CSCs) have been reported to have high PAK4 expression levels as compared to non-CSCs [98]. shRNA mediated PAK4 knockdown in PC cells significantly reduced expression of several stemness-associated markers. Unlimited self-renewal ability to maintain an undifferentiated state and resistance to chemotherapy are key characteristics of CSCs, and high PAK4 expression in Pancreatic CSCs was associated with increased sphere-forming potential (representative of self-renewal ability) and chemoresistance. Signal transducer and activator of transcription 3 (STAT3), that plays an important role in proliferation and self-renewal of CSCs, had an increased nuclear level in PAK4 overexpressing PC cells, resulting in increased STAT3 transcriptional activity. Thus, PAK4 mediates stemcell-like phenotype of PC cells through activation of STAT3 signaling [98].

PAK4 mRNA and protein expression levels were found to be very high in Hepatocellular Carcinoma (HCC) cells [99]. IHC staining indicated a strong

cytoplasmic staining pattern for total PAK4 and a strong nuclear staining pattern for p-PAK4 (Ser474), suggesting that the activated PAK4 preferentially localizes to the nuclei of HCC cells. PAK4 overexpression was associated with venous association, liver invasion, poor tumor cell differentiation and the late pTMN stage, suggesting a more aggressive tumor behavior and a higher incidence of metastasis. Overexpression of PAK4 promoted cell migration and invasiveness of HCC cells, while shRNA mediated PAK4 knockdown significantly reduced cell migration. PAK4 was also shown to interact with and phosphorylate p53 on Ser215, in HCC cells. PAK4 mediated p53 phosphorylation resulted in significant reduction of p53 tumor suppressive, transactivating and DNA binding activities, with the nuclear localization of PAK4 augmenting p53 inhibition, suggesting that PAK4 is an inhibitory kinase of p53. PAK4 mediated p53 inhibition is likely to be responsible for protecting HCC cells from DNA damaging drug-induced cell death. Unphosphorylated p53 could suppress HCC cell invasiveness, but was abolished by PAK4 phosphorylation, suggesting that p53 mediated inhibition of cell migration is PAK4 and Ser215 phosphorylation dependent in HCC cells. A qRT-PCR analysis suggested that PAK4 mediated p53 phosphorylation resulted in significant down regulation of 3 metastasis suppressors, cadherin 6 (CDH6), cyclin-dependent kinase inhibitor 2A (CDKN2A), and kisspeptin-1 receptor (KISS1R), while levels of 2 metastasis enhancers, fibroblast growth factor receptor 4 (FGFR4) and vascular endothelial growth factor A (VEGFA), were up regulated [99].

PAK5 overexpression has been seen in some colorectal cancers, where PAK5 plays a role in invasiveness of colorectal cells [100]. PAK6 protein levels are elevated in some prostate and breast cancer cell lines [101], and PAK6 mRNA levels are also higher in some cancer cells [12]. PAK6 levels are elevated in prostate tumors that relapsed after androgen deprivation therapy, and it plays a role in motility and stress response of tumor cells [101]. Inhibition of PAK6, combined with irradiation, decreases survival of prostate cancer cells [102]. This indicates that PAK6 is linked to radiosensitivity in prostate cancer cells. However, the role of PAK6 is complicated in prostate cancer as it was also shown to inhibit prostate cancer growth via phosphorylation of androgen receptor and tumorigenic E3-ligase murine double minute-2 (Mdm-2) [103]. PAK6 was also identified as a gene that is hypermethylated in prostate cancer, which is often associated with suppression of tumorigenesis [104]. Thus, the exact role of PAK6 in prostate cancer needs to be fully clarified.

6. Breast Cancer:

Breast Cancer is the leading cause of death among women in most developed countries [105]. Breast cancer can be categorized into different subtypes based on different histological and biological features. Accurate grouping of breast cancers into clinically relevant subtypes is important for developing efficient therapeutic strategies.

Conventionally, immunohistochemistry (IHC) markers such as ER, PR and HER2 expression, along with clinical-pathologic variables including tumor size,

tumor grade and nodal involvement, are used for disease prognosis [106, 107]. High throughput platforms for gene expression analysis such as microarrays and RNA-seq, have helped in stratifying and categorizing breast cancer patients, to improve the accuracy of disease prognosis and clinical outcome [108-110]. Breast cancer can be broadly classified into five intrinsic subtypes with distinct clinical outcomes: Luminal A (ER+, PR+, HER2-, Ki67-), Luminal B (ER+, PR+, HER2-, Ki67+), HER2 *neu* positive, triple negative (ER-, PR-, HER2-), and normal-like tumors [111, 112]. Here is the detailed analysis of each subtype:

Luminal Tumors: Luminal-like tumors express hormone receptors, estrogen receptor (ER) and progesterone receptor (PR), with expression profiles similar to that of normal luminal breast epithelium [111]. These tumors commonly express luminal cytokeratins 8/18, ER and genes associated with ER activation such as *LIV1* and *CCND1* [111-113]. Luminal-like tumors can be further classified into two subtypes, luminal A and luminal B. Luminal A tumors exhibit overexpression of ER-regulated genes and a lower expression of HER2 gene cluster and proliferation-related genes. Luminal B tumors have a much lower expression of ER-related genes, a variable expression of HER2 gene cluster and a relatively higher expression of proliferation related genes [114]. Luminal-like tumors are the most common amongst breast cancer patients, with luminal A being the majority [115].

Luminal-like tumors have a good prognosis, and respond well to hormone therapy but poorly to cytotoxic chemotherapy [116]. Treatment response differs

between luminal subtypes. Luminal A tumors can be effectively treated with endocrine therapy, while luminal B tumors being more proliferative, may benefit from a combination of chemotherapy and hormone therapy [117]. Other targeted approaches have been shown to be effective against luminal-like tumors. For example, in 2012, the mTOR inhibitor, Everolimus, in combination with Exemestane was approved for treating ER+, HER2- advanced breast cancer that recurs on standard therapies [118]. Another drug, Palbociclib, a CDK 4/6 inhibitor, developed by Pfizer, is currently under Phase II clinical trials for the treatment of this breast cancer population [119].

HER2-enriched tumors: HER2 enriched tumors, identified by gene expression array, are similar to ER-/PR-/HER2+ group, identified by immunostaining or fluorescence in situ hybridization (FISH) [106]. However, HER2 overexpression tumors exhibit gene expression changes not exhibited by all HER2 positive tumors. For example, HER2 overexpression tumors are characterized by overexpression of HER2 amplicon genes such as GRB7 and PGAP3 [120], and 40-80% of these tumors also exhibit TP53 mutation [117].

Although HER2 overexpression tumors tend to exhibit a poor prognosis, they are more sensitive to anthracycline and taxane-based neoadjuvant chemotherapy than luminal breast tumors [113, 116]. The poor prognosis of this subtype seems to arise from a higher risk of early relapse due to partial eradication of tumor cells. HER2 monoclonal antibody, Trastuzumab, is a commonly used targeted treatment option for HER2 overexpression tumors.

However, mutations in the HER2 receptor combined with PTEN loss [121] and CXCR4 [116] up regulation, have been implicated in trastuzumab resistance. Consequently, combination therapies to inhibit multiple targets are utilized to improve clinical outcome of treatment for this subtype.

Triple Negative Breast Cancer: Triple negative breast cancer (TNBC) refers to tumors that lack expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor type 2 (HER2). Approximately 12 to 17% of women with breast cancer have TNBC, and as a group, patients with triple negative breast tumors have a poor prognosis and do not respond to endocrine therapies or therapies targeting HER2 receptor [122]. This reflects the growing need to devise efficient clinical therapeutic option for this disease. Although, a majority of triple negative breast cancers exhibit a basal-like phenotype (lacking ER and HER2 expression and gene expression pattern usually found in basal or myoepithelial cells in normal breast epithelium) and a majority of basal-like breast cancers are also triple negative [110], TNBC does encompass molecular features of other types of breast cancer. These include the claudin-low tumors, which comprise of cells that exhibit stem-cell like phenotype and can undergo epithelial-to mesenchymal transition (EMT); the interferon rich subgroup, which comprises of tumors that have a considerably better prognosis than that of triple negative tumors and the normal breast-like subgroup, that comprises of samples that contain a disproportionately high level of stromal and normal cells [110].

Triple negative and basal-like tumors account for about 15% of all invasive breast cancers, and usually comprise a high histologic grade [123, 124]. More than 75% of tumors arising from BRCA1 mutation, an important breast-cancer susceptibility gene, have a triple-negative like or basal-like phenotype or both [123, 124]. Triple-negative and basal-like breast cancers are characterized by a poor correlation between the size of the primary tumor and the probability of the survival [125, 126]. Patients with TNBC are associated with a relatively poorer prognosis as compared to those with other breast cancer subtypes due to an inherently aggressive clinical behavior, lack of available drug targets and a higher risk of relapse [127]. Follow up studies have also revealed a time-dependent survival profile for these tumors, with a very poor, early outlook diminishing after 5 years [122, 127]. Their rapid growth and frequent occurrence in young women can make mammographic detection difficult. Triple negative and basal-like breast cancers are more likely than other types of breast cancer to metastasize to viscera, particularly to the lungs and brain, as compared to the bone [128, 129]. Multiple studies have indicated that triple-negative and basal-like breast cancers are associated with a poor prognosis, due to lack of targeted therapy.

Thus, it seems that TNBC is not a single clinical entity, but a heterogeneous collection of different subtypes of breast cancers. Research has focused on identifying TNBC subtypes that are clinically relevant and illustrate the true degree of its molecular heterogeneity. Identifying novel biomarkers that drive this heterogeneous disease is extremely important to devise therapeutically relevant treatment regimens.

Treatment: Since TNBC patients do not benefit from endocrine therapy or targeted HER2 therapy (for example, Trastuzumab), chemotherapy is the current mainstay of treatment. Despite the lack of available biomarkers and poor prognosis, patients with TNBC have a higher response to chemotherapy than patients with other breast cancer subtypes. This is referred to as the TNBC paradox, because of its high risk of recurrence without any treatment but also a higher likelihood of benefit from treatment [130]. Despite the high response rate to systemic chemotherapy, almost all women with metastatic TNBC are likely to die of their disease [131]. Combination chemotherapy regimens have been utilized to improve response rates in TNBC patients, however this is usually accompanied with increased toxicity and minimal benefits in patient survival.

Platinum salts, including cisplatin and carboplatin, lead to DNA crosslink strand breaks, that result in apoptosis, specifically in cells unable to efficiently repair these lesions. A phase II study to evaluate platinum monotherapy reported moderate efficacy in metastatic TNBC patients [132]. Patients with mutations in BRCA 1/2 had a higher response rate than those with wild type BRCA 1/2. In the Triple Negative Breast Cancer Trial (TNT), patients with BRCA 1/2 mutations had a higher response rate to carboplatin treatment than docetaxel treatment, while there was no benefit observed in metastatic TNBC patients [133]. On the other hand, clinical studies have shown that patients with early stage TNBC, however, respond well to platinum monotherapy. In summary, the overall clinical efficacy of platinum-based agents in patients with metastatic TNBC has been modest, with a higher response rate observed in patients with BRCA 1/2 mutation; while there is

strong evidence to support the addition of platinum-based agents to standard taxane-anthracycline-based neoadjuvant regimens for patients with high-risk BRCA-mutated TNBC. However, there is limited clinical utility to this combination because of the added toxicities and an unclear effect on improvement in disease-free survival and overall survival [134].

Because of the heterogeneity of TNBC, research has been focused on identifying tumor-specific molecular biomarkers to improve TNBC targeted therapy. A majority of TNBCs that persist following chemotherapy have altered pathways. Consequently, inhibitors that target these pathways including, PARP inhibitors, PI3K inhibitors, MEK inhibitors, heat shock protein 90 (Hsp90) inhibitors and histone deacetylase (HDAC) inhibitors are currently under clinical investigation [135]. A subtype of TNBC, that overexpresses Androgen Receptor (AR), was shown to exhibit sensitivity to AR antagonists *in vitro* and *in vivo* [136]. Other molecular targets include EGFR, which is overexpressed in 2% of breast cancers and consequently, clinical trials with EGFR inhibitors such as cetuximab, have yielded positive results in TNBC patients [134]. Using the immune system as a biomarker can also be helpful to reduce the risk of tumor spreading or maintaining tumor dormancy [137].

Thus, a number of promising therapeutic options are under investigation for targeting TNBC treatment, and identifying novel biomarkers will help in improving treatment of this, so far, therapeutically elusive group of breast cancers.

7. PAK signaling in Breast Cancer

PAK1 is frequently overexpressed in breast cancer, and expression levels of endogenous PAK1 levels correlate with invasiveness and increased survival [41]. PAK4 is also frequently overexpressed in breast cancer [12, 87, 88] and is found in its wild-type form in most tumors [80, 93].

Transgenic mice that express a constitutively active PAK1 mutant (PAK1 T423E) in the mammary gland, form mammary tumors, but at a low penetrance and a long latency period, suggesting that other genetic events are required for this transformation [138]. Dominant negative PAK1 (PAK1 K299R) and a PAK1 inhibitor (PAK1 inhibitory domain: PID) lead to a significant reduction in tumors formed by the breast cancer cell line, MDA-MB-631, when injected in to the flanks of severe combined immune deficiency mice (SCID). These results indicate that PAK1 kinase activity is required for tumor formation in this breast cancer cell line [139].

The MCF10A progression cell line series, consisting of MCF10A, neoT, ATI and DCIS cells, is useful as an *in vitro* model of breast cancer. MCF10A cells represent normal breast epithelium [140] and the other cells are models for increasing level of oncogenic transformation [141-143]. When grown under 3D culture conditions, normal mammary epithelial cells form spherical acinar structures, while the transformed cells form disorganized acinar structures. Both PAK1 and PAK4 protein levels are elevated in the malignant cells of the series [144, 145]. Dominant negative PAK1 can partially reverse the abnormal morphologies of the malignant cells, and it also inhibits proliferation and

migration of all cells in the series. However, overexpression of exogenous, wild-type or activated PAK1 has no effect on cell growth, migration or acinar structures [145]. On the other hand, overexpression of PAK1 in MCF7 cells, a ER/PR + cell line, leads to abnormal centrosome number and abnormal spindle organization. This leads to aneuploidy, which can result in loss of tumor suppressor genes and accumulation of oncogenes [41].

HER2/Neu/ErbB2 is a growth factor receptor frequently overexpressed in breast cancer and studies have suggested an important role of PAK1 in HER2 + breast cancer. HER2 activation in MCF10A cells leads to leads to increased cell proliferation and a decrease in apoptosis, in 3D cultures, and this corresponded with increased PAK1 kinase activity. Expression of a constitutively active PAK1 mutant (PAK1 L107F) has similar effects, while inhibition of PAK1 kinase activity blocks the effects of HER2. An activated PAK1 mutant can bypass the requirement of HER2 activity for oncogenic transformation. Blocking PAK1 also inhibits the ability of HER2 positive breast cancer cell lines to form tumors in mice. PAK1 is activated in breast cancer cells that are estrogen receptor (ER) negative and that overexpress HER2 [139].

The mouse mammary epithelial cell line iMMEC has been used as model to study the role of PAK4 in breast cancer [146]. Under 3D culture conditions, iMMECs form spherical acini that resemble the acinar structures formed by normal breast epithelia [147]. Wild-Type iMMECs, similar to normal breast epithelial cells, have very low levels of PAK4. However, WT iMMECs transfected with wild-type PAK4, form disorganized acinar structures that are usually

associated with oncogenic transformation. The acini are abnormally large, and their lumens are never completely empty. They have a larger layer of epithelial cells surrounding the lumen, and the cells within the acini have higher levels of proliferation and decreased apoptosis [146]. Many of these abnormalities are characteristics of changes that occur during pre-cancerous conditions and early tumorigenesis. Features such as partial filling of luminal space with cells are reminiscent of atypical hyperplasia and DCIS [148]. When iMMECs transfected with wild-type PAK4 are implanted on to the mammary fat pads of mice, the mice develop mammary tumors at a high frequency [146], indicating that wild-type PAK4 can play a central role in mammary transformation. Oncogenes such as Ras and HER2 also cause oncogenic transformation in iMMECs [147, 149], interestingly, they result in upregulated PAK4 protein levels. These studies suggest that PAK4 can play an important role in driving mammary tumorigenesis.

In contrast to its role in carcinogenesis when overexpressed, PAK4 silencing using RNAi in the human triple negative breast cancer cell line MDA-MB-231 results in a significant reduction in cell proliferation and migration [150]. While cancer cells are less susceptible to apoptosis, PAK4 knockdown dramatically induces apoptosis in these cells. Most strikingly, when these PAK4 siRNA knockdown MDA-MB-231 cells are implanted in to the mammary fat pads of mice, tumor formation is significantly reduced. Additionally, the microRNA, mir-199a.b-3p, which is downregulated in several types of aggressive cancers, was found to directly target PAK4. mir-199a.b-3p can function as a tumor suppressor and specifically suppresses cell proliferation in breast cancer cells. It also alters

the cell cycle while reducing the migration and invasiveness of breast cancer cells, most likely due to its role in down-regulating PAK4 [151]. These data indicate that inhibiting PAK4 can restore many aspects of normal growth in breast cancer cells suggesting a central role for PAK4 in mammary cell transformation.

8. PAKs as drug target in cancer

8.1 Inhibitors of Group I PAKs

Among the PAK families, the rationale for targeting Group I PAKs has been established due to their regulatory role in multiple signaling pathways. The ATP-binding pocket of PAK1 has been extensively studied and hence has helped to design structure based inhibitors. Several approaches for designing Group I PAK inhibitors have been explored and some of them have been successful. These approaches are described below: To target the large ATP-binding pocket in PAK1, Meggers and co-workers designed octahedral ruthenium complexes and by screening through a focused library of 48 such ruthenium complexes, they identified an initial lead, GSK3/Pim1 inhibitor DW12 [152]. By adding bulkier substituents to this compound, they were able to enhance PAK1 selectivity and binding. However, no further information is available on the cell activity, efficacy or pharmacokinetics for this series of compounds, and their drug properties and toxicity concerns render the utility of this series of compounds questionable [153]. Several fine-design ATP competitive inhibitors have been identified that lock the

movements of the active kinase-site in Group I PAKs. Given the diversity and overlapping nature of PAK regulators and effectors, the specificity of some of these inhibitors pose critical problems, consequently resulting in the delayed use of these inhibitors in clinical studies. To circumvent the kinase selectivity issues arising from the conserved nature of the PAK1 catalytic pocket, Deacon et. al conducted a high throughput screen to identify non-ATP-like, uncompetitive allosteric PAK1 inhibitors [154]. Full-length PAK1 protein activated *in vitro* with recombinant Cdc42-GTP γ S was targeted by screening a library of 33,000 compounds, which led to identification of iPA-3. iPA-3 was shown to inhibit PAK1 only in its basal (inactive) state, but did not inhibit Group II PAKs, consistent with the absence of autoinhibitory domain in the latter. Because of selectively stabilizing the PAK1 autoinhibitory conformation, iPA-3 can only prevent PAK1-mediated auto-phosphorylation and activation. While iPA-3 did exhibit good Group I PAK specificity and cell permeability, its chemically and metabolically labile nature greatly limited its usefulness [153].

8.2 Inhibitors of Group II PAKs

Group II PAKs, although share certain sequence homology with Group I PAKs, are very different from Group I PAKs. PAK4 is structurally distinct from PAK1 in terms of its regulatory and catalytic domain. PAK4, unlike PAK1, exists as a monomer instead of a dimer, in its inactive state. While PAK1 is activated by a direct interaction with Rho GTPases, interaction with GTPases seems mainly a target function for PAK4, instead of being essential for its kinase activity. PAK4

also has different substrates of phosphorylation than that of PAK1. Most importantly, PAK4 has been shown to have both kinase-dependent and – independent functions in promoting tumorigenesis. Consequently, the mechanisms by which PAK1 inhibitors work might not be able to efficiently target PAK4. Taken together, structural and regulatory differences between PAK1 and PAK4 indicate that the strategy for development for PAK4 inhibitors should be different from that of PAK1 inhibitors.

The PAK4 gene has been shown to be amplified in a number of cancers including squamous cell carcinomas, pancreatic cancer, endometrioid and ovarian tumors. Our lab and other groups have observed PAK4 protein levels to be high in breast cancer cells and primary breast cancer tissue [12, 82, 88, 146, 155, 156] with the PAK4 gene being frequently amplified in basal like breast cancers [92]. PAK4 expression in cancers, with its key role in regulating cell growth, cell survival and cytoskeletal organization, makes it an attractive drug target. One of the first PAK inhibitors to be generated was PF-3758309, by Pfizer. High throughput screening of kinase focus library compounds helped identify PF-3758309, that demonstrated an excellent profile, leading to its selection as a clinical candidate. PF-3758309 is an ATP- competitive inhibitor of PAK4 kinase domain. PF-3758309 inhibited PAK4 dependent phosphorylation of its substrate GEFH1. It strongly inhibited cell proliferation, cell survival and anchorage independent growth of HCT116 cells. When it was tested on a panel of 92 tumor cell lines, half of them exhibited IC₅₀ values of less than 10 nM. PF-3758309 showed robust tumor inhibition in five of the seven models tested:

HCT116, A549, MDA-MB-231, M24met, and colo205. Although it was designed as a PAK4 inhibitor, it turned out to be broadly active against both Group I and Group II PAKs, and also other kinases, including AMPK (AMP-dependent kinase) and RSK (Ribosomal S6 kinase) [157, 158]. Human clinical trials were terminated due to undesirable PK characteristics of the drug (<1% bioavailability), adverse side effects, and consequent lack of tumor responses [159, 160]. A second PAK4 kinase inhibitor, LCH-779944, inhibits PAK4 kinase activity more modestly [161, 162]. It also displays inhibitory activity towards PAKs 1, 5 and 6 and reduces EGFR and c-Src phosphorylation. LCH-779944 reduces proliferation and invasion of gastric cancer cells *in vitro*, and reduces filopodia formation and cell elongation, but it has not been tested for *in vivo* studies.

A third compound, Compound 17, generated by Genentech, displays good biochemical potency and enhanced Group II selectivity. This is due to the presence of an open back pocket, which is more energetically accessible in PAK4 (and other group II PAKs) than it is in PAK1. Compound 17 reduces the viability of breast cancer cell lines and decreases tumor cell migration and invasion in two human triple negative breast cancer cell lines [163], and it enhanced tamoxifen sensitivity in MCF7 cells [155]. However, it has poor bioavailability due to poor permeability and/or high efflux [153]. Glaucarubinone, a natural product isolated from the seeds of the tree, *Simarouba glauca*, has been recently shown to demonstrate anti-cancer properties. Glaucarubinone was originally developed as an anti-malarial agent, but was shown to inhibit both PAK4 and PAK1 levels [164]. Glaucarubinone inhibited cell proliferation and

migration of human pancreatic cells *in vitro*, and reduced tumor growth *in vivo*. Glaucarubinone can also synergize with gemcitabine, to result in a more significant reduction of PAK4 and PAK1 protein levels and consequently, tumor growth inhibition *in vivo*. Being a natural agent, Glaucarubinone is likely to have pleiotropic effects which can complicate its development as a therapeutic agent. A research group recently identified a novel PAK4 inhibitor, KY-04031, using a high throughput screening. Unfortunately, KY-04031 had low PAK4 binding affinity and required high drug concentration to inhibit cell proliferation in a PAK4-dependent manner [165]. GL-1196 is another small molecule inhibitor that inhibits PAK4 kinase activity and suppresses cell proliferation through downregulation of PAK4/c-Src/EGFR/cyclin D1 pathway and CDK4/6 expression and inhibits invasiveness by blocking PAK4/LIMK1/cofilin pathway in human gastric cancer cells [166].

8.2A KPT inhibitors, KPT-8752 and KPT-9274:

KPT inhibitors were first identified as small molecules which are able to interact with PAK4. This interaction with PAK4 was first discovered by using the SILAC (stable-isotope labeling in cells) technique. This series of PAK4 inhibitors consists of several analogues, we studied KPT-8752 and KPT-9274, with KPT-9274 being our clinical candidate. These compounds are different from the other PAK4 inhibitors because they reduce steady state PAK protein levels, instead of inhibiting PAK4 kinase activity. They are most likely to function by binding specifically to and destabilizing PAK4 protein, however, the exact mechanism is

not yet understood. PAK4 protein kinase has been shown to promote tumor formation through both kinase dependent and independent mechanisms. For example, PAK4 can promote cell survival by a mechanism independent of its kinase activity [66, 72, 73, 82, 167, 168]. Likewise, other PAK family members have also been shown to have kinase independent functions. Kinase independent functions are critical to consider for any protein kinase that is to be used as a drug target. Drugs designed against protein kinases are sometimes not as successful as hoped, and over time cancer cells have developed resistance even towards initially successful drugs. We propose that one reason for this may be that kinase independent functions of the protein kinase remain intact. Consequently, drugs designed to inhibit PAK4 kinase activity alone will be insufficient in blocking its tumorigenic potential. Hence, we believe KPT inhibitors provide a novel and effective way of inhibiting PAK4 function in promoting tumor formation.

In addition to PAK4 inhibition, KPT-8752 and KPT-9274 were also shown to inhibit synthesis of NAD (nicotinamide adenine dinucleotide), by blocking activity of the enzyme NAMPT (nicotinamide phosphoribosyltransferase), a rate-limiting enzyme in a NAD biosynthesis salvage pathway [169]. NAD is an essential metabolite required for sustaining energy production (TCA cycle) and regulating cellular processes, including DNA repair (PARP), epigenetics (sirtuins) and cell signaling in rapidly proliferating cancer cells [170]. Inhibition of NAMPT results in a significant depletion of NAD, making NAMPT an attractive potential drug target. Thus, a dual inhibition of

PAK4 signaling and NAD biosynthesis has the potential for a successful therapeutic strategy in cancer. Recent studies have shown that KPT-9274 inhibits both PAK4 and NAD biosynthesis pathways and significantly reduces Renal Cell Cancer (RCC) tumor growth in a mouse xenograft model [169]. KPT-8752 and KPT-9274 were also shown to inhibit both PAK4 and NAMPT activity in breast cancer cell lines, however inhibition of NAMPT did not correlate with the ability of these compounds to block cell proliferation [171]. Thus, anti-tumorigenic effects of KPT inhibitors, KPT-8752 and KPT-9274, can be attributed to dual inhibition of PAK4 signaling and NAD biosynthesis, however more work will be required to determine which effects of the compound can be attributed to PAK4 and which can be attributed to NAMPT.

KPT-8752 and KPT-9274 have been shown to inhibit cell growth and cell survival of breast cancer cell lines. KPT-9274, the orally bioavailable KPT inhibitor, has been shown to inhibit tumor growth in mouse xenograft model of 3 human triple negative breast cancer cell lines [171]. Treatment with KPT-9274 was shown to inhibit cell proliferation, stem cell like phenotype and chemoresistance in Pancreatic ductal Adenocarcinoma (PDAC) cells, both *in vitro* and *in vivo* [172]. KPT-9274 was also shown to inhibit proliferation and clonogenic growth of oesophageal squamous cell carcinoma cells (OSCC) and suppress tumor growth and induce apoptosis in OSCC mouse xenografts growth [173]. These studies identify PAK4 as a novel and effective therapeutic target in different types of cancers and KPT-9274 was capable of specifically inhibiting PAK4 and PAK4 associated downstream targets, and significantly reduce tumor

growth in preclinical animal tumor models. This validates the use of KPT-9274, either alone or in combination with other chemotherapeutic agents, as a clinical therapeutic option for cancer therapy. KPT-9274 is currently in phase I clinical trial for patients with advanced solid malignancies and non-Hodgkin lymphoma (NHL; NCT02702492).

9. High Throughput mRNA sequencing:

High throughput mRNA sequencing (RNA-seq) is a powerful analytical tool that can be used to discover novel genes and transcripts and accurately quantify transcript expression in response to a specific stimulus. RNA-seq can reveal the full repertoire of alternative splice isoforms in a transcriptome and identify the rarest and most cell-specific transcripts. RNA-seq analysis generates enormous amounts of raw sequencing reads per sample, with the number of reads from a RNA transcript corresponding to that transcript's abundance. The resulting RNA-seq data can be analyzed using bioinformatics tools which provide a robust and efficient method of interpreting this data [174].

Recent studies have confirmed the utility of RNA sequencing to identify study gene expression pattern in different types of cancers. A research group has recently identified differential patterns of transcript isoform expression to stratify different subtypes of breast cancer [175]. Differential isoform expression pattern revealed by RNA-seq analysis was sufficient to distinguish ER+ and Triple Negative Breast Cancers (TNBC). Thus, RNA-seq data can be used to accurately distinguish subtypes of breast cancers and this is significant because

it helps identify subtype-specific therapeutic targets. Devising the most effective and targeted chemotherapy requires the accurate identification of breast cancer subtype and thus, RNA-seq can serve as a predictable tool to achieve this goal. A research group utilized RNA-seq to identify PAK4 as a potential drug target in Oesophageal Squamous Cell Carcinoma (OSCC) [173]. Super Enhancers (SE), a special group of enhancers, were found to be associated with a number of oncogenic transcripts that drive pathogenesis of OSCC. Treatment with a novel CDK7 inhibitor, THZ1, selectively inhibited SE-associated oncogenes in OSCC cells. RNA-seq analysis of the inhibited SE-associated transcripts, identified novel oncogenes that were most actively expressed in OSCC cells, with PAK4 being one of the identified genes. Targeting PAK4 using a novel PAK4 inhibitor, KPT-9274, significantly reduced cell proliferation and tumor growth of OSCC cells, validating RNA-seq data that PAK4 can serve as a novel and potential drug target in OSCC.

Thus, High Throughput mRNA sequencing is a powerful and useful analytical tool that can be used to reveal a gene expression signature specific to a pathological condition. RNA-seq data can also help uncover novel biomarkers that can serve as potential therapeutic targets for cancer therapy, and help devise a more effective and targeted approach for chemotherapy.

9. Concluding Remarks.

PAK4 plays an important role in regulating cell growth, cell survival and cytoskeletal organization; aberrant signaling in these cellular functions are often associated with cancer growth and progression [176]. PAK4 is frequently overexpressed in different types of cancers, while its levels are low in normal tissue. This expression pattern makes it an attractive drug target. Consequently, PAK4 inhibitors have been developed in the past, but their use has been limited. This is attributed to the fact that PAK4 has both kinase-dependent and-independent functions. In this thesis, we describe a novel PAK4 inhibitor, KPT-9274, that functions by reducing PAK4 protein levels, and can inhibit tumor growth in mouse xenograft models of human triple negative breast cancer cell lines. This work shows for the first time that PAK4 can serve as a novel drug target in triple negative breast cancer therapy and KPT-9274 can have clinical applications for the triple negative breast cancer population.

To get a better understanding of PAK4 signaling pathway in breast cancer, we ran a Next Generation Sequencing of RNA samples collected from non-transformed iMMECs (WT iMMECs) and iMMECs overexpressing PAK4. We were able to identify a novel gene expression pattern and predict gene regulatory networks impacted by PAK4 overexpression. This study reveals the PAK4 transcriptome profile in mammary tumor forming cells, and is an important step towards delineating PAK4 signaling pathway in breast cancer. This study will help reveal novel biomarkers in breast cancer, and identify potential therapeutic targets for breast cancer therapy.

Chapter II

Therapeutically targeting PAK4 for tripe negative breast cancer treatment

Abstract

Breast cancer is a heterogeneous disease consisting of several subtypes. Among these subtypes, triple negative breast cancer is particularly difficult to treat. This is due to a lack of understanding of mechanisms behind the disease, and consequently a lack of druggable targets. PAK4 is a signaling protein with key roles in cell proliferation, cell survival and cell morphology. PAK4 protein levels are high in breast cancer cells and breast tumors, and the gene is often amplified in basal like breast cancers, which are frequently triple negative. Overexpression of PAK4 is sufficient to cause oncogenic transformation of non-transformed mouse mammary epithelial cells, while siRNA knockdown of PAK4 in a human triple negative breast cancer cell line blocks its tumorigenic potential. This makes PAK4 a promising drug target. Inhibition of PAK4, however, is complicated because of its kinase-dependent and -independent functions in promoting tumorigenesis. Thus, drugs that are designed to inhibit PAK4 kinase activity alone might be insufficient in blocking its tumorigenic potential. A new family of PAK4 inhibitors that include the structural analogues, KPT-8752 and KPT-9274, inhibit PAK4 by a novel mechanism: reducing steady state PAK4 protein levels in the cell. We have found that KPT inhibitors inhibit cell growth and cell migration and promote apoptosis in breast cancer cells, most notably in triple negative breast cancer cells. Most importantly, oral administration of KPT-9274 significantly reduced tumor growth in mouse xenograft models of three human triple negative breast cancer cell lines. Our results show that KPT inhibitors hold promise for inhibiting the growth of breast cancer cells, both in

vitro and in vivo, and KPT-9274 can serve as a novel therapeutic option for triple negative breast cancer therapy.

Introduction

Breast cancer is a heterogeneous disease and can be classified into at least 5 subtypes: (1) luminal A (usually ER and/or PR+, HER2-, low Ki67), (2) luminal B (usually ER+ and/or PR+, HER2+, or HER2- with high Ki67), (3) HER2 positive, (4) basal like, and (5) normal breast like [111] [177]. These 5 sub-divisions can be even further subdivided. Most basal like tumors are triple negative (ER, PR and HER2 negative) [178] and most triple negative tumors display basal like phenotype. Triple negative cancers are difficult to treat due to lack of available biomarkers and poor prognosis; hence new effective treatment for this breast cancer subtype is urgently needed. The mechanism behind triple negative breast cancer continues to be poorly understood, and hence the identification of novel biomarkers is essential to provide effective druggable targets and improve clinical therapy. PAK4 inhibition is significant because of the important links that have been found between PAK4 and different types of cancer, including breast cancer. DNA analysis has revealed that the chromosomal region containing the gene for PAK4 protein kinase (19q13.2) is frequently amplified in basal like breast cancer [92], raising the possibility that PAK4 may have an important role in breast cancer. Our lab along with others have found PAK4 protein and mRNA levels to be high in a number of breast cancer cell lines, as well as rat and human mammary tumor samples [12, 82, 88, 146, 155, 156, 179]. Furthermore, in a

study of 80 breast cancer patients with different stages of disease, PAK4 levels were shown to increase as breast tumors progressed, and the highest expression was associated with advanced stage disease [156]. In another study of 93 invasive breast carcinoma patients, high PAK4 levels were associated with advanced stage cancer, large tumor size, lymph node metastasis and poor survival [179]. In another panel of 300 human breast cancers, PAK4 protein was highly expressed in the more severe grade carcinomas [82].

Previous research in our lab has shown that when PAK4 is stably overexpressed in wild-type mouse mammary epithelial cells (iMMECs), these cells undergo oncogenic transformation and form tumors when transplanted in to mammary fat pads of mice [146]. While PAK4 overexpression leads to transformation and tumorigenesis in mice, PAK4 knockdown using siRNA resulted in a strong inhibition of tumorigenic potential of the human triple negative breast cancer cell line, MDA-MB-231 [150]. Additionally, the microRNA, mir-199a.b-3p, which is down-regulated in several types of aggressive cancer, was found to directly target PAK4. mir-199a.b-3p can function as a tumor suppressor and specifically suppresses cell proliferation in breast cancer cells. It also alters the cell cycle while reducing the migratory and invasive activity of breast cancer cells, most likely due to its role in down-regulating PAK4 [151]. These data indicate a central role for PAK4 in mammary cell transformation and make it a potential therapeutic target.

PAK4 is overexpressed in breast cancer tissue, while its levels are very low in normal mammary tissue. PAK4 has several cellular functions that may explain

its frequent link to cancer. It has been shown to have key roles in promoting cell survival and proliferation [66, 72, 73], prolonged activation of the ERK/MAP Kinase pathway [146], and regulation of cytoskeletal changes [8], all of which are frequently linked to cancer. Because of the link to cancer, there has been considerable interest in generating PAK inhibitors. PF-3758309 was one of the first PAK4 inhibitors to be developed by Pfizer and undergo clinical trial. Although designed as a PAK4 inhibitor, it inhibited both Group I and Group II PAKs, along with other kinases [157, 158]. Clinical trials were terminated due to undesirable PK characteristics of the drug, adverse side effects and consequent lack of tumor responses [159, 160]. Another PAK4 inhibitor was developed by Genentech, referred to as Compound 17, which inhibited PAK4 kinase activity, and was shown to inhibit cell growth and invasiveness of two human triple negative breast cancer cell lines [163]. However, it has poor bioavailability, likely due to poor permeability and/or high efflux [153]. GL-1196 is another small molecule that inhibits PAK4 kinase activity, and suppresses the invasive capability of gastric cancer cells [179].

Karyopharm Therapeutics has developed a new family of PAK4 inhibitors that include two structural analogs: KPT-8752 and KPT-9274. KPT inhibitors function differently from other PAK4 inhibitors in that they reduce the steady state level of PAK4 protein in cells. This mechanism is important because PAK4, like other PAK family members, has kinase-independent functions in promoting tumorigenesis [72, 73, 82, 167-169]. For this reason, inhibitors that reduce PAK4

protein levels and not just inhibit its kinase activity are needed to more efficiently block PAK4 function in cancer.

The goal of this study is to validate the use of PAK4 protein kinase as a drug target in breast cancer therapy. In this study, we have found that treatment with KPT-8752 and KPT-9274 inhibit cell growth, cell survival and cell migration in several breast cancer cell lines. After examining several subtypes of breast cancer cell lines, we were encouraged to observe that KPT treatment was most effective against triple negative cancer cells. Most importantly, oral administration of KPT-9274 significantly inhibited tumorigenesis in mouse xenograft models of human triple negative breast cancer cell lines. These results are significant because triple negative breast cancer therapy is less responsive to many of the current therapies and there is an urgent need to devise more effective therapies. Since KPT-9274 is in a Phase 1 human clinical trial of patients with advanced solid malignancies (NCT02702492), this data has practical applications to the breast cancer population.

Materials and Methods

Reagents and Cell culture: KPT-9274 and KPT-8752 from Karyopharm Therapeutics Inc (Newton, MA) were dissolved in dimethyl sulfoxide (DMSO). MCF7, MDA-MB-231 and SkBr-3 cells were maintained in DMEM/F-12 medium supplemented with 10% FBS serum and 1% penicillin/streptomycin. SUM159 cells were maintained in Ham's F12 medium supplemented with 5% FBS; MDA-MB-468 cells were maintained in RPMI medium supplemented with 10% FBS

serum and 1% penicillin/streptomycin. BT-474 cells were maintained in DMEM medium supplemented with 10% FBS serum, 1% penicillin/streptomycin and 1% glutamine. iMMECs were maintained in Hams F-12 medium supplemented by 10% FBS, 1% penicillin/streptomycin and other supplements. NIH3T3 cells were maintained in DMEM medium supplemented with 10% Bovine Calf Serum, 1% penicillin/streptomycin and 1% glutamine. All cells were maintained at 37°C and 5% CO₂.

SILAC and identification of the KPT inhibitors: KPT-7523 was immobilized on a resin using amino-coupling to a poly-ethylene glycol (PEG) linker. This method is described in more detail in [180, 181]. The PEG linker was used to create space and flexibility between compound and resin. MS-751 cells (cervical cancer) were labeled with heavy/light arginine and lysine for at least 6 doublings. MS-751 cells were used because they are sensitive to KPT-7523 in vitro (MTT assay IC₅₀ = 30 nM). Labeled cells were lysed in modified RIPA buffer and treated with DMSO or excess KPT-7523 for 2 hours. The pre-treated lysates were then incubated and rotated with KPT-7523-resin overnight at 4°C to pull-down interacting proteins. The next day, light and heavy resins were washed then mixed in equal proportions. The resin samples were boiled and the purified proteins were run on SDS-PAGE. Proteins were cut from the gel, trypsin digested then identified through Mass Spectroscopy. KPT-7523 interacting proteins were identified as those having a heavy/light ratio with >2 fold enrichment. PAK4 was identified as the strongest interactor with ~32-fold

enrichment across 4 different replicates. A similar SILAC experiment in U-2 OS cells ($IC_{50} = 20 \text{ nM}$) confirmed these results. Follow-up biophysical assays (isothermal titration calorimetry, surface plasmon resonance and x-ray crystallography) confirmed the interaction between PAK4 and KPT-7523 (data not shown). Using exogenous, endogenous, and purified protein from cells, KPT-7523 showed interaction to PAK4 and not PAK5 or PAK6. There was no interaction with group I PAK proteins. The interaction between PAK4 and KPT-7523 did not change the kinase activity of PAK4, as assessed by autophosphorylation and phosphorylation of Histone H-4. KPT-8752 and KPT-9274 are structural analogues of KPT-7523 with better optimized ADME/PK properties. The interaction between KPT inhibitors disrupts steady state levels of PAK4 in cell lines and reduces overall PAK4 activity. Ultimately, PAK4 downstream signaling is modulated by treatment with KPT inhibitors.

Western Blot analysis: Cell lysates (25 μg) were resolved by SDS-PAGE and transferred to PVDF membrane. The membrane was blocked in TBS/T containing 0.1% Tween-20 (TBS/T) and 5% non-fat milk for 1 h. After washing with TBS/T, the membrane was incubated with primary antibody in TBS/T containing 0.1% Tween-20 (TBS/T) and 5% BSA overnight. After washing three times with TBS/T, the membrane was probed with HRP conjugated secondary antibody for 1 h. After washing three times with TBS/T, the part of membrane corresponding in size to the bands of interested protein was excised, and the immunocomplexes were visualized by Luminata Western HRP substrates from

Millipore (Billerica, MA). Primary antibodies against PAK4, Cofilin, Phospho-cofilin (Ser3), β -Catenin and Phospho- β -Catenin (Ser675) and β -actin (Rabbit) and HRP-conjugated anti-rabbit antibodies were obtained from Cell Signaling Technologies (Danvers, MA, USA). Primary antibodies were diluted into TBS/T containing 5% bovine serum albumin at 1:1000. Secondary antibody was diluted into TBS/T containing 5% non-fat dry milk at 1:5000. The blots were analyzed either exposing the blots to X-Ray film, or by using the GeneGnome XRQ-NPC bioimaging system from SYNGENE (Cambridge, UK). This system utilizes a software GeneSys (Version 1.5) which automatically selects the right imaging conditions for each blot, backgrounds are adjusted as necessary, and the results are displayed digitally, without the use of X-Ray film. Quantitation of western blots were carried out using image J software. Protein is normalized to β -actin and results are plotted as percent of control, where the band intensity for control is set as 100% for each protein.

MTT assays: MDA-MB-231, MDA-MB-468, SUM159, MCF7, SkBr-3, BT-474, WT IMMEC and NIH3T3 were seeded into 96-well plates at 2000 cells/well. Cells were treated with KPT-9274 or KPT-8752 from Day 0 to Day 4. At each time point, 10 μ l of MTT-I solution (thiazolyl blue tetrazolium bromide, M2128, Sigma-Aldrich, St, Louis, MO) was added into each well and incubated for 5 h, followed by addition of 100 μ l of MTT-II solution (distilled water with 10% SDS and 0.01M HCl). The plate was then incubated overnight and the absorbance was measured with a spectrophotometer (Tecan US, Durham NC) at 560 nm.

Apoptosis assay: MDA-MB-231, MDA-MB-468, SUM159, MCF7, BT-474, SkBr-3 and WT iMMECs were incubated with 15 μ M of DMSO or 3 μ M KPT-8752 or 1 μ M KPT-9274 for 72 h. Apoptosis was assessed by staining with Annexin V and Propidium iodide. Annexin V is a membrane phosphatidylserine (PS) binding protein. It binds to the cells early in apoptosis, which is characterized by PS being flipped to face the outer membrane of the cells. Propidium iodide can enter the cell and bind to nucleic acid, but only after the membrane has begun to rupture, a characteristic of more advanced apoptosis. To assess binding by Annexin V and propidium iodide, cells were trypsinized into single cell suspension, counted, washed with 1X Annexin V binding buffer and stained with Annexin V and Propidium Iodide (BD Pharmingen FITC Annexin V Apoptosis Detection Kit II, BD Biosciences, Franklin Lakes, NJ). The cells (1×10^5) were incubated with Annexin V and Propidium iodide for 15 minutes in the dark at room temperature, then washed with 1X Annexin V binding buffer and analyzed by flow cytometry using a Gallios Cytometer (Applied Biosystems, Foster City, CA).

Scratch Assay: MDA-MB-231, MDA-MB-468, SUM159, MCF7 and SkBr-3 cells were plated at 100 000 cells per well in a 6 well plate and allowed to grow to confluence. The medium was aspirated and the monolayers were wounded by scratching with a sterile pipet tip. After washing with PBS, growth medium containing 10% fetal bovine serum was added. Phase contrast microscopic images were recorded at the indicated time points.

Animal Studies: All animals were approved by the Institutional Review Board for the Animal Care and Facilities Committee of Rutgers University. All methods were approved by the guidelines at Rutgers University, and methods were carried out according to the guidelines and regulations of the animal care and facilities committee at Rutgers. Female nude mice (5-6 weeks old, weighing 20-25 grams) were purchased from Charles River Laboratories (Wilmington, MA). They were allowed to acclimatize to the facilities for two weeks following which MDA-MB-231, MDA-MB-468 and SUM159 cells were injected subcutaneously in both the flanks of the mice, at 10⁶ cells per site, in a 100 μ l mixture containing Matrigel (BD Biosciences) and Hank's Buffer (Gibco) at a 1:1 ratio. Seven days post injection, mice were treated with placebo or KPT-9274 (100 mg/kg or 150 mg/kg) orally twice a day/ four days per week. Tumor size and total body weights were measured twice weekly. Tumors were measured with a vernier caliper, and tumor volume (V; mm³) was calculated using the equation $V = \frac{D \cdot d^2}{2}$ where D (mm) and d (mm) are the largest and smallest perpendicular diameters. After sacrificing the animals, tumors were excised, weighed, and snap frozen in liquid nitrogen for western blot analysis.

Statistical Analyses: Statistical analysis was done using a two-tailed t-test assuming unequal variance with error bars representing SD. * represents a P value of < 0.001 and is considered significant

Results

PAK4 protein levels are high in multiple breast cancer cell lines.

We assessed the steady state level of PAK4 protein in several breast cancer cell lines by western blot analysis. As observed in Figure 1, PAK4 levels are high in MDA-MB-468, SUM159, BT- 549 and MDA-MB-231 (all triple negative), MCF7 (ER+/PR+), SkBr-3 (HER2+), and BT-474 (PR+/HER2+) breast cancer cell lines. These results are consistent with previous results where the PAK4 levels were high in primary breast cancer tissue [82, 88, 156].

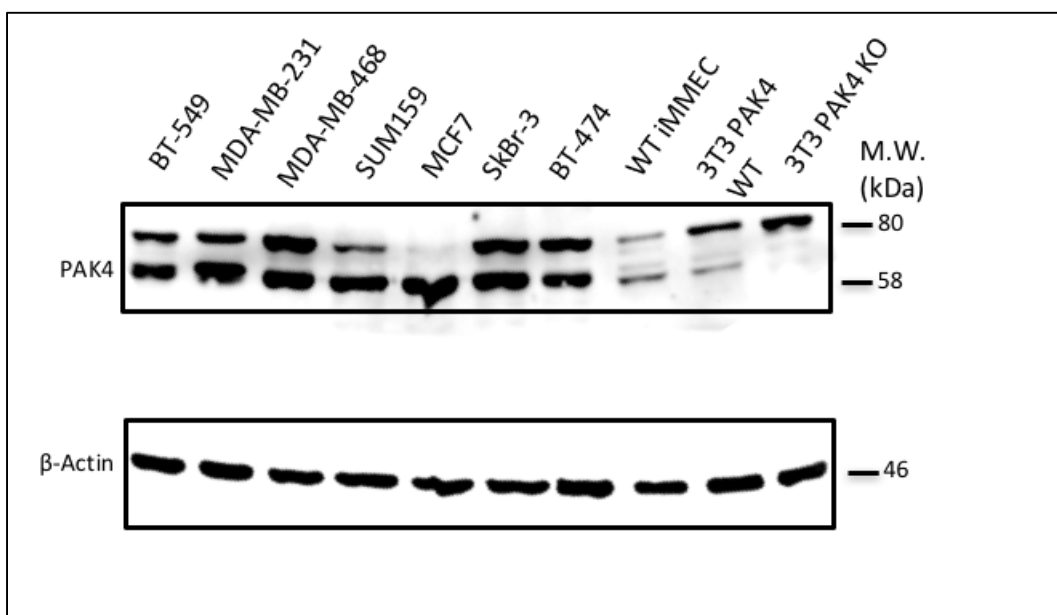


Figure 1: PAK4 is highly expressed in breast cancer cell lines.

PAK4 protein levels in seven breast cancer cell lines were assessed by western blot analysis. β -actin was used as a loading control. 3T3 PAK4 WT and 3T3 PAK4 KO are 3T3 cells isolated from wild-type and PAK4 knockout mice, respectively. Knockout cells are used here only for the accurate identification of the PAK4 band [171].

KPT-8752 and KPT-9274; a novel series of small molecules that can reduce cellular PAK4 protein levels and PAK4 associated signaling pathways.

A new family of compounds were identified as small molecules which are able to bind to PAK4 from cellular lysate. This interaction with PAK4 was first discovered by using the SILAC (stable-isotope labeling in cells) technique, as described in the Materials and Methods section (32), and the compounds are referred to as KPT inhibitors. Our group investigated two structural analogues in the PAM series; KPT-9274 and KPT-8752. The structures of KPT-8752 and KPT-9274 are shown in Figure 2A. KPT-8752 or KPT-9274 treatment of SUM159 cells, a triple negative breast cancer cell line, reduced PAK4 protein substantially after 72 h of treatment (see Figure 2B, C). In contrast, a previously published PAK4 inhibitor, compound 17 [163], which blocks PAK4 kinase activity, does not reduce PAK4 protein. In addition to PAK4, we also analyzed the level and phosphorylation status of several PAK4 downstream targets. Serine 675 of β -catenin was shown to be a direct phosphorylation substrate of PAK4 [57]. We found that phospho-S675- β -catenin was sharply reduced in response to either KPT-8752 or KPT-9274. The phosphorylation of Cofilin also occurs downstream to PAK4 signaling [6]. We found that KPT-8752 and KPT-9274 treatment of breast cancer cells reduced Phospho-Cofilin as expected. In addition to SUM159 cells, KPT-9274 also reduced PAK4 protein in two other triple negative breast cancer cell lines; MDA-MB-231 and MDA-MB-468 cells, which was most noticeable after 48 - 72 hours of treatment (Figure 2D). KPT-8752 had a similar effect in these cells (data not shown).

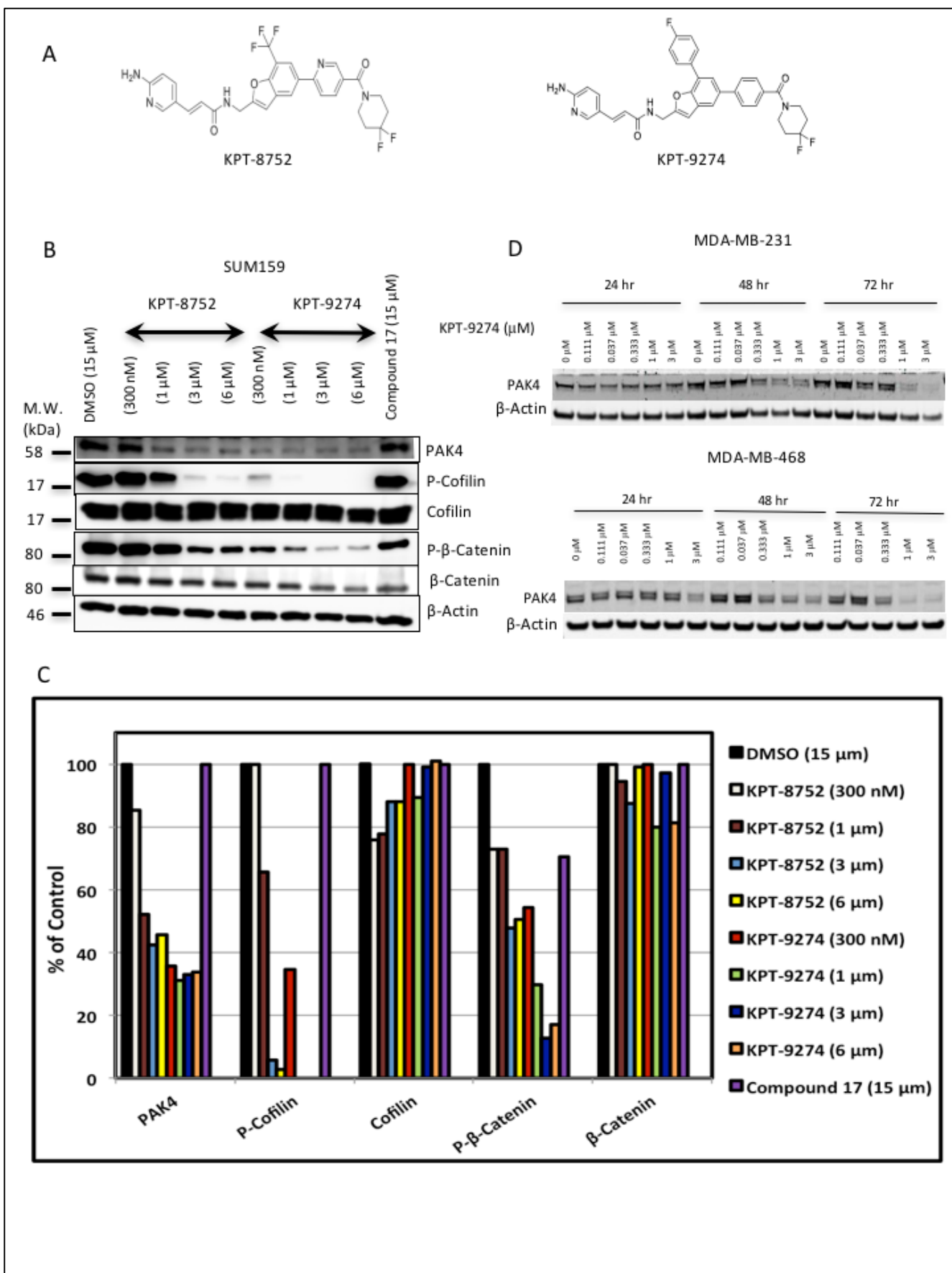


Figure 2: KPT-8752 and KPT-9274 reduce PAK4 protein levels and reduce the phosphorylation of PAK4 downstream targets.

A. Structures of KPT-8752 and KPT-9274. B. Western blot analysis of SUM159 cells treated with either KPT-8752, KPT-9274, or compound 17 (72 hr). Western blots were probed with anti PAK4 and anti PAK1 antibodies, and with antibodies against the PAK4 downstream targets β -catenin and Cofilin. β -actin was used as a loading control. C. The intensity of the bands in the western blot in panel B were quantitated using Image J software, and the bands were normalized to the β -actin control. Results are plotted as a percent of control, where the control represents the band intensity for DMSO, and set as 100% for each protein (PAK4, PAK1, Phospho-Cofilin, Cofilin, Phospho- β -Catenin and β -Catenin). This data was from a single experiment, where multiple doses of the inhibitor are represented. D. Western blot analysis of PAK4 levels in MDA-MB-231 cells and MDA-MB-468 cells treated with KPT-9274. (In the top panel of Figure 2B, an upper band that represents a non-specific band that reacts with the PAK4 antibody, is spliced out of the figure, in order to focus on the PAK4 band) [171].

KPT-8752 and KPT-9274 block cell growth in several human breast cancer cell lines. The effects of KPT-8752 and KPT-9274 on cell growth was analyzed by using a variation of an MTT cell proliferation assay. The assay is colorimetric and measures the change in the number of metabolically active cells over time as an indicator of cell proliferation (see Figure 3). Several breast cancer cell lines with high levels of PAK4 protein were plated in tissue culture cluster plates. They

were treated with different concentrations of KPT-8752 or KPT-9274 and incubated with MTT solution, and absorbance was measured at different time points as described in materials and methods. After KPT-8752 and KPT-9274 treatment, three of the cell lines, MDA-MB-231 (Figure 3A), MDA-MB-468 (Figure 3B), and SUM159 (Figure 3C), displayed significantly reduced colorimetric change, or viability, over time. For MDA-MB-231 cells, growth was almost completely inhibited in the presence of 3 μ M KPT-8752, or as little as 1 μ M KPT-9274. For MDA-MB-468 and SUM159 cells proliferation was completely inhibited with 1 μ M KPT-8752 or 300 nM KPT-9274. In contrast, the decrease in viability was less pronounced in MCF7 cells (an ER+/PR+ cell line, Figure 3D), and the inhibitory effect was even lower for SkBr-3 cells (HER2+, Figure 3E) and BT-474 cells (PR+/HER2+, Figure 3F). Treatment with KPT-8752 or KPT-9274 had no significant effect on cell viability of WT iMMEC or wild-type NIH-3T3 WT cells. It is of interest that the triple negative breast cancer cells (MDA-MB-468, MDA-MB-231, and SUM159) were the most responsive to treatment with these compounds, responding to even the lowest concentrations. These results are consistent with previous studies from our lab indicating that PAK4 knockdown with siRNA reduces proliferation of MDA-MB-231 cells [150].

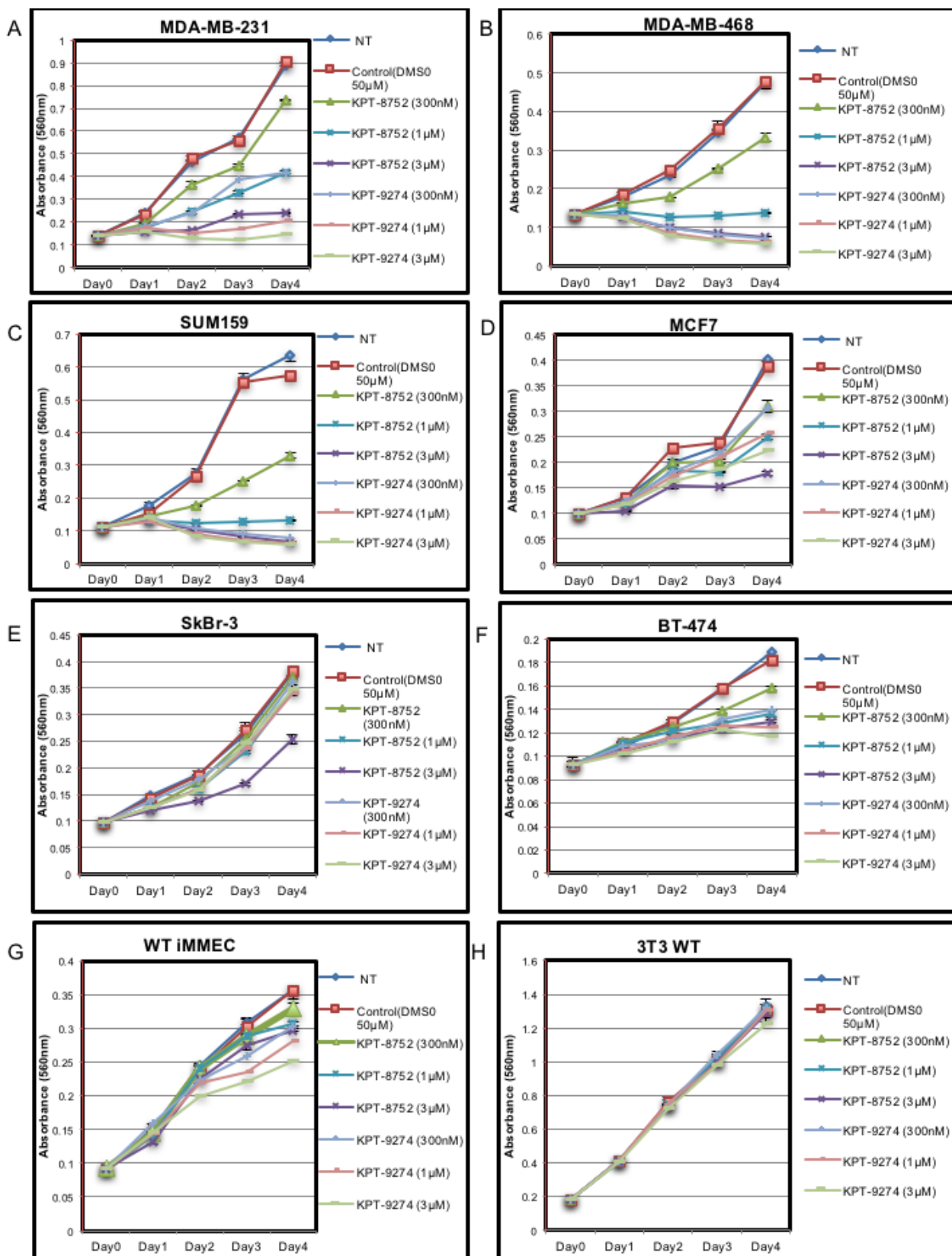
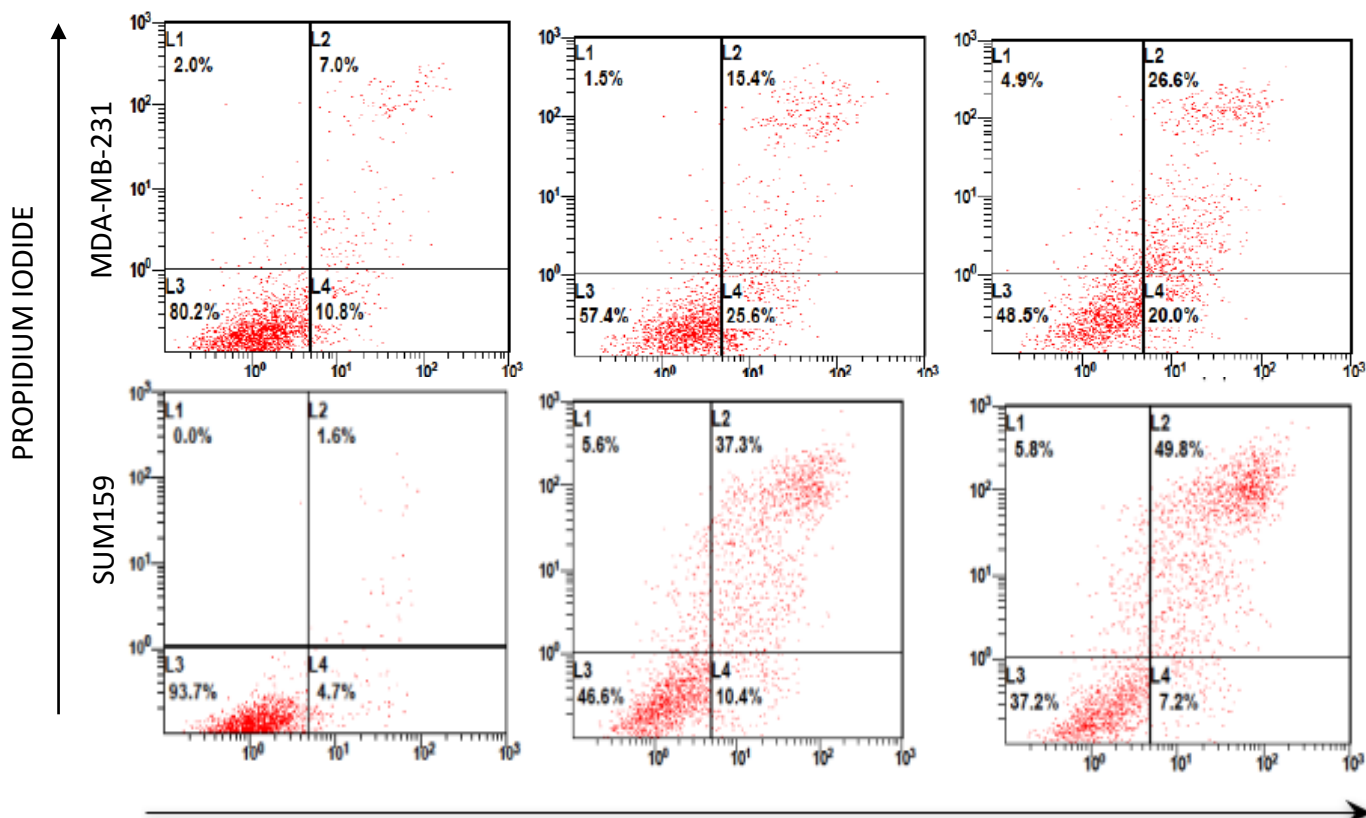


Figure 3: Treatment with KPT-8752 and KPT-9274 leads to a decrease in cell proliferation in several breast cancer cell lines.

Cells were plated overnight and treated with DMSO (control), KPT-8752 or KPT-9274 from Day 0 to Day 4. The MTT colorimetric assay was then conducted where the cells were incubated with MTT solutions at different time points. MTT absorbance curves were then analyzed in MDA-MB-231 (A) MDA-MB-468 (B) SUM159 (C) MCF7 (D) Sk-Br3 (E) BT-474 (F) WT iMMEC (control) (G) and NIH3T3 (control) cells (H). The results, presented as change in absorbance over time, correlate with the number of viable cells over time and can be considered as an indicator of cell proliferation. (Note, the amount of MTT-I absorbed by each cell type varies, and therefore the overall growth rates cannot be compared from one cell type to another). Error bars represent SEM. Data shown is representative of three separate repeat experiments [171].

KPT-8752 and KPT-9274 induce apoptosis in human breast cancer cell lines. Since previous studies indicate that PAK4 siRNA knockdown induces apoptosis in MDA-MB-231 cells ¹³, we next studied whether KPT-8752 or KPT-9274 could also affect the survival of additional breast cancer cell lines with various etiologies. The cells were treated with either vehicle control (DMSO), KPT-8752, or KPT-9274 for 72 hours, and apoptosis was measured by staining with Annexin V and propidium iodide (see Figure 4). The proportion of apoptotic cells corresponds to the number of Annexin V positive and propidium iodide positive cells (L2), and drug induced change in apoptosis is assessed by

quantitating the change in L2 in the treated versus untreated cells. MDA-MB-231, SUM159 and MDA-MB-468 cells (Figure 4A, B and C), (all triple negative), showed a strong increase in apoptosis after treatment with the inhibitors as indicated by high Annexin V and propidium iodide staining (L2). In MCF7 (ER+/PR+; Figure 4D). although some basal level of apoptosis was seen in the DMS treated cells, the increase in apoptosis after treatment with KPT-8752 or KPT-9274 was lower than what was observed for the SUM159 cells. For SkBr-3 (HER2+; Figure 4E) and BT-474 cells (PR+/HER2+; 4F), treatment with either compound resulted in almost no increase in apoptosis. WT iMECs (Figure 4G) had a basal level of apoptosis, but no increase in apoptosis was observed following KPT-8752 or KPT-9274 treatment. These results are consistent with the MTT assay results demonstrating that the triple negative cells showed the greatest response to inhibitor treatment.



PROPIDIUM IODIDE

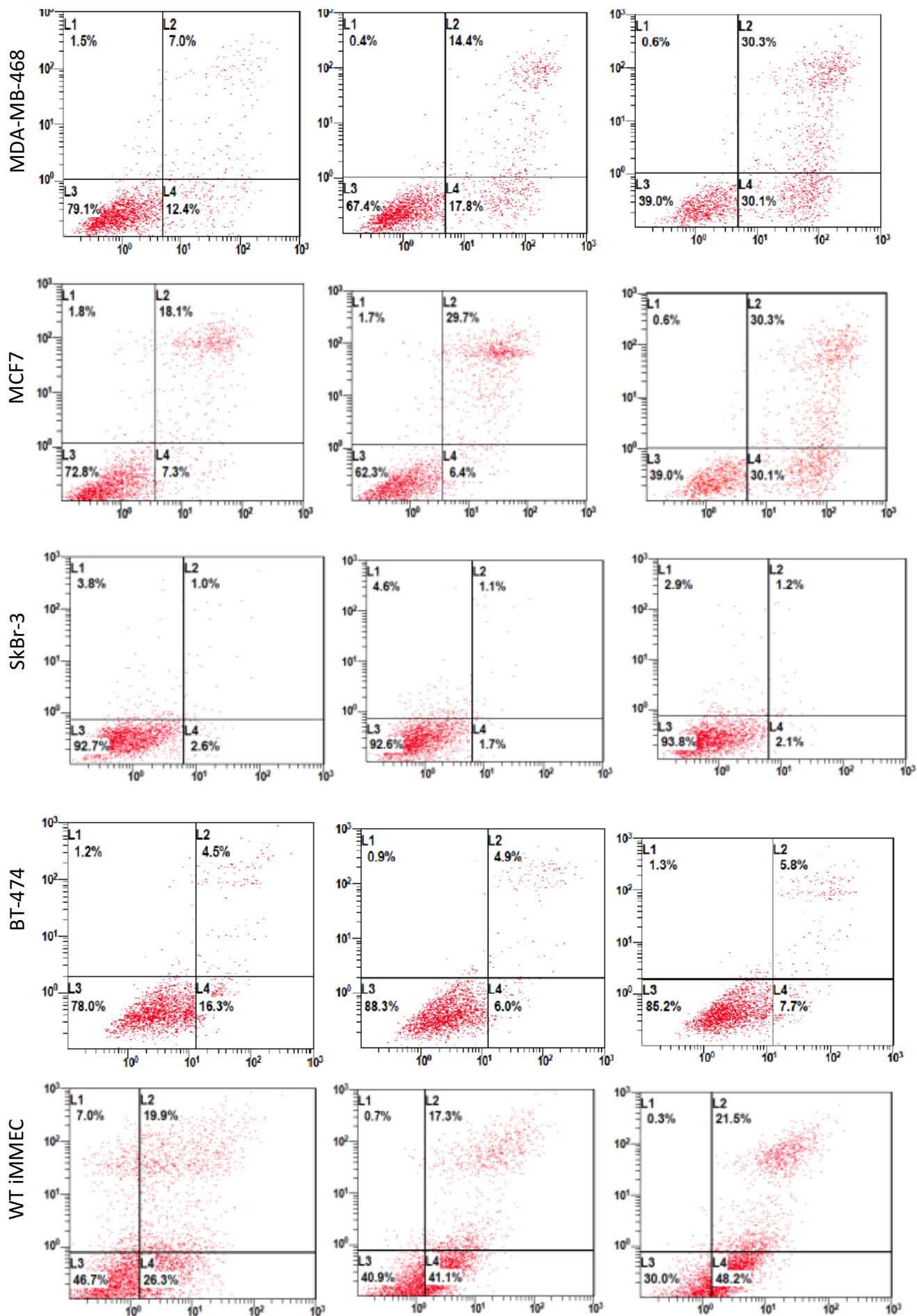
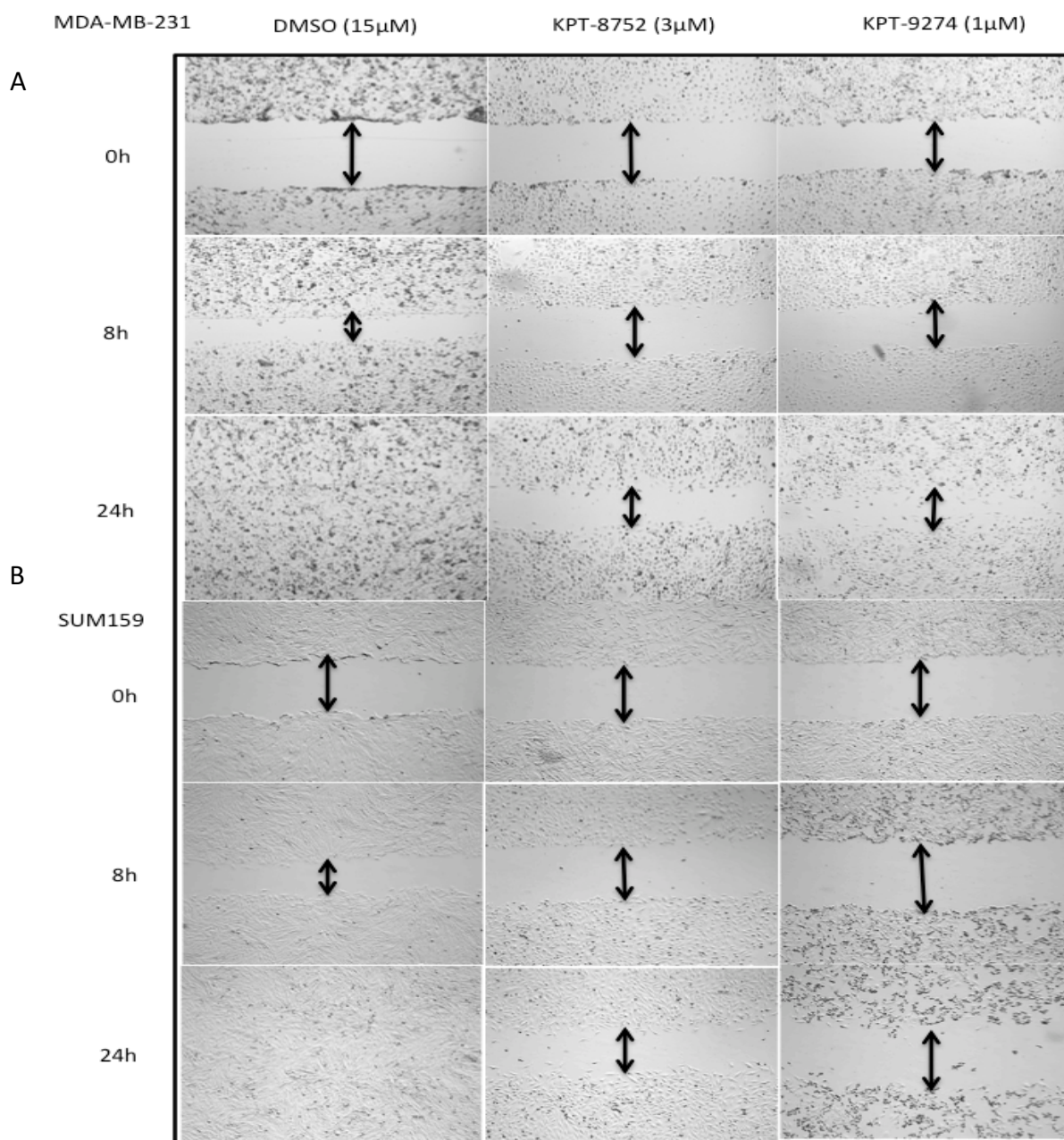


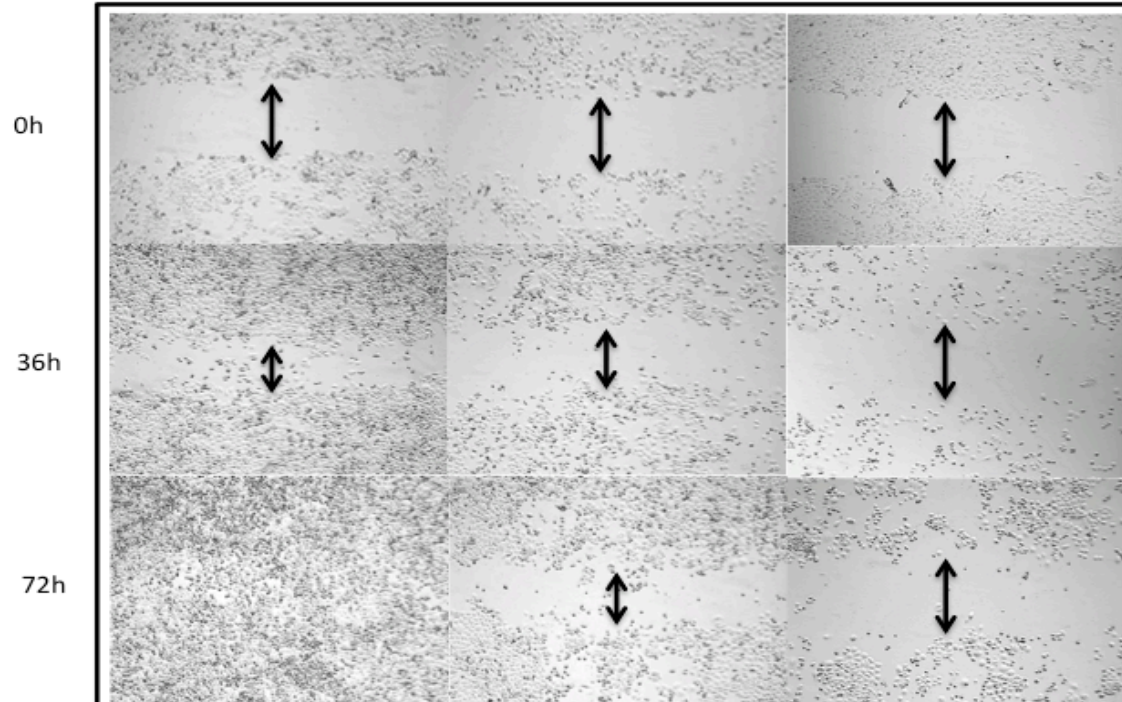
Figure 4: KPT-8752 and KPT-9274 cause an increase in cellular apoptosis in breast cancer cells. MDA-MB-231 (A), SUM159 (B) and MDA-MB-468 (triple negative) (C), MCF7 (ER+/PR+) (D), SkBr-3 (HER2+) (E), BT-474 (PR+/HER2+) (F) and WT iMMEC (control) cells (G) were treated with either (i) DMSO (15 μ M), (ii) KPT-8752 (3 μ M), or (iii) KPT-9274 (1 μ M) for 72 hours followed by staining for Annexin V / Propidium Iodide (PI). For all cell types, L3 represents the proportion of cells that have low intensity of Annexin V and PI staining and hence have low apoptotic activity, L4 represents the proportion of cells that stain more intensely for Annexin V indicating the early stages of apoptosis, and L2 represents cells that have high levels of Annexin V and PI representing highly apoptotic cells [171].

KPT inhibitors block cell migration of breast cancer cells: PAK4 has a functional role in regulating cytoskeletal organization, which is an important process during cell migration. This is significant because increased cellular migration is an important characteristic of cancer cell metastasis. We tested whether treatment with KPT inhibitors has any effect on motility of breast cancer cells using the in vitro scratch assay [182] (Figure 5). Cells were pre-treated with DMSO, KPT-8752 or KPT-9274 for 72 hours before wounding. Twentyfour hours post wounding, DMSO treated MDA-MB-231 (A), SUM159 (B) and MDA-MB- 468 cells (C) migrated completely into the wounded area, while the same cells treated with KPT-8752 or KPT-9274 had significantly reduced cell motility. MCF7 cells (Figure 5D) were less migratory and did not completely close the wound even

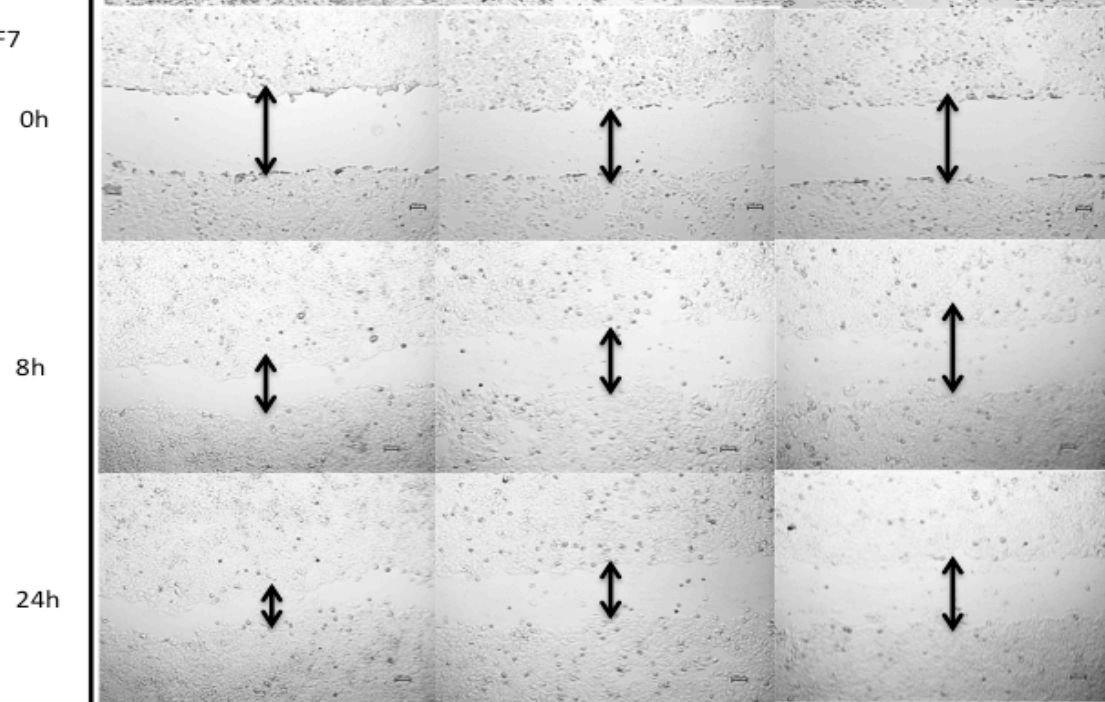
after 36 hours with no treatment. However, treatment with KPT-9274, did lead to some inhibition in migration by 36 hours post wounding. SkBr-3 cells were not nearly as migratory as the other cells, and consequently are not significantly affected by the KPT inhibitors (Figure 5E). These results indicate that KPT inhibitors can substantially block cell migration of the MDA-MB-231, MDA-MB-468 and SUM159 cells (triple negative), and that they have a partial inhibitory effect on MCF7 cells.



C MDA-MB-468 DMSO (15 μ M) KPT-8752 (3 μ M) KPT-9274 (1 μ M)



D MCF7



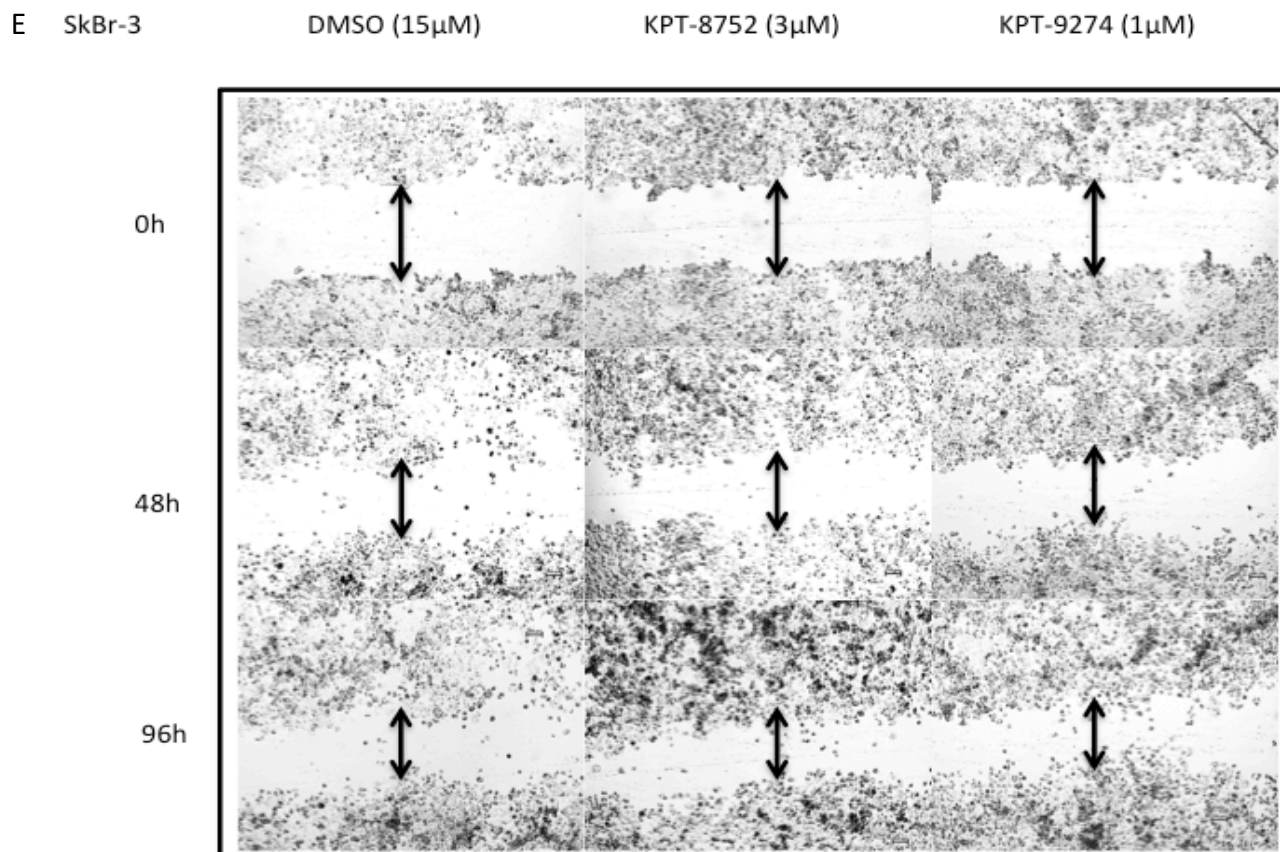


Figure 5: KPT inhibitors impair motility of breast cancer cells. Scratch assay was used to assess the effect of PAK4 inhibition on cell motility of MDA-MB-231 (A), SUM159 (B), MDA-MB-468 (C), MCF7 (D) and SkBr-3 cells (E). Confluent monolayers of cells treated with DMSO (control), or with KPT-8752 or KPT-9274 for 72 h and were scratched using sterile pipet tips. The cells were then allowed to recover in medium containing 10% fetal bovine serum. Phase contrast micrograph images were recorded at the indicated time-points after wounding to monitor migration of cells into the wounded area [171].

Oral administration of KPT-9274 blocks tumorigenesis in mouse xenograft

studies. The triple negative breast cancer cells MDA-MB-231, SUM159 and MDA-MB- 468 were the most responsive to treatment with KPT-9274 *in vitro* and represent a subtype of breast cancer that is in particular need of novel therapies. We therefore used these cell lines as a model to test the *in vivo* efficacy of the orally bioavailable clinical candidate KPT-9274 (Figure 6). MDA-MB-231, SUM159 and MDA-MB-468 cells were injected into the flanks of female nude mice. Seven days following tumor cell injection (when tumors were approximately 50 – 100 mm³) mice were administered KPT-9274 or placebo. Treatment continued twice per day for four days per week. Treatment with orally administered KPT-9274 resulted in a significant reduction in the tumor volumes in all three models of the treatment groups as compared to the control groups, and in the tumor weights, which were measured for MDA-MB-231 and SUM159 (see Figure 6 and Table 1). Treatment did not significantly affect the body weights of the mice (Figure 6C, 6F, and 6H).

Table 1

Cell line	No. of tumor (n)		Dosing Regimen	Average Tumor Volume (mm ³) (Endpoint)		Average Tumor Weight (mg)	
	KPT-9274	Placebo		KPT-9274	Placebo	KPT-9274	Placebo
MDA-MB-231	16	20	150 mg/kg PO bidx4	128 ± 21.7	515.1 ± 81.6	64.2 ± 9.6	300.2 ± 55.8
MDA-MB-468	8	7	100 mg/kg PO bidx4	398.7 ± 41	1053.8 ± 76	-	-
SUM159	10	10	150 mg/kg PO bidx4	187 ± 33.7	524.2 ± 90.2	160.8 ± 19	463.1 ± 81.1

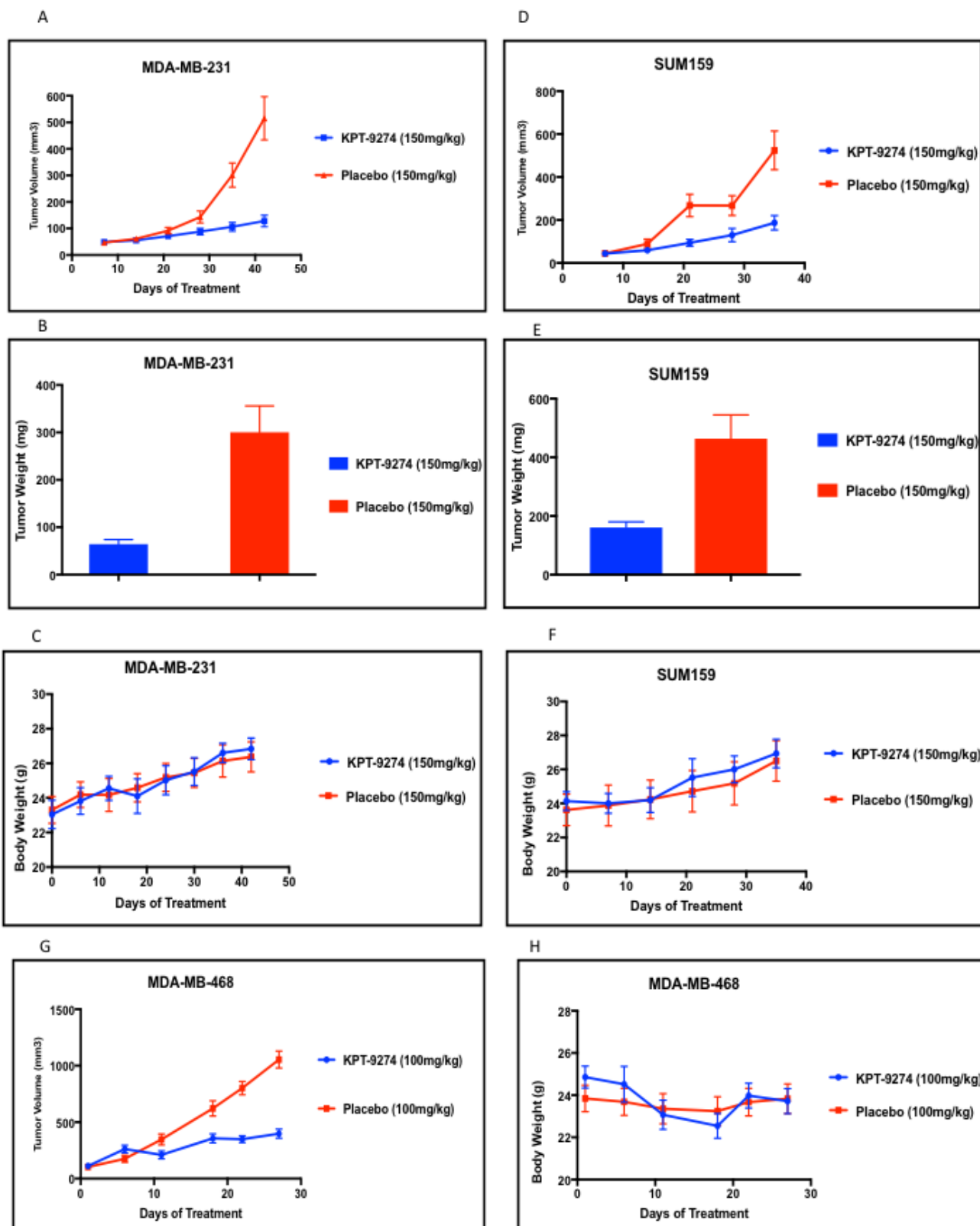


Figure 6: Oral administration of KPT-9274 blocks tumor growth in mice.

10^6 cells (MDA-MB-231 (A, B, C), SUM159 (D, E, F), or MDA-MB-468 (G, H), all of which are triple negative breast cancer cell lines, were injected into both flanks of female nude mice. (MDA-MB-231: n = 8, treatment group; n = 10, control group; MDA-MB-468: n = 8, treatment group; n = 7, control group; SUM159: n = 5 treatment group; n = 5, control group). Seven days following injection, mice were treated with orally administered KPT-9274 or Placebo (150 mg/kg PO bidx4 for the MDA-MB-231 and SUM159 cells, or 100 mg/kg PO bidx4 for the MDA-MB-468 cells). Tumor volume (V; mm³) was calculated for each cell line (see A, D, and G). Tumor weight was assessed for the MDA-MB-231 cells and the SUM159 cells (see B and E). Body weight of mice (see C,F,H) was monitored throughout the course of dose administration [171].

After treatment concluded, the MDA-MB-231 and SUM159 tumors were excised and immunoblotting was performed to measure PAK4 protein levels (Figure 7). We observed a significant decrease in PAK4 levels in excised tumors from the treatment group, when compared to those from the control (placebo) group. Compared to PAK4, the levels of an off-target protein, PAK1, were not significantly changed after treatment (Figure 7). Thus, our results indicate that orally administered KPT-9274 reduces the steady state level of PAK4 protein and is capable of reducing growth of the triple negative breast cancer cells MDA-MB-231, MDA-MB-468 and SUM159. These results are also consistent with previous studies indicating that PAK4 knockdown with siRNA blocks tumorigenesis caused by MDA-MB-231 cells.

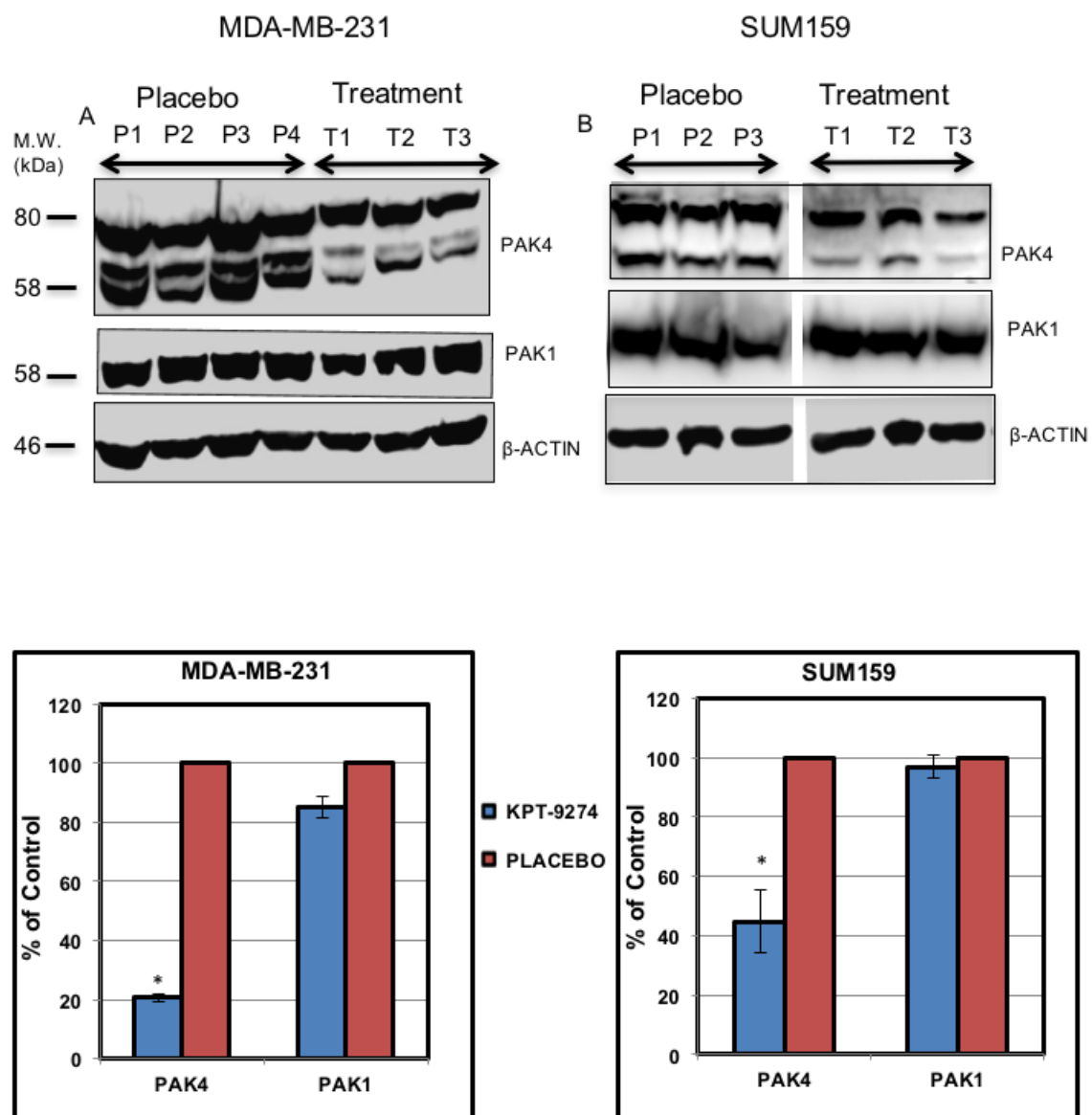


Figure 7: Orally administered KPT-9274 reduces PAK4 protein levels.

As the treatment concluded, MDA-MB-231 and SUM159 xenograft tumors were excised and analyzed by Western Blot (A). For the MDA-MB-231 mice, tumors from three independent mice that were treated with the KPT-9274 (T1, T2, T3), and from four independent mice that were treated with placebo (P1, P2, P3, P4) were analyzed by western blot, while for the SUM159 cells, tumors from three independent mice treated with KPT-9274 (T1, T2, T3) and three independent mice treated with placebo (P1, P2, P3) were analyzed by western blot. Blots were probed with anti PAK4, anti PAK1, or anti β -actin antibody as a loading control. The intensity of the bands in the blot in panel A were quantitated using Image J software, and the bands were normalized to the β - actin control (B). Results are plotted as a percent of control, where the control represents the band intensity for placebo, and set as 100% for each protein (PAK4 or PAK1). Data shown is representative of three separate repeat experiments. (The membranes were cut prior to exposure so that only the portion of gel containing bands in the size range of PAK4, PAK1, or β -actin would be visualized, as described in materials and methods. The top band in the PAK4 panel is a nonspecific band that appears in response to our PAK4 antibody, regardless of the presence of PAK4) [171].

DISCUSSION

In this study, we have found that KPT-8752 and KPT-9274 reduce cell proliferation, cell survival and cell migration in several triple negative breast cancer cell lines *in vitro*. Most importantly, we found that KPT-9274 can inhibit breast cancer tumorigenicity *in vivo*, in three independent mouse xenograft models using human triple negative breast cancer cell lines. We examined several subtypes of breast cancer cell lines in the current study and were encouraged to find that treatment with these inhibitors blocks the growth of three triple negative breast cancer cell lines. Because this subtype is generally less responsive to many of the current therapies, the data presented here provides a path forward to address the urgent need for novel treatment options for triple negative breast cancer patients.

KPT-8752 and KPT-9274 were identified as small molecules that bind to and reduce the steady state level of PAK4 protein in cells, and they were subsequently shown to inhibit the activity of NAMPT [169]. PAK4 has been found to be elevated in both breast cancer cells and primary breast tumors [12, 82, 88, 146, 155, 156]. Furthermore, in basal like breast cancer (a type that is usually triple negative) the chromosomal region containing the PAK4 gene is frequently amplified [92]. However, not all of the cancer cell lines described here were responsive to KPT-8752 or KPT-9274 despite their having high levels of PAK4. Specifically, in contrast to the triple negative cells, treatment with the compounds had only moderate effects on MCF7 cells (ER+/PR+), BT-474 (PR+/HER2+) and SkBr-3 cells (HER2+) *in vitro*, even though all of these cell lines have high levels

of PAK4. Investigation of additional breast cancer cells will help to determine whether the different responses we observed were in fact related to the backgrounds of the different cell types, and whether or not they are dependent on PAK4 status. From a clinical perspective, it will be vital to understand the reason why KPT-9274 was unable to reduce the viability of HER2+ and ER+ cells. One possibility is that KPT-9274 may operate through an additional target(s), in addition to, or possibly even instead of, PAK4, which may play a critical role in the biology of the triple negative cancer cells. Alternatively, the results may indicate that HER2 and ER may promote tumorigenesis by mechanisms that are independent of PAK4. If this is the case, the use of KPT-9274 in combination with HER2 inhibitors or estrogen blockers may be warranted.

The PAK family of protein kinases are important signaling molecules connected to many cellular functions including cell proliferation, migration, and cytoskeletal organization. Aberrant signaling in these pathways are often associated with cancer development and progression [176]. Because of this link, many PAK inhibitors have been developed in the past, as mentioned before. While clinical studies involving several of the compounds described above have been terminated due to poor bioavailability, improved second generation derivatives of some of these compounds may hold more promise. KPT-9274 is a unique type of inhibitor in that it reduces the steady state level of the PAK4 protein, although the exact mechanism by which it reduces PAK4 levels is not completely understood. Reduction in PAK4 protein is important because like

other PAK family members, PAK4 has been shown to have certain functions that are independent of its kinase activity [72, 73, 82, 167, 168]. Therefore, reduction of PAK4 protein advantageously reduces or inhibits any oncogenic process that requires the presence of the protein.

While our results indicate that KPT-9274 is a promising agent for triple negative breast cancer treatment, new data is emerging that it may also be effective against other types of cancers [169] [173]. As a result, KPT-9274 is currently under phase I clinical trials to evaluate its safety, tolerability, and efficacy (NCT02702492), in patients with solid tumors and lymphomas. Among the other available PAK4 inhibitors, we have found that Compound 17 [163] can also block the growth of breast cancer cell lines in our system, however it was less effective than KPT-9274 (Rane and Minden, unpublished results). In the future, it would be important to determine whether inhibiting PAK4 simultaneously with different types of inhibitors, could result in even stronger inhibition of PAK4 and inhibition of cancer cell growth. In addition to PAK4, other PAK family members such as PAK1 and PAK2 are often linked to breast cancer [41, 78, 138, 139, 183, 184]. Therefore, in future studies it will be interesting to determine whether combinations of inhibitors against the different PAK isoforms may be even more effective, particularly in cells that are unresponsive to single isoform inhibition.

Although KPT-9274 and its analogs inhibit PAK4, other PAK isoforms could still be affected. In this study, we found that the reduction in PAK4 levels by KPT-9274 is significantly stronger than the reduction in PAK1, suggesting

specificity for the group II PAKs. However, we have not tested the effects of KPT-9274 on the other group II PAK family members, PAK5 and PAK6, in breast cancer cells. Since PAK5 and PAK6 proteins are less frequently associated with breast cancer, our focus for this study was PAK4, but further investigation is warranted.

It is important to consider that in addition to PAK4, KPT-9274 and KPT-8752 also reduces the synthesis of NAD (nicotinamide adenine dinucleotide), by blocking the activity of the enzyme NAMPT (nicotinamide phosphoribosyltransferase) [169]. NAD is involved in a wide range of cellular processes, including DNA repair, and cell signaling, which are also thought to be important in cancer [170]. A direct link between PAK4 inhibition and NAD has not been established, but NAMPT has been reported to activate Cdc42, a known activator of PAK4, during cytoskeletal organization [185]. It is important to consider that many of the effects that we have seen in response to KPT-9274 could also be attributed to NAMPT inhibition, and more work will be required to distinguish the effects of the compound attributed to PAK4 from those attributed to NAMPT or possibly even other targets. It should be noted, however, that in breast cancer cell lines, we have seen that KPT-8752 and KPT-9274 do block NAMPT activity, but that inhibition of NAMPT did not correlate with the ability of the compounds to block cell proliferation (Minden lab, unpublished results). While it is important to consider the possibility that KPT-9274 has pleiotropic effects and impacts multiple signaling pathways, it is interesting to note that nearly all of the effects observed with the inhibitor are consistent with what has been reported

with PAK4 knockdown via siRNA. In particular, our previous work indicated that siRNA knockdown of PAK4 in the triple negative breast cancer cell line MBA-MB-231 reversed many aspects of tumorigenesis. siRNA mediated PAK4 knockdown resulted in inhibition of cell proliferation, increased apoptosis, and most importantly, decreased tumorigenesis in mice [150]. These studies strongly support the idea that blocking PAK4 correlates with inhibition of tumorigenesis in triple negative breast cancer. Although more work will be required to determine the exact mechanism by which KPT- 9274 operates, our results provide support for the use of this promising clinical candidate in triple negative breast cancer, and also validates the use of PAK4 as a novel drug target in triple negative breast cancer therapy, a disease that is refractory to most of the current treatments, due to lack of available biomarkers and poor prognosis.

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Chapter III

Decrypting the PAK4 transcriptome profile using Next Generation Sequencing

Abstract

The PAK4 (p21-Activated Kinase 4) protein kinase has long been associated with cancer. The PAK4 gene is amplified in different types of cancers, including breast cancer, and PAK4 has key roles in regulating cell proliferation, cell cycle progression and cell morphology. PAK4 overexpression is associated with oncogenic transformation in several breast cancer cell lines, while PAK4 inhibition reduces the tumorigenic potential of several types of cancer cells. There is limited information available, however, on the molecular mechanism by which PAK4 can promote tumorigenesis. To gain insight into the long term gene expression changes that occur downstream to PAK4, we performed Next Generation Sequencing (NGS) on RNA samples collected from non-transformed immortalized mouse mammary epithelial cells (WT iMMECs) and iMMECs overexpressing PAK4. Unlike WT iMMECs, iMMECs overexpressing PAK4 are transformed, and form tumors when injected into mammary fat pads of mice, suggesting that PAK4 regulates key signaling pathways that are important for oncogenic transformation. While many previous studies have focused on PAK4 substrates, here we address the long term changes in gene expression that are impacted by PAK4 overexpression. In response to PAK4 overexpression, we found that 1,672 genes were differentially expressed with at least a log₂ fold change of 0.9, relative to untransformed WT iMMECs. Most of the genes were previously not known to be regulated by PAK4. qPCR analysis of 8 of these genes validated the sequencing data. These results shed new light on the PAK4 transcriptome profile involved in tumorigenesis, particularly in mammary cells,

and will be helpful in delineating the mechanism by which PAK4 functions in other types of cancers.

Introduction

The p21-Activated kinases (PAKs) were originally identified as downstream effectors of Rho GTPases, Cdc42 and Rac, though they can be activated by multiple mechanisms [26, 186-191]. The PAKs fall into 2 categories, Group I PAKs and Group II PAKs, based on their structures and sequences [1-3]. PAK4, a Group II PAK, is an important regulator of many cellular functions that get dysregulated in tumorigenesis, such as cell proliferation, survival, and morphology [8, 66, 72, 73, 146]. The PAK4 gene is amplified in different types of cancers, including breast cancer [92], pancreatic cancer [80, 93, 94], squamous cell carcinomas [95], esophageal squamous cell carcinoma (ESCC) [173], endometrioid tumors, ovarian tumors and cell lines [192], as well as prostate cancer [193]. Recent studies have shown that KPT-9274, a compound that inhibits PAK4, reduces tumorigenesis in triple negative breast cancer, as well as renal adenocarcinoma, and squamous cell carcinoma [169, 171, 173]. These studies support the idea that PAK4 is an important regulator of tumorigenesis. Previous studies directed at studying PAK4 signaling have focused on identifying substrates phosphorylated by PAK4, and some of these studies have relied on *in vitro* assays. Information about the long-term effect of PAK4 overexpression on gene expression, however, is limited. A better understanding of the mechanism by which PAK4 modulates gene expression is warranted, in order to understand

how it contributes to cancer when it is overexpressed, and to identify the best ways for its targeted inhibition.

High throughput mRNA sequencing (RNA-seq) is a powerful analytical tool that can be used to accurately quantify transcript expression in response to a specific stimulus. RNA-seq analysis generates enormous amounts of raw sequencing reads per sample, with the number of reads corresponding to the transcript's abundance. The resulting RNA-seq data can be analyzed using bioinformatics tools which provide a robust and efficient method of interpreting this data [174]. Here we have used RNA-sequencing as a valuable tool that can be used to reveal the differential expression pattern of genes in response to PAK4 overexpression. These studies provide critical and de novo information on the PAK4 transcriptome in transformed mammary epithelial cells.

Immortalized mouse mammary epithelial cells (iMMECs) are a model that represent normal mammary epithelial cells. Previous studies in our lab have shown that although iMMECs are non-transformed cells, PAK4 overexpression in iMMECs result in oncogenic transformation. PAK4 transformed iMMECs display hallmarks of precancerous conditions and most importantly, they form tumors when transplanted in the mammary fat pads of mice [146]. Conversely, inhibition of PAK4 using siRNA blocks tumorigenesis of MDA-MB-231 cells, a triple negative breast cancer cell line [150]. Furthermore, the microRNA, mir-199a.b-3p, can function as a tumor suppressor by downregulating PAK4 and was shown to inhibit cell proliferation, cell migration and invasiveness of breast cancer cells [151]. These data point to a central role for PAK4 in promoting mammary

tumorigenesis. A better understanding of how PAK4 operates in the cell, and the long term changes in gene expression that occur in response to PAK4 will be crucial for developing more effective inhibitors of PAK4 signaling pathways. To address the gene expression changes that occur when mammary epithelial cells are transformed by PAK4, we isolated RNA samples from non-transformed iMMECs (WT iMMECs) and from iMMECs overexpressing PAK4, which were previously shown to be tumorigenic [146]. Sequencing Analysis was run by RUCDR, a Rutgers University based institution, that utilizes cutting edge Next Generation Sequencing (NGS) platforms for gene profiling studies. The resulting sequencing data was analyzed using Top Hat, Cufflinks, and Cuffdiff. Analysis of the sequencing data provided the differential expression pattern of thousands of genes in response to PAK4 overexpression. Utilizing several statistical parameters, as described in the Materials and Methods section, we generated a list of 1,672 genes whose expression was significantly regulated in response to PAK4 overexpression. By using IPA (Ingenuity Pathway Analysis) software, we were able to categorize the results and predict which regulatory networks are impacted by PAK4 overexpression. This study helps shed light on the PAK4 transcriptome profile in a mouse mammary tumor cell line, and it is an important step towards delineating the PAK4 signaling pathway in breast cancer. This type of transcriptome study will be instrumental for the identification of novel biomarkers in breast cancer, and for determining which biomarkers have the most potential therapeutic targets for breast and other cancers.

Materials and Methods

RNA sample preparation and collection: Total RNA was collected WT iMMECs and iMMECs overexpressing PAK4 (three plates for each condition, 2 million cells each plate), using RLT plus buffer (Qiagen). The RNA samples were further processed by RUCDR, a Rutgers University based institution, that utilizes Next Generation Sequencing (NGS) platforms for gene profiling studies. At first, the samples were homogenized using the Qiagen TissueLyser, and extracted in the QIAasympohony using the QIAasympohony RNA kit. Following extraction, samples were quantified using the Trinean Dropsense96 and quality was assessed using the Caliper LabChip GX.

Library preparation for transcriptome sequencing: RNAseq libraries were prepared using Illumina RNA Library Prep Kit v2 according to manufacturer's user guide with 400ng of RNA as input. The libraries were then quantified using KAPA Library Quantification kit according to manufacturer's user guide and pooled. The pooled libraries were sequenced on NextSeq 550 system, using NextSeq 500/550 Mid Output v2 kit. The sequencing parameters used were 150 bp, single-end with 20 million reads per sample.

Sequencing Data Analysis: Raw sequencing data was generated in the Fastq format. The reads were analyzed and mapped as described in [194]. Briefly, the reads were mapped to a mouse reference genome (mouse mm9) using TopHat software. Cufflinks was used to assemble the transcript and calculate the

fragment abundance, followed by use of Cuffdiff, a part of the Cufflinks package, to determine the differential gene expression pattern between the two conditions. Transcript abundance was expressed as FPKM (fragments per kilobase of transcript per million mapped fragments). The FPKM for each condition was expressed as an average, and the log₂ fold change between the two conditions was indicated. Our analysis generated a list of 25,692 genes, of which 14,055 were tested for differential expression. Of these, we selected 1,672 genes which showed a differential gene expression pattern between the two conditions with a log₂ fold change over 0.9 or under -0.9 (corresponding to a 1.87 fold increase or decrease). While log₂ fold change of 0.9 was used as the cutoff, many of the genes had significantly larger fold changes, ranging from a log₂ fold change of -7.425 to +4.966 (corresponding to a 171.8 fold decrease to a 31.25 fold increase). A p value cutoff of .0269 or less and a q value of 0.0483 or less was used, though 1,447 out of the 1,672 genes have a p value of 5.0 E-5 and a q value of 1.5 E-4).

Quantitative Real-Time PCR: RNA was reverse transcribed to cDNA using the One-Taq RT-PCR kit from New England Biolabs (Ipswich, MA). cDNA was then amplified using PrimeTime® qPCR primers from Integrated DNA Technologies (Coralville, Iowa) and SYBR® Green PCR Master Mix from Applied Biosystems using the ABI Prism 7000 Sequencing Detector (Applied Biosystems, Foster City, CA). Thermal cycling conditions were: 1 cycle of 50 degrees for 1 min, 1 cycle of 95 degree for 10 min and 40 cycles of 95 degree for 15s and 60

degree for 1 min. GAPDH was used as an internal control and the relative changes of gene expression were calculated by the following formula, fold change = $2^{-\Delta\Delta Ct} = 2^{-\{\Delta Ct \text{ (test)} - \Delta Ct \text{ (control)}\}}$ where $\Delta Ct = Ct \text{ (PAK4)} - Ct \text{ (GAPDH)}$ and Ct = threshold cycle number.

IPA analysis of differentially expressed genes: Sequencing data was analyzed by using Ingenuity Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity), which is a database resource for categorizing genes and analyzing their relationships with multiple cellular functions, diseases, and signaling pathways. The list of differentially expressed genes was analyzed by IPA which predicted their relationships with various gene regulatory networks.

Results

Analysis of differentially expressed genes in PAK4 overexpressing iMMECs compared with wild-type iMMECs: Previous studies from our lab showed that mouse mammary epithelial cells (iMMECs) stably transfected with PAK4 were transformed and formed tumors in mammary tissue in mice [146]. To study the gene expression changes induced by PAK4 overexpression in these cells, RNA samples were isolated from three separate plates each of wild-type iMMECs, and iMMECs overexpressing PAK4. RNA was sequenced using Illumina NextSeq kit, and the sequencing reads were then analyzed as described in the materials and methods section and in [194], which included mapping to a mouse reference genome using TopHat, followed by assembly and differential expression analysis

using Cufflink and Cuffdiff. This generated a list of 25,692 genes, of which 14,055 were tested for differential expression. Of these, 1,672 genes showed a differential gene expression pattern between the two conditions, according to the parameters described in the materials and methods section. Using IPA (Ingenuity Pathway Analysis) software, we found that 1,606 of the genes mapped to known genes (see Supplemental Figure 1) and these were analyzed further. A list of the top 10 upregulated and downregulated genes is shown in Tables 1A and 1B.

Table 1A: Top 10 genes whose expression is up regulated in response to PAK4 overexpression.

gene_id	gene	locus	sample_1	sample_2	value_1	value_2	Log2(fold change)	Fold_change	p_value	q_value	significant
XLOC_015037	Ifi44	chr3:151393886-151412923	wt	pak4	1.00798	32.1581	4.99564	31.903	5.00E-05	0.000157052	yes
XLOC_021384	Trim30a	chr7:111557349-111613707	wt	pak4	1.3588	35.1368	4.69258	25.858	5.00E-05	0.000157052	yes
XLOC_021385	Trim30d	chr7:111618528-111656382	wt	pak4	0.938263	23.0572	4.61908	24.574	5.00E-05	0.000157052	yes
XLOC_014060	Ccdc169	chr3:54941260-54976858	wt	pak4	0.0805637	1.85026	4.52146	22.966	5.00E-05	0.000157052	yes
XLOC_022032	Ddx60	chr8:64399627-64517022	wt	pak4	1.01876	22.071	4.43726	21.664	5.00E-05	0.000157052	yes
XLOC_018728	Usp18	chr6:121195923-121220935	wt	pak4	5.57581	120.617	4.43511	21.632	5.00E-05	0.000157052	yes
XLOC_022060	Mef2b	chr8:72676676-72691749	wt	pak4	1.72558	29.6001	4.10045	17.153	5.00E-05	0.000157052	yes
XLOC_007945	Apol9a	chr15:77233893-77241522	wt	pak4	0.592146	9.3107	3.97487	15.723	5.00E-05	0.000157052	yes
XLOC_007519	Apol9b	chr15:77559501-77566998	wt	pak4	0.823683	11.6661	3.82409	14.163	5.00E-05	0.000157052	yes
XLOC_014454	Gbp2	chr3:142283626-142300972	wt	pak4	1.79099	25.3576	3.82359	14.158	5.00E-05	0.000157052	yes

Table 1B: Top 10 genes whose expression is down regulated in response to PAK4 overexpression.

gene_id	gene	locus	sample_1	sample_2	value_1	value_2	Log2 (fold change)	Fold Change	p_value	q_value	significant
XLOC_012699	Wfdc2	chr2:164388215-164394006	wt	pak4	50.6128	0.294445	-7.42536	-172.117	5.00E-05	0.000157052	yes
XLOC_013319	Mdk	chr2:91762345-91772454	wt	pak4	10.3472	0.187773	-5.78411	-55.248	5.00E-05	0.000157052	yes
XLOC_001509	Lama4	chr10:38685320-38829994	wt	pak4	2.53409	0.0489298	-5.69461	-51.813	5.00E-05	0.000157052	yes
XLOC_004948	Crip1	chr12:11439022-2-114392090	wt	pak4	48.4252	0.952021	-5.66862	-51.02	5.00E-05	0.000157052	yes
XLOC_011267	As3mt	chr19:46781932-46815585	wt	pak4	7.03768	0.193031	-5.1882	-36.496	5.00E-05	0.000157052	yes
XLOC_000206	Wnt10a	chr1:74838592-74850749	wt	pak4	4.04463	0.113863	-5.15063	-35.587	5.00E-05	0.000157052	yes
XLOC_004990	Fam84a	chr12:14154403-14158844	wt	pak4	3.22968	0.120089	-4.74922	-26.954	5.00E-05	0.000157052	yes
XLOC_003566	Grb10	chr11:11830501-11937423	wt	pak4	1.89963	0.0723973	-4.71364	-26.246	5.00E-05	0.000157052	yes
XLOC_019329	Slc2a3	chr6:122677826-122692763	wt	pak4	2.38254	0.0943554	-4.65825	-25.252	5.00E-05	0.000157052	yes
XLOC_011518	Pcsk5	chr19:17507321-17912122	wt	pak4	0.801974	0.031827	-4.65523	-25.252	5.00E-05	0.000157052	yes

Validation of differential gene expression data using qPCR: To validate the RNA-seq data, we ran a qPCR analysis of 8 genes that were identified in the sequencing analysis. These genes were selected on the basis of their functional relevance in cancer, their likelihood of being associated with PAK4, and consistent gene expression pattern in each of the three samples. The genes consisted of FoxC2, Xaf1, Apc2, Stat1, and Traf1, which were up-regulated by PAK4 overexpression, and ParvB, Mmp14, and Wnt10A, which were down regulated by PAK4 overexpression. The expression patterns for all of the 8 genes, including the fold change and statistical analysis, is shown in Table 2.

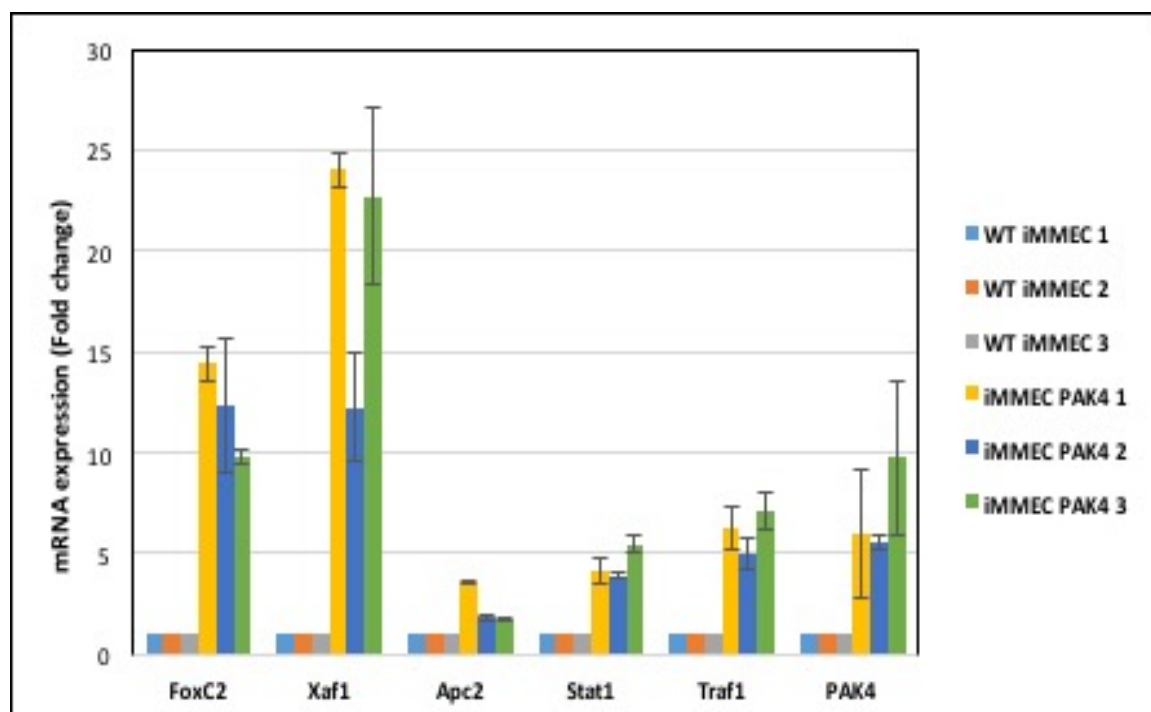
qPCR analysis revealed that there was a strong correlation between the RNA-seq results and qPCR data (Figure 1). This data shows that the RNA-seq results are reproducible, and that RNA-seq is a reliable tool for studying the PAK4 transcriptome profile in transformed mammary epithelial cells.

transformed mammary epithelial cells.

Table 2: List of genes whose expression was validated using qPCR.

gene id	gene	sample 1	sample 2	value 1	value 2	Fold Change	p value	q value	significant
XLOC_013001	Traf1	wt	pak4	0.102809	0.971884	9.453	5.00E-05	0.000157052	yes
XLOC_022364	Foxc2	wt	pak4	0.234227	1.95065	9.453	5.00E-05	0.000157052	yes
XLOC_003016	Xaf1	wt	pak4	3.86891	31.3782	8.110	5.00E-05	0.000157052	yes
XLOC_001720	Apc2	wt	pak4	0.115739	0.74756	6.458	5.00E-05	0.000157052	yes
XLOC_000112	Stat1	wt	pak4	10.6055	60.2673	5.682	5.00E-05	0.000157052	yes
XLOC_000206	Wnt10a	wt	pak4	4.04463	0.113863	-35.587	5.00E-05	0.000157052	yes
XLOC_006646	Mmp14	wt	pak4	11.4912	0.917818	-12.531	5.00E-05	0.000157052	yes
XLOC_007593	Parvb	wt	pak4	8.17028	1.11413	-7.352	5.00E-05	0.000157052	yes

A



B

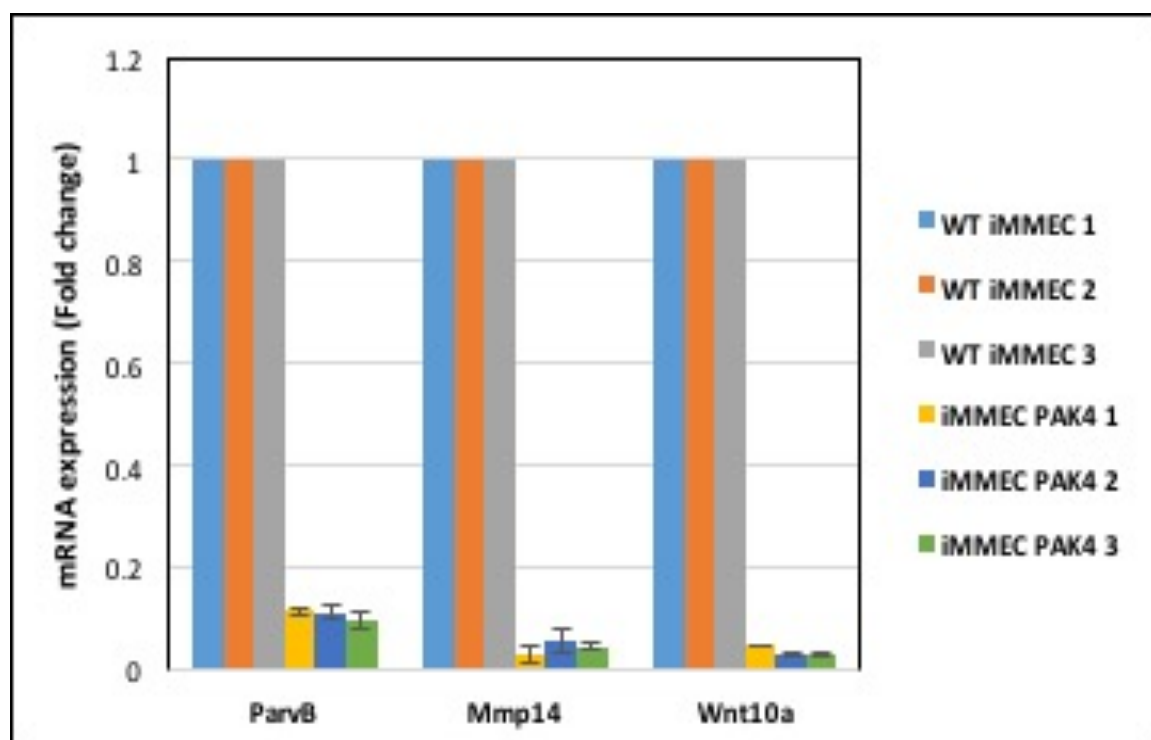


Figure 1: qPCR validation of sequencing data. Expression of (A) upregulated genes (FoxC2, Xaf1, Apc2, Stat1, Traf1) and (B) downregulated genes (ParvB, Mmp14, Wnt10a) was monitored using qPCR analysis. mRNA levels were normalized to GAPDH. Data representative of three separate repeat experiments. Error bars represent S.D.

Gene expression pattern in response to PAK4 inhibition. KPT-9274, developed by Karyopharm Therapeutics, is a dual specific inhibitor that blocks PAK4 levels as well as the activity of the enzyme NAMPT [195, 196 , 197]. KPT-9274 has been shown to inhibit growth of several different types of cancer cells in vitro and in vivo [195, 196 , 197], including triple negative breast cancer (TNBC) cells [197]. We tested whether treatment of the TNBC cell line SUM159, with KPT-9274 has the converse effect on the transcripts described above, compared with PAK4 overexpression in the IMMECs. We found, in fact, that FOXC2 and XAF1, which were upregulated in response to PAK4 overexpression, were downregulated, as was PAK4 (see Figure 2). In contrast, ParvB and MMP14 which were downregulated by PAK4 overexpression, were also downregulated by KPT-9274 treatment.

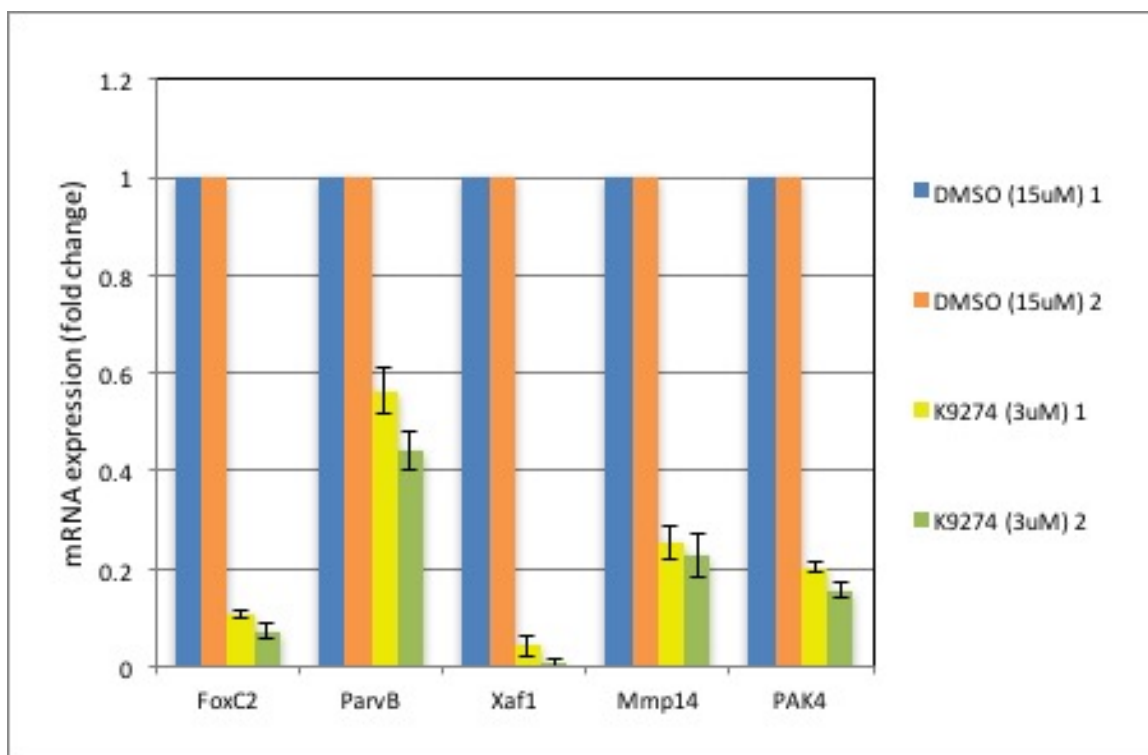


Figure 2: Treatment with of breast cancer cells with KPT-9274 modulates the expression of FoxC2, ParvB, Xaf1, and Mmp14. Expression of FoxC2, ParvB, Xaf1, Mmp14 and PAK4 were monitored in SUM159 cells following treatment with KPT-9274 or control (DMSO). mRNA levels were normalized to GAPDH. Data are representative of three separate repeat experiments.

Gene	Cancer Prediction	Expr Log Ratio	Gene Expression
IL18	Increased	3.141	Increases (14)
PARP14	Increased	2.021	Increases (26)
IKBKE	Increased	1.985	Increases (10)
CCL5	Increased	1.699	Increases (4)
PGF	Increased	1.67	Increases (93)
IL23A	Increased	1.624	Increases (3)
CSF3	Increased	1.6	Increases (21)
SCUBE3	Increased	1.493	Increases (15)
GLI1	Increased	1.456	Increases (79)
ATP7B	Increased	1.423	Increases (12)

Table 3: Gene expression of Top 10 genes that predict Cancer. The number in parenthesis, in the gene expression column, corresponds to the number of published scientific articles that delineate the role of the respective gene in cancer.

The transcriptome in PAK4 overexpressing cells predicts cancer and cellular changes that are frequently associated with oncogenic transformation. The transcriptome profile of PAK4 overexpressing cells, when analyzed by IPA, predicted that the pattern of gene expression strongly predicts cancer, as well as other cellular processes such as increased cell growth and proliferation, regulation of cell death and survival, and tissue morphology. Figure 3 shows a graph indicating the top cellular processes predicted to be upregulated in response to PAK4 overexpression. A total of 1,302 genes out of the total

number of genes analyzed, have been linked to an increase in cancer. Of these, 99 are upregulated or downregulated in a direction that predicts cancer. The top 10 of these genes are listed in Table 3. Based on this profile, cancer is predicted to be increased with a significant Z score of 2.905, and an overlap p-value of 3.09×10^{-14} .

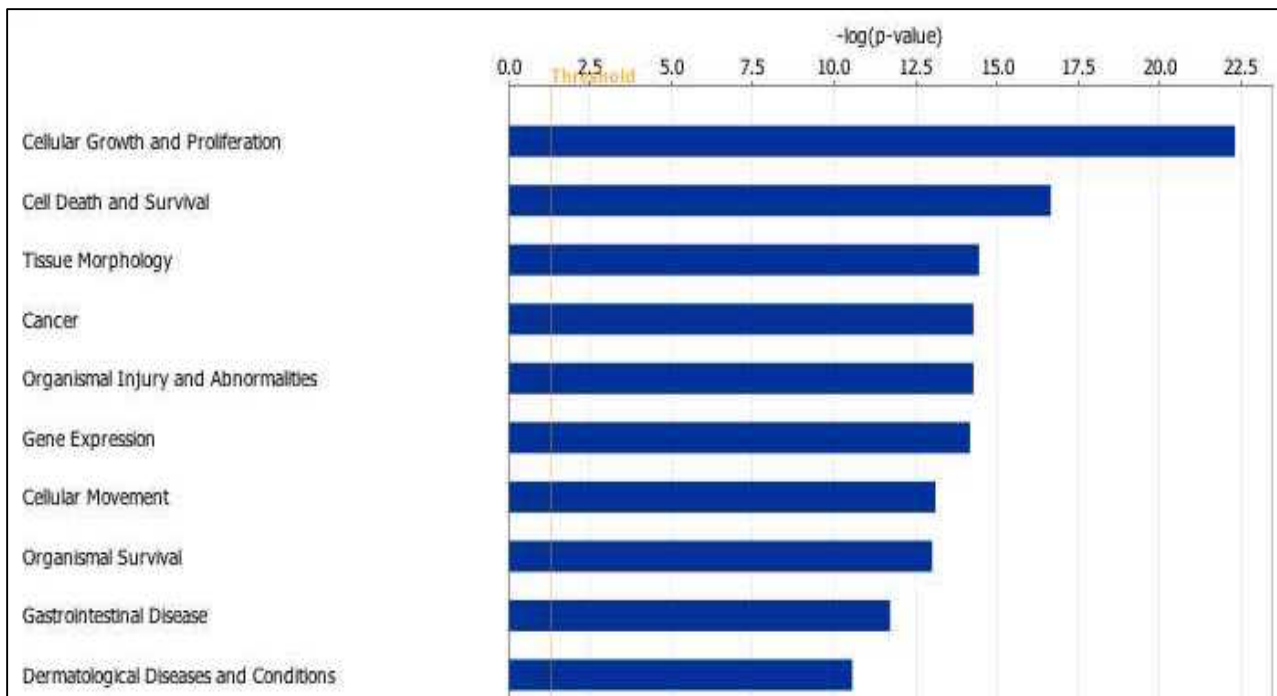


Figure 3: Top 10 cellular processes activated in response to PAK4 overexpression. The differentially expressed genes were analyzed using IPA analysis. The top 10 cellular processes predicted to be upregulated according to the PAK4 transcriptome are shown. The $-\log(p\text{-value})$ represents the statistical significance of the outcome.

IRF7 and TNF are predicted to be activated. Based on the RNA sequencing results, we were able to predict the upstream regulators that are most likely to drive the gene expression pattern observed in our dataset, and to illustrate which target genes are most likely to be impacted by these upstream regulators. Our results predict that the most activated upstream regulator is IRF7. Figure 4A illustrates a network indicating which target genes in our network are most likely regulated by IRF7. For this network the Z score is 6.07, (strongly predicting IRF7 activation) and the expression patterns of 48 genes are consistent with the activation of IRF7. The Z score is a statistical test that measures the match between the observed gene expression and the expected relationship (bioinformatics.cancer.gov). While activation of IRF7 has the highest Z score of any activator in this study, TNF is the upstream regulator with the largest number of target genes. The expression pattern of 198 target genes predict activation of TNF, with a Z score of 1.644. An illustration of TNF and the target genes that predict its activation, is shown in Figure 4B.

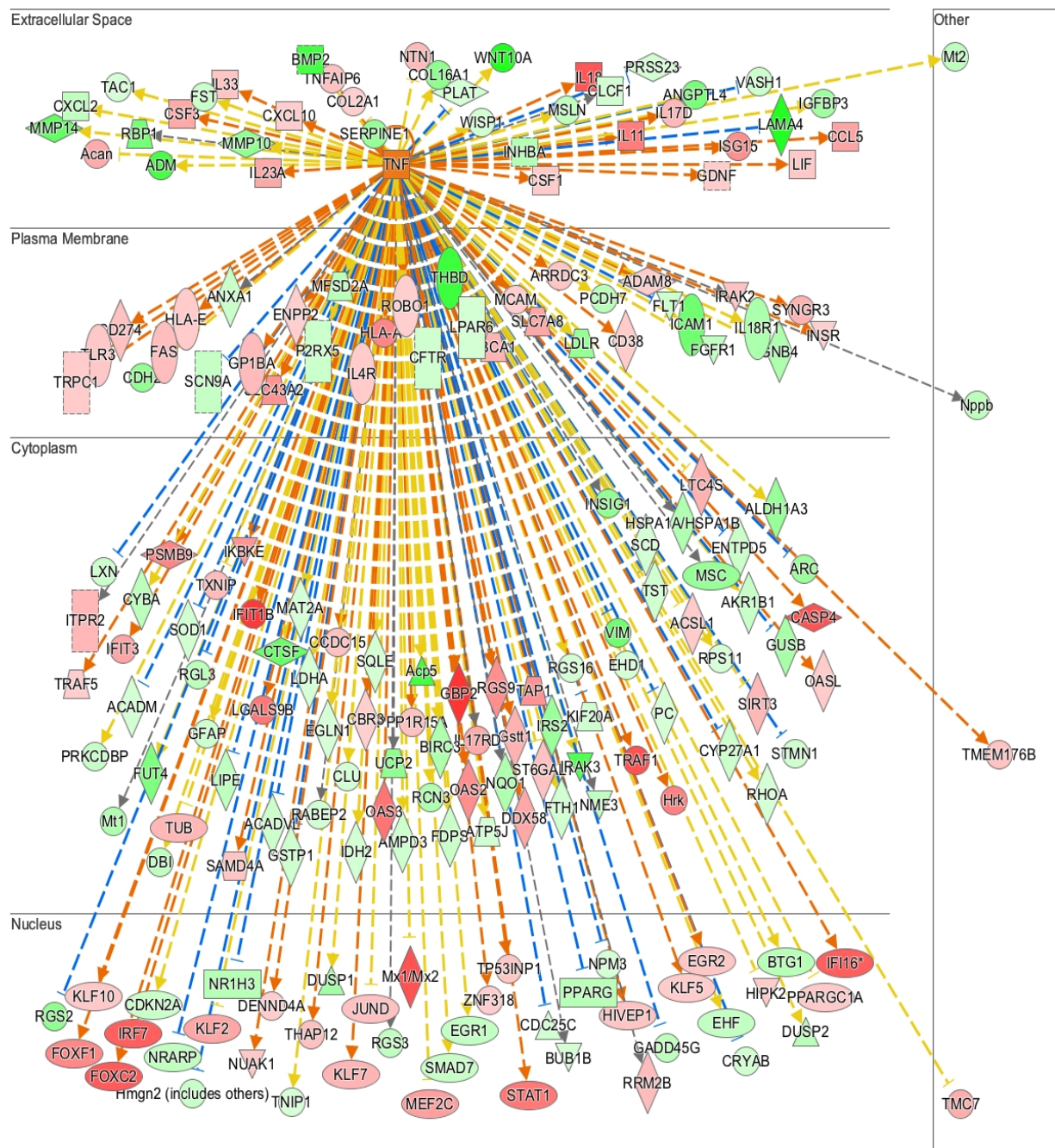


Figure 4: Most activated upstream regulators of gene expression in response to PAK4 overexpression. A. Analysis of the differentially expressed genes using IPA strongly predicts that IRF7 is activated. The expression of 48 genes in the dataset predict the activation of IRF7, with a Z score of 6.07.

B. TNF is predicted to be activated with a Z score of 1.644. The expression of 198 genes in the dataset predict its activation.

TNF 1

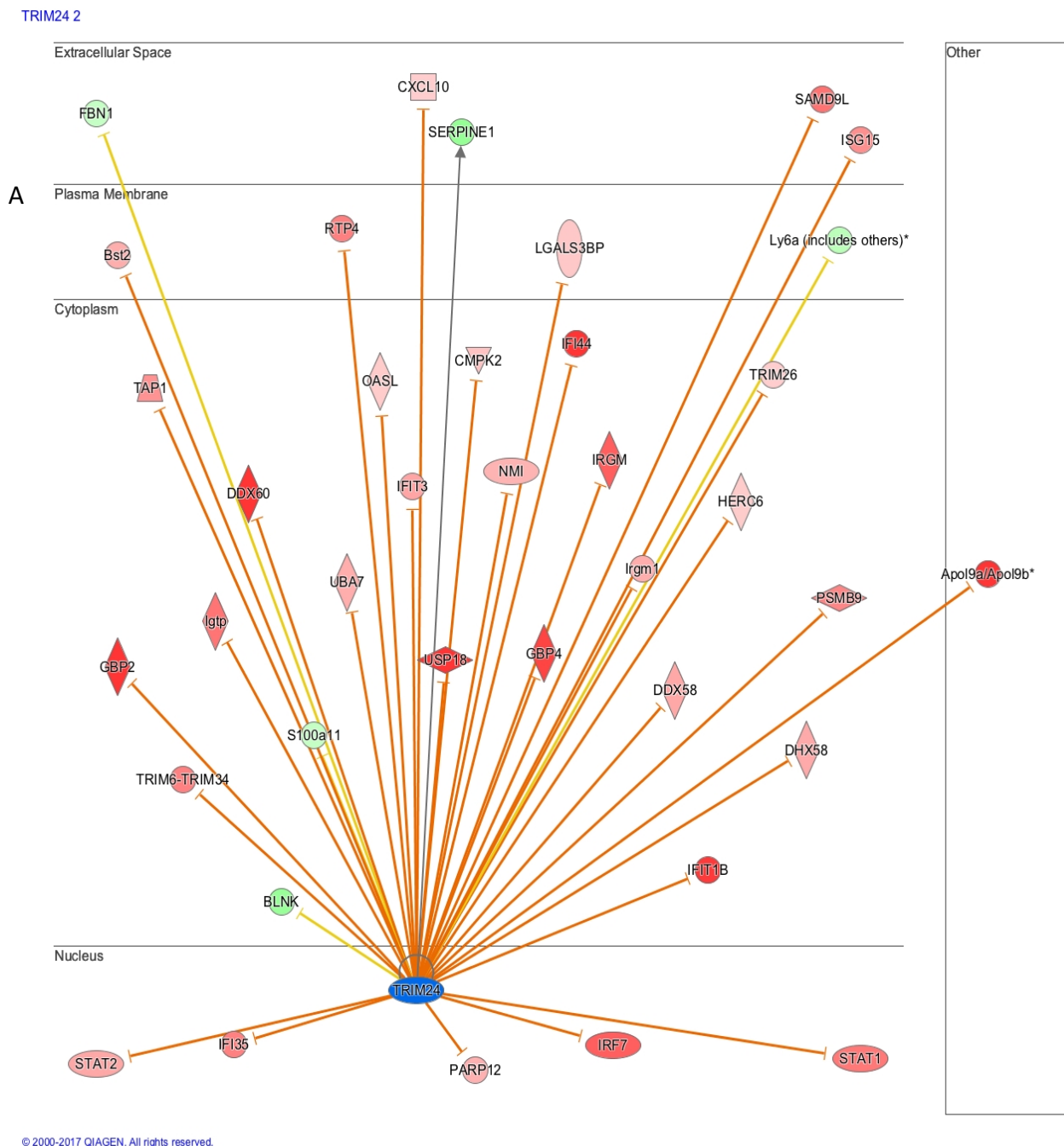
B



TRIM24 and TGFB1 are predicted to be inactivated: In contrast to IRF7 and TNF, TRIM24 is predicted to be the most inactivated upstream regulator, where 38 genes are regulated in a direction that predicts TRIM24 inhibition, with a Z score of -4.776 (Figure 5A). While TRIM24 is predicted to be the most inhibited regulator according to the Z score, TGFB is also predicted to be a strongly inhibited upstream regulator, and has the highest number of target genes which predict its inhibition. In this case 186 genes are expressed in a direction that predicts TGFB1 inhibition, with a Z score of -1.477. A network of TGFB1 and the target genes regulated in this dataset is illustrated in Figure 5B.

Mechanistic networks predicted to be modulated in response to PAK4 overexpression: The upstream regulators predicted in this study are all known to interact within broader networks with other regulators. By using IPA software, we were able to predict the activation states of mechanistic networks of upstream regulators that could account for the differential gene expression pattern observed in our dataset. Figures 6A and 6B show examples of regulatory networks that contain IRF7 and TRIM24, respectively. These figures also illustrate the top downstream target genes (those that have a log2 fold change of 4 or more, corresponding to a fold change of 16 or more) in our dataset, regulated by these upstream regulators. The IRF7 network (Figure 6A) includes 14 upstream regulators and 203 target genes, 22 of which are shown here. The TRIM24 network includes 7 upstream regulators and a total of 169 downstream

target genes whose expression in the database could be explained by such a network (a total of 11 of these target genes are shown here) (Figure 6B).



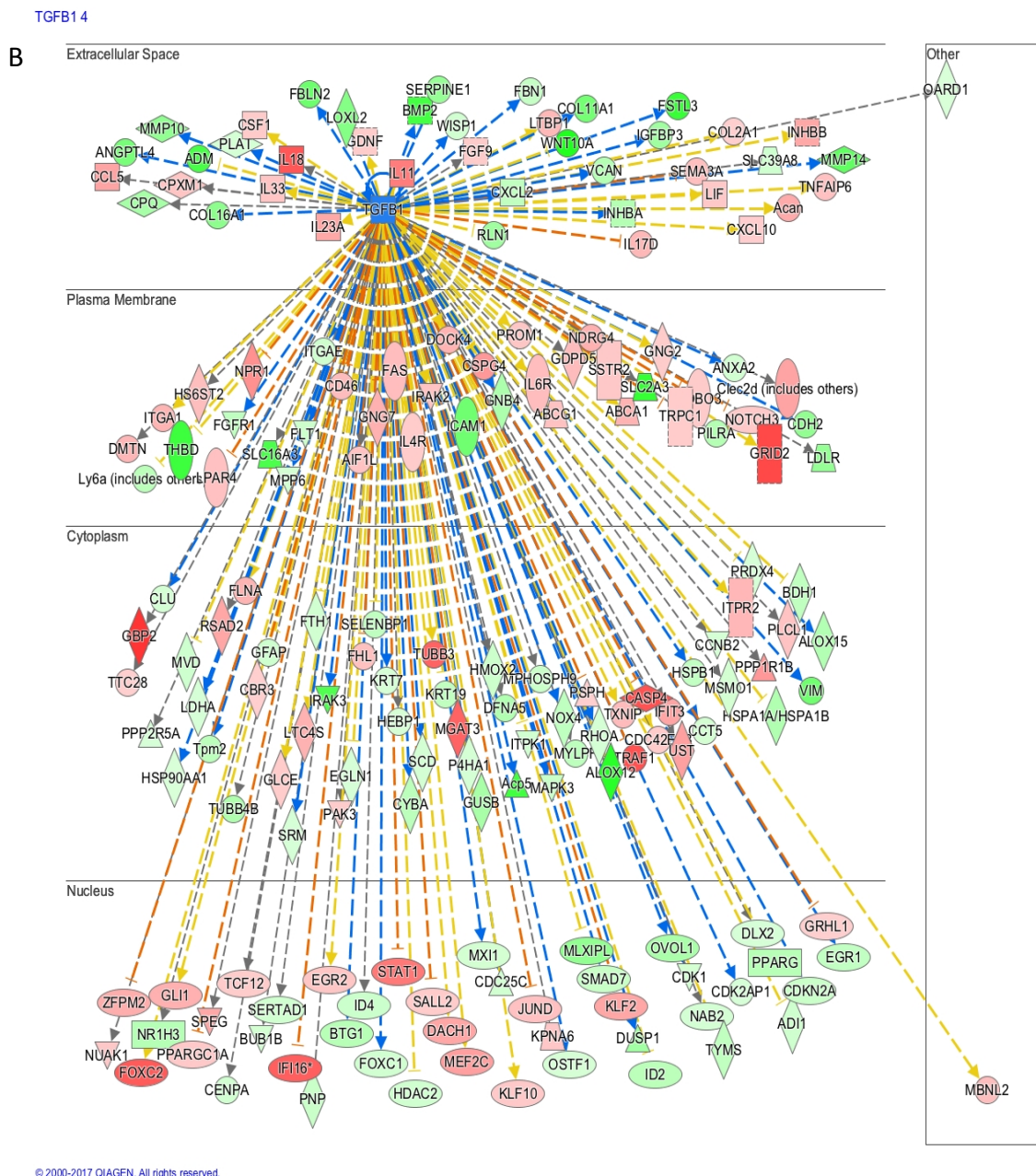


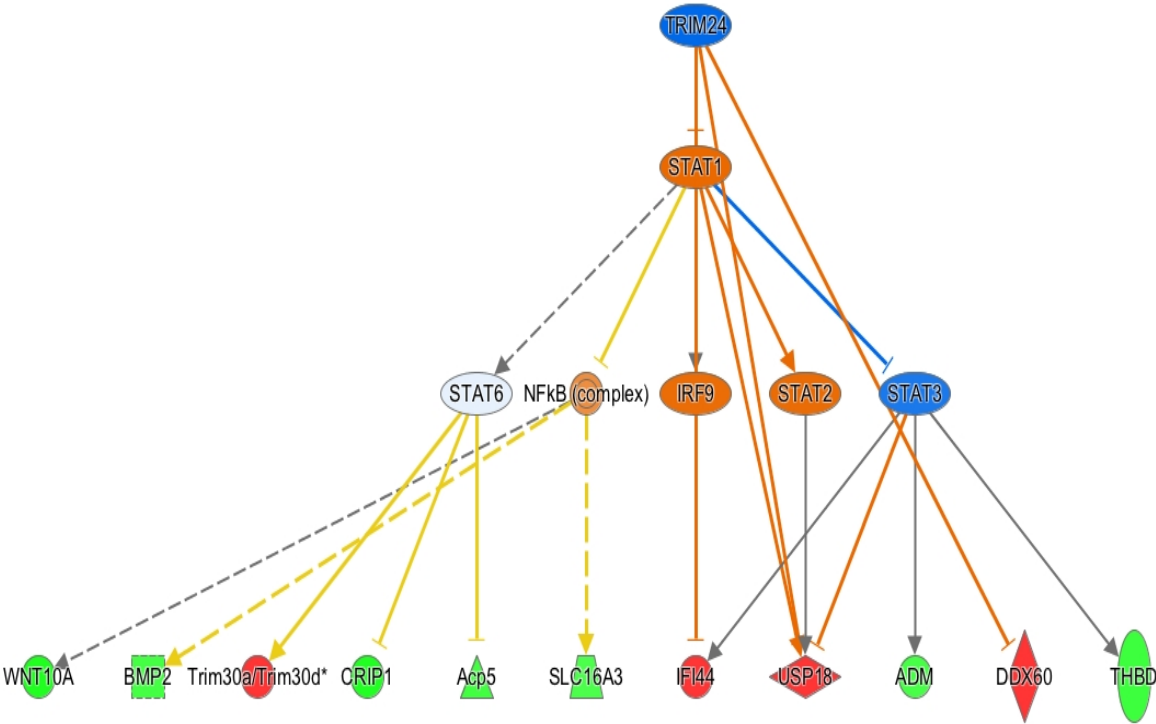
Figure 5: Most Inhibited Upstream Regulators of gene expression in response to PAK4 overexpression. A. TRIM24 is predicted to be strongly inhibited with a Z score of -4.776. 38 target genes predict its inhibition. **B.** TGF β 1 predicted to be inhibited with a Z-score of -1.477. 186 genes in the dataset predict its inhibition.

A. IRF7 regulatory network. The PAK4 transcriptome predicts that IRF7 is activated. IRF7 is predicted to function as part of a regulatory network, as shown here, in which IRF7 interacts with 14 other upstream regulators, predicting the expression of 203 target genes found in the dataset. 22 of the target genes, which have a log2 fold change of at least 4 (corresponding to a fold change of 16), are shown here.

B. TRIM24 regulatory network. The PAK4 transcriptome predicts that TRIM24 is inhibited. TRIM24 is predicted to function as part of a regulatory network, as shown here, in which it interacts with 6 other upstream regulators, predicting the expression of 169 target genes found in the dataset. 11 of the target genes, which have a log2 fold change of at least 4 (fold change of 16), are shown here.

TRIM24 39

B



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Prediction Legend

more extreme in dataset	less
Increased measurement	
Decreased measurement	
more confidence	less
Predicted activation	
Predicted inhibition	

Glow Indicates activity when opposite of measurement

Predicted Relationships

- Leads to activation
- Leads to inhibition
- Findings inconsistent with state of downstream molecule
- Effect not predicted

Discussion

The PAK4 protein kinase is frequently implicated in cancer when it is overexpressed [80, 92-95, 150, 151, 192, 198, 199]. Currently, most studies designed to delineate the molecular mechanisms by which PAK4 functions have focused on its substrates, some of which have been validated only *in vitro*. PAK4 substrates include β -catenin, which leads to activation of the Wnt pathway [200], Raf1, which activates the MAPK pathway [65], GefH1, which inhibits Rho [35], LIM Kinase 1 (LIMK1) [36], which regulates actin polymerization [37, 38], Ran, which regulates complexes on the mitotic spindle [47], and β -5 integrin, which is involved in cell adhesion and migration [50]. Most of these substrates are directly phosphorylated by PAK4, within minutes of PAK4 activation. The long term transcriptional changes triggered by PAK4, however, are poorly understood. Here we carried out High Throughput mRNA sequencing (RNA-seq) analysis of iMMECs transformed with PAK4, compared with wild-type iMMECs. Our goal was to uncover information about PAK4 induced transcriptional changes, in order to learn more about the mechanism by which PAK4 regulates cell growth and leads to cancer when it is overexpressed.

Our sequencing results revealed a list of genes that are differentially expressed in response to PAK4 overexpression. It is noticeable that PAK4 is absent from the list of differentially expressed genes. This is to be expected because our iMMECs were stably transfected with the human PAK4 gene [146], while the reference genome used to map the sequencing reads was mouse, since iMMECs are murine cells. Human and mouse PAK4 protein are 92%

identical to each other, and therefore we expected that either human or mouse PAK4 would lead to the same transcriptome changes. However, unlike the protein sequence, the human and mouse PAK4 mRNA sequences contain stretches of 84% sequence identity, especially in the coding region, but have overall only approximately 69% identity, and thus human PAK4 RNA was not detected in the mouse reference genome. Increased PAK4 mRNA levels in iMMECs stably transfected with PAK4 is demonstrated by q-PCR (Figure 1), however, and increased PAK4 protein levels were demonstrated previously [146].

Transcriptome analysis of the PAK4 overexpressing and wild-type cells led to the identification of genes not previously known to operate downstream to PAK4. For example, PAK4 overexpression leads to downregulation of ParvB, a member of the parvin family. The parvins are actin-binding proteins involved in cytoskeletal organization and adhesion [201]. They also bind and inhibit Integrin Linked Kinase (ILK). In mammary epithelial cells, overexpression of ILK is associated with anchorage independent growth and mammary hyperplasia, and with elevated levels of AKT, GSK-3 β , and MAPK phosphorylation. ParvB has been proposed to suppress oncogenic ILK activity [202]. ParvB may also be linked to Cdc42 activation, via its interaction with alpha pix, a Cdc42 exchange factor [203]. Reduction in ParvB levels by PAK4 could thus suggest an inhibitory loop, since PAK4 is a Cdc42 binding protein [8]. Inhibition of ParvB is associated with an increase in invasion through the extracellular matrix, and overexpression of ParvB in breast cancer suppresses cancer cell growth and transformation.

Importantly, ParvB is significantly downregulated in breast cancer and breast cancer cell lines [204], and may be involved in tumor suppression. Our finding that ParvB is downregulated in PAK4 overexpressing mammary cells, supports a model by which inhibition of ParvB leads to increased cell growth and migration, and mediates the decrease in cell adhesion seen in response to PAK4, an important hallmark of cancer.

In contrast to ParvB, FOXC2 (forkhead box protein 2) is upregulated by PAK4 overexpression. FOXC2 is a transcription factor that regulates cell proliferation, migration, motility and metastasis [205, 206]. Its expression is increased during the epithelial-mesenchymal transition (EMT), and it has been linked to metastasis in cancer [205]. FOXC2 was shown to be overexpressed in highly metastatic forms of breast cancer [207], and conversely, inhibition of FOXC2 reduces metastatic activity [207]. FOXC2 may also have an important role in angiogenesis, via vascular formation and remodeling [208-210]. Thus FOXC2 may also contribute to cancer, downstream to PAK4, by increasing metastatic activity and by encouraging angiogenesis. The XAF1 (X linked inhibitor of apoptosis) gene, also strongly upregulated by PAK4 overexpression, is a member of a family of proteins that bind to caspases and inhibit their activity. XAF1 is overexpressed in some cancers and may be important in mediating apoptosis resistance. A role for PAK4 in regulating XAF1 expression may help explain how PAK4 inhibits apoptosis and promotes cell survival [88], which are important steps in the oncogenic process.

While the expression of FoxC2, Xaf1, and ParvB may provide a link between PAK4 overexpression and cancer, some of the findings were more surprising. For example, the MMP14 (matrix metalloprotease 14) gene was strongly reduced in response to PAK4 expression. Yet MMP14 has been shown to be overexpressed in human cancers [211-214]. One possible explanation for our unexpected findings is that MMP14 is downregulated as a compensation mechanism, in response to PAK4 overexpression and cell growth pathways. Alternatively, our results could also be due to *in vitro* culture conditions, or MMP14 may have other unknown functions. In fact, it is not uncommon for proteins to have both tumor promoting and tumor inhibitory functions in different situations [215]. It should also be noted that our results reveal mRNA changes, but protein changes have not been analyzed in our system. Our results emphasize the importance of RNA sequencing, as a way to identify new and unexpected transcriptional patterns in cancer and as a starting point for discovering new gene functions.

KPT-9274 is a dual specific inhibitor of PAK4 and NAMPT [195-197]. We treated the breast cancer cells SUM159 with KPT-9274 and used qPCR to analyze 4 of the genes shown to be differentially expressed in response to PAK4 overexpression. We found that treatment with KPT-9274 led to downregulation of FOXC2 and XAF1. This was the anticipated result, because PAK4 overexpression led to upregulation of these genes. Inhibition of FOXC2 and XAF1 may help explain the mechanism by which KPT-9274 can block tumorigenesis in breast and other cancers. In contrast, ParvB and MMP14 that

were downregulated by PAK4 overexpression, but were also downregulated by KPT-9274. The reason for these unexpected results are unknown, but it should be noted that KPT-9274 blocks not only PAK4 but also NAMPT, and therefore may target multiple pathways.

While we have identified a list of genes that are differentially expressed, we still do not know the exact mechanism by which these genes are regulated, and whether they are directly or indirectly regulated by PAK4. By using IPA, we were able to predict which upstream regulators would most likely correlate with the transcriptome profile that corresponded to PAK4 overexpression. For example, we found that IRF7 (Interferon Regulatory Factor 7) is predicted to be active based on the expression of a subset of genes in the PAK4 overexpressing cells (see Figure 4A). IRF7 is a transcription factor known mostly for its role in the immune response to pathogens [216], where it is implicated in the regulation of chemokines and cytokines. The finding that IRF7 leads to upregulation of many of the same genes seen in PAK4 overexpressing cells, and that many of these are also involved immunity and inflammation, may have important implications. This would suggest that certain genes involved in the immune response and inflammation are also important for PAK4 induced tumorigenesis. In fact, *Ifi44* (Interferon induced gene 44), which we found to be the top upregulated gene in response to PAK4 overexpression (see Table1A), was one of the genes that predict activation of IRF7. Although its function is not clearly defined, *Ifi44* is thought to function downstream to interferons in the immune response. Our results raise the interesting possibility that PAK4 overexpression in cancer leads

to the regulation of cytokine and chemokine production and secretion, which in turn dictates the presence or absence of immune cells in the tumor microenvironment (TME). This could have a major impact on tumor growth and viability. This is an important area of future investigation, and demonstrates how RNA sequencing, combined with pathway analysis software, can lead to new hypotheses and new areas of investigation.

Two examples of genes whose regulation by PAK4 predict IRF7 activation are CXCL10 and CCL5. CXCL10 is a chemokine that is secreted by many cells, often in response to Interferons. It plays a role in T cell activation and in chemotaxis in the immune system, but also plays important roles in controlling cell growth in various cell types [217]. It has both proliferative and anti-proliferative activities, and can have both pro and anti-tumor effects. It is overexpressed in many cancers including some breast cancers, although it is downregulated in other cancers. CCL5 is also an inflammatory cytokine, which is also known to be involved in cancer. Many tumors overexpress and secrete CCL5, and which in turn can act in an autocrine or paracrine manner to promote or maintain cancer cell proliferation, or to recruit cells that have an immunosuppressive effect. CCL5 can also activate pathways that lead to angiogenesis and metastasis.

In addition to IRF7, activation of TNF was also predicted to correlate with the transcriptome profile in PAK4 overexpressing cells (see Figure 4B). TNF is also a cytokine that is involved in the immune response and inflammation. It is often associated with cancer, although it can have both tumor growth inhibitory

and stimulatory functions [218]. Interestingly, PAK4 was previously shown to have a role in the control of cell survival downstream to TNF [66, 72, 73]. TNF can activate NF- κ B [218], a transcription factor that has also been linked to PAK4 activity [66] and that is linked to cell survival and cancer.

In contrast to TNF and IRF7, the PAK4 transcriptome is predicted to be associated with inactivation of the transcriptional regulator TRIM24. TRIM24 binds to nuclear receptors such as the estrogen receptor, and regulates their transcription, and it is a negative regulator of p53 by functioning as an E3 Ligase [219, 220]. The role for TRIM24 in cancer is complex, because while in some cancers, including breast cancer, it is associated with tumorigenesis, in other cancers, such as liver cancer, it may have a tumor suppressive role [219]. It is not yet clear how TRIM24 inactivation would be linked to PAK4 and cancer, but it is important to note that the prediction of TRIM24 inactivation is based in part on the expression patterns of many of the same families of genes described above, that may be associated with the immune system and the TME. These include IRF7, CXCL10, and STAT1 (see Figure 5A), which can all be regulated by TRIM24. It is also important to note that while TRIM24 is predicted to be inactivated, other members of the TRIM family (such as TRIM30a and TRIM30d) were found to be upregulated at the mRNA levels (see Table 1A). TRIM proteins are inducible by interferons so that they may also be linked to immunity and inflammation [221].

TGF-beta is also predicted to be inactivated, according to the PAK4 transcriptome (see Figure 5B). This is based on the expression of a large number

of genes including STAT1 and FOXC2. TGF-beta has been shown to have both a positive and negative effect on the growth of breast cancer cells, depending on the stage and type of cancer [222]. Our results predicted a tumor suppressive role for TGF-beta in PAK4 overexpressing cells.

Finally, IPA predicts the interaction of multiple signaling networks, that could explain the transcriptome results, as shown in Figures 6A and 6B. Figure 6 shows the predicted interactions between several of the proteins addressed here, such as TRIM24 and STAT1, and the genes predicted to be regulated by these pathways, as identified in our sequencing results. The regulation of these pathways would be consistent with regulation of genes such as Wnt10A, which was strongly downregulated in our study, ACP5, which has important roles in the bone and in immune cells, and BMP2, which was downregulated by PAK4 and which has tumor inhibitory activity in some cancers [223]. While the regulatory networks include diverse sets of pathways and genes, a frequent theme is the regulation of pathways that are often associated with chemokines, the immune response, and inflammation (such as STAT1, IRF7, NF- κ B). These pathways are central in regulating the tumor microenvironment (TME) and further studies to validate PAK4 role in TME regulation is warranted.

Our results reveal the PAK4 transcriptome profile in a mammary tumor background and may help understand PAK4 M.O.A. in promoting mammary tumorigenesis. Previous studies in our lab have shown that PAK4 is overexpressed in several subtypes of breast cancer. However, targeting PAK4 using a novel PAK4 inhibitor, KPT-9274, was efficacious in triple negative breast

cancer only. Other breast cancer subtypes, including ER/PR + and HER2 + breast cancer cells, which exhibited high PAK4 levels, had a significantly diminished response to treatment with KPT-9274. This data suggests that PAK4 might function through different signaling pathways to promote mammary tumorigenesis. Delineating the varied effectors of PAK4 signaling pathway will help uncover novel biomarkers for breast cancer, with some serving as potential therapeutic targets. Our study identifies the PAK4 transcriptome profile in mammary tumor forming cells, and can provide translational utility in other types of cancers as well. This will help understand the role of PAK4 in different types of cancers and thereby, help in designing more effective PAK4 targeting compounds.

Acknowledgments

We would like to thank Dr. Li Cai, Dr. Tony Kong and Misaal Patel for providing technical help with utilizing RNA-seq analysis software.

Chapter IV

Significance and Future Prospects

In this thesis, we show for the first time that PAK4 can serve as a novel drug target in triple negative breast cancer (TNBC) therapy. Research from our lab and other groups has shown that PAK4 is overexpressed in different types of cancers, including breast cancer. In my thesis, I have shown that PAK4 is overexpressed in different subtypes of breast cancer cells, while its levels are low in normal mammary tissue. This expression pattern makes PAK4 an attractive drug target.

To validate PAK4 as a drug target in breast cancer, we collaborated with Karyopharm Therapeutics, to test a novel series of PAK4 inhibitors that include the isoforms, KPT-8752 and KPT-9274. My research shows that treatment with KPT inhibitors significantly inhibit PAK4 protein levels and PAK4 associated downstream signaling pathways, and this effect was most significant in triple negative breast cancer cells. Further studies to test the *in vivo* efficacy of KPT-9274, proved that orally bioavailable KPT-9274 is very potent in inhibiting tumor growth in mouse xenograft models of human triple negative breast cancer. This work shows for the first time that PAK4 can be targeted for triple negative breast cancer therapy.

Research to study the effects of PAK4 overexpression in cancer has mostly focused on identifying direct substrates of PAK4 phosphorylation. However, these studies do not take into account the long term effect of PAK4 overexpression on the gene expression pattern responsible for oncogenic transformation. We carried out High Throughput mRNA sequencing (RNA-seq) to reveal the PAK4 transcriptome profile in iMMECs overexpressing PAK4. We

show that PAK4 overexpression in WT iMMECs results in a differential expression pattern of several genes, that were previously unknown to be regulated by PAK4. This study can help understand how PAK4 mediates oncogenic transformation in breast cancer and can provide translational utility to study PAK4 functions in other types of cancers as well.

1. PAK4 as a drug target in triple negative breast cancer therapy

1.1 PAK4 is overexpressed in different breast cancer subtypes.

We carried out western blot analysis of several subtypes of breast cancer cell lines to monitor PAK4 protein expression. We found that PAK4 is overexpressed in BT-474, MDA-MB-231, MDA-MB-468 and SUM159 (Triple Negative), MCF7 (ER+/PR+), SkBr-3 and BT-474 (HER2+) cells, when compared to WT iMMECs (Chapter I, Figure 1).

1.2 Treatment with KPT inhibitors block PAK4 protein levels.

In the past, many PAK4 inhibitors have been developed that target PAK4 catalytic activity. However, PAK4 has both kinase dependent and independent functions in promoting tumor formation. Consequently, inhibitors that block PAK4 kinase activity alone do not completely block its tumorigenic potential. We collaborated with Karyopharm Therapeutics to test a novel series of PAK4 inhibitors which include KPT-8752 and KPT-9274. The KPT inhibitors are different from other PAK4 inhibitors, because they reduce steady state PAK4

protein levels, without inhibiting PAK4 kinase activity. We observed that treatment with KPT inhibitors significantly reduced PAK4 protein levels in TNBC cell lines (Chapter II, Figure 2). Treatment with KPT inhibitors also inhibited PAK4 associated downstream targets, including P-Cofilin and P- β -catenin, and this effect was also the most significant in TNBC cells.

1.3 KPT treatment inhibit cell growth, survival and motility

PAK4 is known to play an important role in regulating cell growth, cell survival and cytoskeletal organization. We monitored the effects of PAK4 inhibition, following treatment with KPT inhibitors, on several breast cancer cells. Our results show that KPT treatment significantly inhibit cell growth and cell motility and strongly induce apoptosis in all the TNBC cells we tested (Chapter II, Figure 3,4 and 5). Interestingly, KPT treatment had a moderate effect on the ER+/PR+ cell line, MCF7, while the HER2+ cells, SkBr-3 and BT-474 did not respond to treatment with KPT inhibitors. These results show for the first time that inhibiting PAK4, using KPT inhibitors, can block cellular processes that are often dysregulated in cancer, including cell proliferation, cell survival and cell migration, specifically in TNBC cells.

1.4 KPT treatment inhibits TNBC tumor growth

Our *in vitro* results clearly suggested that PAK4 inhibition followed by KPT treatment, had the most significant impact on TNBC cells. To validate if KPT-9274 exhibits *in vivo* efficacy against triple negative breast cancer, we treated

mice, subcutaneously implanted with MDA-MB-231, MDA-MB-468 and SUM159 cells, with orally administered KPT-9274. It was very encouraging to see that KPT-9274 significantly inhibited tumor growth in the mouse xenograft models of all three TNBC cell lines (Chapter II, Figure 6). We further tested if the observed *in vivo* efficacy of KPT-9274 is mediated through PAK4 specific inhibition. We found that KPT-9274 significantly inhibited PAK4 protein levels in the TNBC tumors (Chapter II, Figure 7). More importantly, we observed that KPT-9274 did not significantly impact PAK1 levels, a Group I PAK. This data shows for the first time that a PAK4 specific inhibitor, KPT-9274, can be used to therapeutically target triple negative breast cancer.

2. Delineating the PAK4 transcriptome profile

PAK4 has been long known to regulate cellular processes including cell growth, cell survival and cytoskeletal organization; aberrant regulation of these processes is concurrent with oncogenic transformation. Studies in the past have mostly focused on identifying PAK4 substrates of phosphorylation. These phosphorylation events represent the immediate effects of PAK4 overexpression. However, the long term effects of PAK4 overexpression on gene expression is unaccounted for. A better understanding of how PAK4 overexpression regulates transcriptional changes to mediate oncogenic transformation, will help understand the PAK4 signaling cascade. This will help us develop more efficient PAK4 targeting compounds that can be used for cancer therapy.

To get a better understanding of PAK4 signaling pathway in breast cancer, we ran a High throughput sequencing analysis of RNA samples collected from non-transformed iMMECs (WT iMMECs) and iMMECs overexpressing PAK4. Research from our lab has shown that unlike WT iMMECs, iMMECs overexpressing PAK4 undergo oncogenic transformation and can form tumors in mice. RNA-seq analysis from our study generated a list of genes that were previously unknown to be regulated by PAK4. Using several statistical parameters, we were able to identify 1,672 genes that were differentially expressed in response to PAK4 overexpression in mammary epithelial cells. A qPCR analysis validated RNA-seq data (Chapter III Figure 1), suggesting that RNA-seq can be utilized to accurately quantify gene expression changes in our system.

Ingenuity Pathway Analysis (IPA) analyzed the RNA-seq data to predict a strong activation of cancer, along with other cellular processes that are often dysregulated in cancer (Chapter III, Figure 3). We were also to identify the most activated upstream regulators, IRF7 and TGFB1 (Chapter III, Figure 4), and the most inhibited upstream regulators, TRIM24 and TNF (Chapter III, Figure 5), in our dataset. We were also able to identify mechanistic networks for IRF7 and TRIM24, that are most likely responsible for the observed gene expression pattern observed in our dataset (Chapter III, Figure 6).

This work shows for the first time how PAK4 overexpression can mediate transcriptional changes to mediate oncogenic transformation in mammary

epithelial cells. This study can provide translational utility to understand PAK4 signaling cascade in other types of cancers as well.

3. Future Prospects

Triple Negative Breast Cancer (TNBC) is a particularly aggressive subtype of breast cancer linked with poor prognosis. The poor prognosis is associated with the fact that there are no discernible biomarkers available for its treatment. Consequently, the mainstay for triple negative breast cancer therapy is chemotherapy, which has a differential response, depending on the histological grade of the tumors. Hence, there is an urgent need to identify novel biomarkers that can serve as potential therapeutic targets to improve the clinical outcome of triple negative breast cancer treatment.

PAK4 was shown to be overexpressed in different subtypes of breast cancer, including TNBC. Interestingly, treatment with the KPT inhibitors, KPT-8752 and KPT-9274, significantly inhibited PAK4 protein levels, along with PAK4 associated downstream targets, in TNBC cells. Treatment with KPT inhibitors also significantly inhibited cell growth, cell survival and cytoskeletal organization of TNBC cells. Moreover, orally administered KPT-9274 could inhibit TNBC tumor growth *in vivo*. These results point out that PAK4 plays a central role in mediating TNBC tumorigenesis. The lack of response to KPT treatment in the ER+/PR+ cell line, MCF7 and HER2+ cells, Sk-Br3 and BT-474 suggest that PAK4 might not be the central oncogenic stimuli in these cells. It is very likely that ER, PR and HER2 receptors can mediate tumorigenesis through both PAK4

dependent and –independent functions. Preliminary data in our lab has shown that HER2 overexpression in WT iMMECs is accompanied with upregulated PAK4 protein levels (Rane and Minden Unpublished Data). Consequently, we observed that KPT treatment alone was not sufficient to reduce PAK4 protein levels, but a combination of Herceptin and KPT treatment, reduced PAK4 protein levels, and also inhibited cell growth of SkBr-3 and BT-474, more significantly than either agents alone (Rane and Minden Unpublished data). Further studies to investigate a combination of PAK4 targeting compounds with hormone therapy or HER2 targeting compounds, are warranted to test their efficacy in other subtypes of breast cancer.

Results from this project are of great significance as they provide evidence for the first time that PAK4 can be used as a drug target for triple negative breast cancer therapy. KPT-9274, the clinical candidate, has been shown to be effective against other types of cancers as well including oesophageal squamous cell carcinoma, renal adenocarcinoma and pancreatic cancer [169] [173] [172]. Currently, KPT-9274 is under Phase I clinical trials for patients with advanced solid malignancies. Future studies will help validate PAK4 targeting compounds, either alone or in combination, as a therapeutic option to improve cancer therapy.

To get a better understanding of PAK4 signaling cascade in breast cancer, we carried out High Throughput Sequencing (RNA-seq) analysis of RNA samples collected from WT iMMECs and iMMECs overexpressing PAK4. RNA-seq analysis provided *de novo* information about the transcriptome profile of PAK4 overexpressing mammary epithelial cells. We identified 1,672 genes whose

expression is differentially regulated in response to PAK4 overexpression. Using IPA analysis, we were also able to reveal mechanistic networks that are activated and most likely responsible for the observed gene expression pattern, in response to PAK4 overexpression. IPA analysis also predicts that PAK4 overexpression modulates expression pattern of genes that are involved in regulation of chemokine and cytokine secretion and immune system. These pathways are central in regulating tumor microenvironment (TME). Understanding the role of TME in tumor growth and resistance to existing treatment, is extremely crucial in developing clinically efficacious therapies for cancer. Further studies to validate the role of PAK4 in regulating TME are warranted and can help delineate a *de novo* function of PAK4 in tumorigenesis.

Our results reveal the PAK4 transcriptome profile in a mammary tumor background and identifies several genes that were previously unknown to be regulated by PAK4. It is difficult to predict whether the observed gene expression pattern is a direct effect of PAK4 overexpression or a secondary effect mediated by oncogenic transformation. Genes such as FOXC2 and ParvB, identified in our dataset, have been previously shown to be involved in regulating cell migration and proliferation in cancer. Future studies to investigate their role in the PAK4 signaling cascade are ongoing and will further validate our sequencing data. Another interesting finding of our study was to identify pathways that play an important role in regulation of tumor microenvironment (TME). The TME is a complex ecosystem, consisting of tumor cells and several other cellular components, that plays an important role in maintaining a chronically inflamed

state to protect tumor cells from immune surveillance and immune destruction.

Whether PAK4 has a direct effect on TME or not, is a question that warrants further investigation. This study proposes a PAK4 signaling cascade that is responsible for the oncogenic transformation of WT mouse mammary epithelial cells, and can provide translational utility in other types of cancers as well.

Delineating the varied effectors of PAK4 signaling pathway will help uncover novel biomarkers for cancer, with some serving as potential therapeutic targets.

Chapter V

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