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## PAK Signaling in Oncogenesis

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### Abstract

The p21-activated kinase (PAK) family of serine/threonine kinases plays a pivotal role in physiological processes including motility, survival, mitosis, transcription and translation. PAKs are evolutionally conserved and widely expressed in a variety of tissues and are often over expressed in multiple cancer types. Depending on structural and functional similarities, the six members of PAK family are divided into two groups with three members in each group. Group I PAKs are activated by extracellular signals through GTPase-dependent and independent mechanisms. In contrast, group II PAKs are constitutively active. Over the years, accumulating data from tissue culture models and human tumors has increased our understanding about the biology of PAK family members. In this review, we have summarized the complex regulation of PAK and its downstream diverse myriads of effectors which in-turn are responsible for the biologic effects of PAK family of kinases in cancer cells.

### Keywords

PAK; cancer; cytoskeleton; survival; mitosis; knockout mice

## INTRODUCTION

The salient features of a cancer cell include loss of homeostasis, altered cytoskeleton dynamics, uncontrolled cell proliferation, escape from apoptotic signals, and deregulated gene products. A plethora of evidence points to roles for the p21-activated kinase (PAK) family of serine/threonine kinases in each of the above processes. PAKs are either up-regulated or hyper-activated in a variety of human cancers such as breast, ovary, colorectal, thyroid and pancreatic (Kumar et al. 2006). In a mouse model, PAK1 hyper-activation is sufficient to form mammary gland tumors (Wang et al. 2006; Wang et al. 2003). Although it appears promising to consider PAKs as potential therapeutic targets for interrupting cancer progression, designing a PAK-specific inhibitor has been a challenge, in part owing to the

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functional similarities among the PAK family members. Effective targeting of PAKs will depend on our knowledge of PAK's activation and its impact on downstream signaling cascades leading to phenotypic changes relevant to tumor development and progression.

PAK was initially identified as a binding partner of the Rho GTPase Cdc42 and Rac1 (Manser et al. 1994). Thus far, six PAK family members have been identified in mammalian cells (Hofmann et al. 2004) (Figure 1). On the basis of structural and functional similarities, the PAK family can be categorized into two subgroups, with three members in each. Group I includes PAK1, PAK2, and PAK3 and group II includes PAK4, PAK5, and PAK6 (Figure 2). All PAKs consist of a C-terminal kinase domain and an N-terminal regulatory domain containing a GTPase binding domain and an inhibitory domain. However, group I and II PAKs share only about 50% of the GTPase binding and kinase domains (Bokoch 2003; Kumar et al. 2006). Group I PAKs are activated by GTPases such as Cdc42, Rac, TC10, CHP, and Wrch-1, as well as in a GTPase-independent manner.

### Group I PAKs

The group I PAKs exist as inactive homodimers, wherein the two kinase domains from two different molecules inhibit one another (Pirruccello et al. 2006). The kinase inhibitory domain (KID) of PAK1 binds to the kinase domain of its counterpart and keeps it in an inactive state. GTP-bound forms of Cdc42 and Rac bind to the regulatory domain of the kinase and displace it, thereby allowing phosphorylation of the kinase domain. Conserved residues within the N-terminal p21-binding domain (PBD) participate in the binding and activation by the small GTPases. There is a short lysine-rich stretch (PAK1 residues 66–68) just upstream of the Cdc42 and Rac interactive binding (CRIB) domain within the PBD region for effective binding of the small GTPases (Lei et al. 2000). In addition to the PBD domain, the regulatory domain contains two canonical PXXP Src homology 3 (SH3) binding motifs and a non-classical SH3 binding site for PAK-interacting exchange (PIX) factor. The first canonical SH3 site has the capability to bind to the adaptor protein Nck, while the second SH3 site can bind Grb2. Membrane recruitment of PAK1 via SH3-containing Nck and Grb2 adaptor proteins results in the stimulation of its kinase activity through either phosphorylation by 3-phosphoinositide-dependent kinase-1 (PDK1) or interaction with lipids such as sphingosine or phosphatidic acid (Bokoch 2003). PAKs can also be activated by G-protein-coupled receptor kinase-interacting target 1 (GIT1), which associates indirectly with PAK via the focal adhesion-associated protein PIX (PAK interacting exchange factor, through a mechanism that does not require the small GTPases (Bokoch 2003; Hoefen and Berk 2006). In addition, PAK1 can be directly activated by Akt, and PAK2 can be activated through cleavage by caspase 3 (Zhou et al. 2003; Walter et al. 1998).

To date, seven autophosphorylation residues have been mapped for PAK1: Ser-21, Ser-57, Ser-144, Ser-149, Ser-198, Ser-203, and Thr-423. Of these, auto-phosphorylation of Thr-423 is important for counteracting auto-inhibition and maintaining a full catalytic function toward its substrates. Phosphorylation of Ser-21 and Ser-144 also contributes to kinase activation, whereas auto-phosphorylation of Ser-198 and Ser-203 in PAK1 serves to down-regulate PIX–PAK interaction (Chong et al. 2001). PAK2 is autophosphorylated at eight sites: Ser-19, Ser-20, Ser-55, Ser-192, and Ser-197 Ser-141, Ser-165, and Thr-402. Of these,

the first six sites are autophosphorylated by MgATP alone while the latter three sites-Ser-141, Ser-165, and Thr-402 are selectively phosphorylated upon PAK2 activation (Jung and Traugh 2005; Gatti et al. 1999). Irrespective of the mode of activation, activated PAKs phosphorylate their substrate/effector proteins, which in turn activate various biological functions (Figure 3).

### **Cytoskeleton remodeling a prerequisite for invasion**

The high mortality rate associated with cancer is due to tumor metastasis, which involves the invasion of primary tumor cells through tissue and the extracellular matrix (ECM) to distant sites, a process that requires cytoskeleton remodeling. A complex interplay of signaling cascades triggers changes in the cytoskeletal dynamics, leading to a motile phenotype, and PAKs are considered prime regulators of the actin cytoskeleton and motility (Sells et al. 1997). However, depending upon the precise protein-protein interactions and the regulation of PAK at the plasma membrane, PAK has different effects on the actin cytoskeleton and cell motility. Overexpression of activated PAK mutants induces loss of stress fibers and focal adhesion complexes, whereas modest levels of PAK mutant expression induces polarized lamellipodia and increased cell motility (Manser et al. 1997; Sells et al. 1997). The basis for these differences in phenotype is not completely understood but involves the phosphorylation of multiple downstream effectors that affect cytoskeletal structure, including myosin light-chain kinase (MLCK), paxillin, filamin A, cortactin, the PIX/COOL guanine nucleotide exchange factors, the LIM-kinases, Arpc1b, stathmin, and tubulin cofactor B, many of which are up-regulated in human tumors (Kumar et al. 2006). Therefore, unraveling the molecular mechanisms responsible for the temporal and spatial regulation of PAK-interacting proteins is important for a better understanding of the mechanistic contribution of PAKs in cellular transformation.

In mammalian cells, non-muscle myosins are regulated by phosphorylation of their light chain (MLC) on Ser-19 by MLCK. The Rho target-p160 Rho kinase phosphorylates and inhibits the MLC phosphatase activity, leading to the accumulation of phosphorylated MLC and, thereby, the increased contractility necessary for actin stress-fiber formation and cell spreading (Totsukawa et al. 2004). Activated PAK phosphorylates MLCK and inhibits its activity towards MLC, leading to reduced stress-fibers (Chew et al. 1998). However, the effect of PAK on myosin activity appears to be cell-type specific.

PAK also regulates cell motility through regulation of focal adhesion dynamics. Paxillin, a multi-domain protein, localizes to focal adhesions and forms a structural link between the extracellular matrix (ECM) and the actin cytoskeleton and serves as an important site for signal transduction. In fact, paxillin functions as a scaffold protein, providing multiple docking sites for an array of structural proteins and signaling kinases such as FAK and Src in the focal adhesion. Phosphorylation of residues in the N-terminus of paxillin by these kinases regulates recruitment of downstream effector molecules like vinculin, ARF, GAP, PKL, the exchange factor PIX, and PAK to the focal adhesion (Turner 2000). Once recruited, PAK phosphorylates paxillin on Ser-273, resulting in increased paxillin-GIT1 binding and localization of a GIT1-PIX-PAK signaling module near the leading edge, which culminates in a dramatic increase in migration, protrusion, and adhesion turnover (Hoefen

and Berk 2006). PAK1 regulates actin dynamics at the leading edge of the motile cells by phosphorylating yet another substrate, filamin A. Interestingly, filamin A, in turn, stimulates PAK1 activity, leading to local activation of PAK (Vadlamudi et al. 2002).

Both PAK1 and PAK2 associate with another downstream effector, LIM-kinase (LIMK), which is overexpressed in breast and prostate cancer (Bagheri-Yarmand et al. 2006; Davila et al. 2003; Yoshioka et al. 2003). PAK activates LIMK by phosphorylating it at Thr-508, a residue within its activation loop. Activated LIMK phosphorylates cofilin, the actin-binding protein, and inactivates its F-actin-depolymerizing activity, thus modulating actin dynamics and cell motility (Edwards et al. 1999; Misra et al. 2005).

Following the loss of contractile constraints, the next phase in cancer cell metastasis involves degradation of the ECM. Actin structures are connected to the ECM via focal adhesions or podosomes. Typically, cells expressing focal adhesions display lower rates of motility, whereas invasive cells show the formation of dynamic F-actin-rich adhesion structures called podosomes. The speed of podosome assembly and disassembly generates high rates of motility, and the release of matrix metalloproteinases (MMPs) from podosomes facilitates degradation of the ECM (Block et al. 2008). Both PAK1 and PAK2 regulate migration and invasion by inhibiting the formation of podosomes via phosphorylation of caldesmon, one of the key regulators of actin dynamics that localizes to the podosomes (Morita et al. 2007). Actin dynamics also depend on Arp2/3 protein complex, an actin filament nucleating and organizing regulator. PAK1 can phosphorylate the Arpc1b subunit of this complex, which stimulates Arp2/3 complex assembly and regulates the directional motility of breast cancer cells (Vadlamudi et al. 2004b). Cortactin, an F-actin binding protein, is enriched in dynamic cytoskeletal organelles such as podosomes, membrane ruffles, and lamellipodia and is a substrate for PAKs (Webb et al. 2006). Furthermore, the *cortactin* gene is often amplified in breast cancer and in head and neck squamous cell carcinoma and is associated with lymph node metastasis and poor prognosis (Buday and Downward 2007). PAK-mediated phosphorylation of cortactin reduces its binding affinity for F-actin and for Arp2/3 and affects the stability of branched actin filaments (Lua and Low 2005).

Migration involves dynamic changes not just in the actin cytoskeleton but also in the microtubule network. Stathmin, also called oncoprotein 18 (OP18), is a microtubule-destabilizing protein that is overexpressed in sarcomas and contributes to local tumor invasiveness (Cassimeris 2002; Belletti et al. 2008). PAK1 phosphorylation of stathmin inactivates it, which results in the stabilization of microtubules at the leading edge of motile cells (Wittmann et al. 2004). PAK1 also regulates microtubule dynamics by phosphorylating tubulin cofactor B, which contributes to  $\alpha$ - and  $\beta$ -tubulin heterodimer assembly and is often upregulated in human breast tumors (Vadlamudi et al. 2005). In recent years, yet another PAK effector, guanylyl cyclase, has been shown to regulate cell motility. Guanylyl cyclases catalyze the conversion of GTP to the second messenger, cyclic GMP (cGMP), which has also been implicated in the regulation of cell motility. Although PAK activity is required to promote guanylyl cyclase activity, guanylyl cyclases do not appear to be phosphorylated by activated PAK. In fact, it appears to be an indirect event, wherein activated Rac promotes a conformational change in PAK that enables it to bind to guanylyl cyclases and, presumably,

promote a subsequent conformational change in the guanylyl cyclase that leads to its activation (Settleman 2007). Thus, PAKs act as signaling nodules that link upstream stimuli to a very complex network of signal transduction pathways, resulting in cytoskeletal remodeling.

### Cytoskeleton-regulated gene expression

In addition to its roles in cytoskeletal remodeling, cell motility, and invasion, PAK also influences cancer cell biology via its nuclear functions. Tumor progression involves the activation and repression of various genes that play roles in essential cellular processes. It is not clear whether PAKs participate in gene expression in the nucleus, but nuclear translocation of PAK in response to stimulus and a direct association between PAK1, presumably as a part of the PAK-multi-protein complex, and specific gene chromatin and enhancer elements partly explain PAK-dependent gene transcription and translation (Singh et al. 2005).

Several transcription factors and transcriptional coregulators have been identified as PAK1-interacting substrates, including the forkhead transcription factor (FKHR), estrogen receptor  $\alpha$  (ER $\alpha$ ), SHARP, C-terminal binding protein 1 (CtBP1), and Snail homologue 1 (SNAIL). Of these, both CtBP1 and Snail play a role in the process of epithelial-mesenchymal transition (EMT) (Come et al. 2004; Grooteclaes and Frisch 2000). Although EMT is important in many developmental processes, such as gastrulation and neural crest migration, its deregulation can lead to tumor progression, and EMT is often seen in cells with PAK overexpression or PAK hyperactivation (Yang et al. 2005). PAK1 also phosphorylates ER at Ser-305 and promotes its transactivation functions, leading to increased cyclin D1 expression and conferring growth advantage and hormone independence to breast cancer cells (Nheu et al. 2004; Rayala et al. 2006). PAK1-mediated phosphorylation of PCBP1 on Thr-60 and Thr-127 also stimulates transactivation of the initiation factor (eIF)4E gene promoter and pre-mRNA splicing (Meng et al. 2007). In addition to its role in the regulation of transcription, PAK1 also regulates translation. Activation of PAK2 leads to the binding and phosphorylation of eIF4G, which inhibits the association of eIF4E with m(7)GTP, reducing translation efficiency (Ling et al. 2005).

### PAK signaling and cell-cycle progression

The cell cycle is a tightly regulated process that involves a coordinated orchestra of multiple regulators, and disruption of such regulatory steps leads to uncontrolled cell-cycle progression. A large body of evidence indicates that phosphorylation plays a pivotal role in the cell cycle checkpoints, and therefore, it is not surprising that kinases such as cyclin-dependent kinases, Plk1, Aurora kinases, and PAKs might contribute to the process of tumorigenesis by affecting cell-cycle progression (Schatten 2008).

PAK1 is phosphorylated at Thr-212 by cyclin B1/Cdc2 during mitosis in mammalian fibroblasts (Banerjee et al. 2002; Thiel et al. 2002). This phosphorylation is unique to PAK1 because Thr-212 is not conserved in PAK2 or PAK3. The mitotic phosphorylation of PAK1 does not alter the ability of the small GTPase to stimulate the PAK kinase, but it alters its association with the binding partners that play a role in morphologic changes associated with

cell division. PAK1 also co-localizes with histone H3 in condensing chromatin and phosphorylates it on Ser-10, which is required for transcription activation as well as for the onset of mitosis (Li et al. 2002). In mitotic cells, phosphorylated PAK1 is preferentially localized on centrosomes and spindles, prompting a long period of speculation that PAK1 might play an important role in centrosome dynamics. Zhao *et al.* showed that the PIX/GIT1 complex (but not Cdc42) plays a role in localizing PAK to the centrosome, where it undergoes activation (Zhao et al. 2005). Once activated, PAK dissociates from the PIX/GIT1 complex and binds to and phosphorylates Aurora-kinase A on Thr-288 and Ser-342, the key sites for Aurora kinase activation and polo-like kinase 1 (Plk1) on Ser-49 in mitosis (Maroto et al. 2008; Zhao et al. 2005). As mitosis progresses, PAK1 localizes to the spindle midbody and finally to the contractile ring during cytokinesis (Li et al. 2002). Thus, PAK might participate in all phases of the cell cycle.

### Cytoskeleton-modulated cell proliferation or apoptosis

In the normal cell, DNA is duplicated during mitosis and distributed equally between the two daughter cells. Cancer cells, however, owing to the over-expression and hyperactivation of mitotic kinases such as PAK, often display aneuploidy due to improper segregation of chromosomes (Vadlamudi et al. 2000). Typically, cells with mitotic catastrophe undergo apoptosis and are eliminated; however, some cells develop tolerance to become polyploidy and continue to proliferate and give rise to cancerous phenotypes. Depending on the cellular context, PAKs have been shown to either promote cell growth or push cells to apoptosis. Multiple mechanisms exist through which PAK1 promotes cell survival. In the presence of a cell survival signal, PAK1 phosphorylates Bad on Ser-112 and Ser-136, inhibits its interaction with the Bcl2 family members, and exerts anti-apoptotic actions (Schurmann et al. 2000). In addition, PAK1 interacts with dynein light chain 1 (DLC1), which typically sequesters BimL and prevents it from inactivating the survival functions of Bcl2 (Vadlamudi et al. 2004a). However, under apoptotic stimuli, DLC1–BimL dimers are released from the dynein motor complex, which frees BimL to inhibit Bcl2 (Bouillet et al. 2002; Puthalakath et al. 1999). PAK1 phosphorylates DLC1 and BimL and triggers their degradation, thus blocking the pro-apoptotic signal of BimL (Vadlamudi et al. 2004a). PAK1 also inhibits apoptosis by phosphorylating and inactivating FKHR. Interestingly, FKHR phosphorylation is accompanied by its cytoplasmic accumulation and its inability to activate pro-apoptotic target genes (Mazumdar and Kumar 2003). PAK1-mediated activation of cellular pathways also leads to an enhanced cell survival.

Unlike PAK1, PAK2 (also known as gamma-PAK) has a dual role and regulates both cell survival and cell death pathways, depending on the milieu. PAK2 is activated through at least two signaling pathways: one requires phosphoinositide 3-kinase (PI3K) and/or AKT activity, while the other requires caspase activity (Roig and Traugh 1999; Walter et al. 1998). Cellular stresses such as DNA damage, hyperosmolarity, and serum starvation activate the PAK2 enzyme to generate a proteolytic fragment, the PAK-2p34 (Roig and Traugh 1999; Ling et al. 2005). Binding of Cdc42 to full-length PAK2 translocates PAK2 to the endoplasmic reticulum, where it is autophosphorylated and activated. Activation of full-length PAK2 promotes cell survival by phosphorylating Bad and reducing the interaction between Bad and Bcl-2 or Bcl-x(L), which increases the association between Bad and



14-3-3tau, leading to cell survival (Jakobi et al. 2001). In contrast, proteolytic activation of PAK-2p34 leads to apoptosis (Jakobi et al. 2003). In addition, this pathway is also negatively regulated by PS-GAP, a GTPase-activating protein (GAP) that inhibits apoptosis by interacting with and suppressing PAK-2p34-associated kinase activity (Koeppel et al. 2004).

The third member of the group I PAKs, PAK3, is primarily expressed in the brain. The *PAK3* gene encodes four isoforms, PAK3a, PAK3b, PAK3c and PAK3cb of 544, 559, 622 and 667 amino acids, respectively. PAK3c and PAK3cb are newly identified isoforms that include insertion of exons in the PBD/KI (p21-binding domain/kinase inhibitory) domain which strongly increase the kinase activity and modify the GTPase binding to these isoforms (Kreis et al. 2008). PAK3b isoform has an insertion in the PBD/KI sequence, but it is outside the GTPase- Cdc42 and Rac binding domain, allowing the kinase to be active in the absence of GTPase binding. While, PAK3a is activated by both Rac and Cdc42 GTPases. Although Rac is a weak activator of PAK3a, it plays an important role in recruiting PAK3a to the membrane. In contrast, Cdc42 recruits and activates PAK3a but only recruits PAK3b to the membrane because it is already active (Rousseau et al. 2003).

Similar to the other two group I PAK members, PAK3 plays a role in the regulation of cytoskeleton dynamics. Of the many focal adhesion proteins, only paxillin- $\alpha$  and - $\beta$  isoforms interact with PAK3 and link both the kinase-inactive and activated forms of PAK3 to integrins (Hashimoto et al. 2001). Another effector of PAK3 is Raf-1. PAK3 phosphorylates Raf-1 on Ser-338, leading to Raf activation independent of Ras GTPase (King et al. 1998). PAK3 also confers a survival advantage to cancer cells by activating NADPH oxidase (an enzyme that regulates the intracellular source of reactive oxygen species [ROS]) and altering the redox potential of the cells. The redox state is governed by the balance between ROS and the antioxidant levels in the cell. PAK1 has a crucial role in the regulation of both NADPH oxidase and metabolic pathways. PAK1 phosphorylates the p47 (phox) subunit (Knaus et al. 1995), whereas PAK3 phosphorylates the p67 (phox) subunit of NADPH oxidase (Ahmed et al. 1998), which leads to the activation of NADPH oxidase.

Interestingly, of all the PAKs, PAK3 is the only one known to be associated with a human genetic disease. Mutations in the *PAK3* gene are associated with X-linked, non-syndromic mental retardation (MRX) syndromes. Three types of mutations have been reported- MRX30, R67C, and A365E. MRX30 mutation generates a truncated, kinase-dead mutant. R67C mutation is expected to affect GTPase binding, whereas the A365E mutation affects a highly conserved region within the protein kinase domain (Bienvenu et al. 2000; Gedeon et al. 2003).

## Group II PAKs

Group II PAKs are structurally distinct from group I PAKs (Figure 2). They contain an N-terminal PBD and a C-terminal kinase domain, but lack other motifs found in group I PAKs. The degree of similarity between the PBD and kinase domains in the group II PAKs is much less than that in the group I PAKs. PAK4-6 bind to activated Cdc42 and, to a lesser extent, to Rac, but the activity of these kinases is not appreciably enhanced upon binding to the GTPases. In fact, interaction with Cdc42 induces the translocation of group II PAKs to

different cellular compartments. Binding to Cdc42 results in PAK4 translocation to the Golgi apparatus (Abo et al. 1998; Callow et al. 2002). PAK5 localizes to mitochondria and the nucleus but in a Cdc42-independent manner (Cotteret et al. 2003; Cotteret and Chernoff 2006; Wu and Frost 2006). Thus, unlike with the group I PAKs, the interaction of group II PAKs with GTPases has no influence on kinase activity. However, similar to the group I PAKs, group II PAKs play important roles in cell motility and survival.

PAK4, the first identified member of group II PAK, is a target for Cdc42 and undergoes autophosphorylation on Ser-474 (Abo et al. 1998). In cellular studies, PAK4 has a role in oncogenic transformation with PAK4 activity is required for Ras-driven, anchorage-independent growth (Callow 2002). Expression of active PAK4 (S474E) mutant has transforming potential, leading to anchorage-independent growth of NIH3T3 cells. A kinase-inactive PAK4 (K350A, K351A) efficiently blocks transformation by activated Ras and inhibits anchorage-independent growth of HCT116 colon cancer cells. Expression of activated PAK4 in fibroblasts leads to a transient induction of filopodia, dissolution of stress fibers, and loss of focal adhesions (Qu et al. 2001). The reorganization of the actin cytoskeleton is dependent on PAK4 kinase activity and on its interaction with Cdc42. PAK4 also regulates cytoskeletal changes through the modulation of LIMK1's activity. Activated PAK4 phosphorylates LIMK1 and stimulates its ability to phosphorylate cofilin (Dan et al. 2001). Interestingly, fibroblasts expressing activated PAK4 show oncogenic transformation (Cammarano et al. 2005). In primary fibroblasts, activated PAK4 inhibits cell proliferation and promotes premature senescence. Activated PAK4 also protects cells against apoptotic cell death by phosphorylating the pro-apoptotic protein Bad and by inhibiting caspase activation (Gnesutta et al. 2001; Gnesutta and Minden 2003). PAK4 mediates morphological changes through its association with the Rho-family guanine nucleotide exchange factor GEF-H1 (Callow 2005). GEF-H1 is involved in microtubule dynamics and when mutated causes oncogenic transformation (Krendel et al. 2002; Brecht et al. 2005). In humans, PAK4 is expressed at low levels in normal tissue, but its expression is up-regulated in a large variety of human tumor cell lines (Callow et al. 2002) and Ras-dependent human tumors (Parsons et al. 2005; Chen et al. 2008; Kimmelman et al. 2008).

PAK5 (also known as PAK7) is highly expressed in the brain and contains an N-terminal CRIB motif and a C-terminal kinase domain but lacks a PAK inhibitory domain. PAK5 also contains an auto-inhibitory fragment similar to that of PAK1, which is absent from the other group II PAK family members (Pandey et al. 2002). PAK5 is structurally related to PAK4 and PAK6 within its CRIB domain. PAK5 binds to the GTPases Cdc42 and Rac, but these GTPases do not regulate PAK5 kinase activity. PAK5 is constitutively active, but its autophosphorylation can be further stimulated by the GTP-bound form of Cdc42. PAK5 operates downstream of Cdc42 and Rac and antagonizes Rho in the pathway, leading to filopodia formation and neurite outgrowth (Dan et al. 2002). PAK5 also stabilizes microtubules by negatively regulating MARK2, a kinase that promotes microtubule disruption by phosphorylating microtubule-associated proteins such as tau (Timm et al. 2006). PAK5 has different effectors depending on its localization. In the cytosol, PAK5 activates the JNK kinase pathway, and in the mitochondria it plays a role in survival signals (Cotteret and Chernoff 2006; Dan et al. 2002). PAK5 protects cells from apoptosis by phosphorylating Bad on Ser-112 and preventing its localization to mitochondria (Cotteret et al. 2003).



Although PAK5 itself is constitutively localized to mitochondria, this localization is independent of kinase activity or Cdc42 binding. The role of PAK5 in cancer was not known until a recent study showed that point-mutated PAK5 contributed to human cancers (Greenman et al. 2007).

On the basis of its homology to the PAK family, PAK6 was originally cloned from prostate cancer cells as an androgen receptor (AR)-interacting protein (Yang et al. 2001). Pak6 is over expressed in both primary and metastatic prostate cancer cells and contributes to prostate cancer development and progression post androgen deprivation therapy (Kaur et al. 2008). PAK6 showed very high, although restricted expression pattern with PAK6 detected in brain, testis, prostate, and placenta (Callow, 2002). In most of the tumor cell lines, PAK6 was expressed at low levels or was not detectable. For example, two of the eight colon cancer cell lines exhibited high PAK6 mRNA levels, while PAK6 expression could not be detected in normal colon tissue (Callow, 2005). Like PAK4, PAK6 possesses constitutive basal kinase activity, and its activity is not modulated by binding to active Rac or Cdc42 but is stimulated by binding to AR. PAK6 interacts not only with AR but also with ER $\alpha$ . PAK6 interaction with ER $\alpha$  leads to the repression of ER $\alpha$  transcriptional activity (Lee et al. 2002). PAK6 is primarily localized in the cytoplasm, but in the presence of ligand AR, PAK6 translocates to the nucleus. Moreover, the binding of activated PAK6 to AR inhibits AR from binding to transcriptional coactivators, as well as inhibiting its transactivation function (Yang et al. 2001); thus, PAK6 plays a crucial role in steroid-hormone-mediated signal transduction.

### Lessons from PAK-null mice

Although data from tissue culture model systems have provided deep insights into the complex regulation and functions of the PAK signaling pathways, we have just begun to utilize PAK-null mice to study the role of PAK in vivo (Figure 4). PAK1 knockout mice are viable, healthy, and fertile but have a defective immune response. In contrast, PAK2 knockout mice are embryonically lethal due to multiple developmental abnormalities (Arias-Romero and Chernoff 2008). Since PAK3 expression is brain specific, it is not surprising that PAK3 knockout mice show impaired synaptic plasticity and deficiencies in learning and memory (Meng et al. 2005). Similar to PAK2 knockout, the PAK4 knockout model is embryonically lethal, but these mice probably die of a heart defect. The embryos from PAK4 knockout mice also show neuronal abnormalities such as defective neuronal differentiation and migration of motor neurons of the developing spinal cord and neural tube. In addition, the neural tube in these mice is improperly folded (Qu et al. 2003). The phenotype of the PAK5-null mice is completely different from that of PAK4-null mice. Owing to the role of PAK5 in neurite outgrowth in neuroblastoma cells, one would expect some degree of neuronal defects in PAK5-null mice, but the mice develop normally and are fertile (Li and Minden 2003). To date, there has been no published report of a PAK6 knockout mouse model. The expression patterns of PAK5 and PAK6 are very similar, and PAK6 mRNA levels are significantly higher than those of PAK4 in the adult mouse brain, indicating that these kinases can have overlapping functions and can compensate for the absence of one another. In brief, among the PAK family, only PAK2 and PAK4 are essential for development.

## Negative regulation of the PAK pathway

Although we have learned a lot about the positive regulation and functions of PAKs, our understanding of the endogenous negative regulators of PAKs continues to lag behind. Two closely related human protein phosphatases, POPX1 and POPX2, efficiently dephosphorylate PAK1. POPX1 and POPX2 bind to various forms of PIX and PAK1-containing complexes, allowing PAK to cycle rapidly between active and inactive states (Koh et al. 2002). Similarly, PAK3 is identified as a substrate for protein phosphatase (PP) 1 and 2A and a Mg(2+)/Mn(2+)-dependent phosphatase(s). PP1 $\alpha$  and PP2A dephosphorylate Thr-421 in the activation loop of PAK3, whereas PP1 $\alpha$ , PP2A, PP2B, and PP2C dephosphorylate PAK3 at Ser-139 (Zhan et al. 2003). One newly identified mechanism is a negative-inhibitory loop generated by Cdc42 homologous protein (Chp). Chp inhibits PAK1 functions through ubiquitination and proteasome-mediated degradation. Chp-induced degradation of PAK depends on its p21-binding domain and kinase activity and on the autophosphorylation of PAK1, but not on the PIX and Nck binding sites (Weisz et al. 2007). Another PAK-interacting protein, hPIP1, negatively regulates PAK kinase activity by blocking auto-activation and/or interactions with GTPases. hPIP1 contains G protein beta-like WD repeats and shares sequence homology with Skb15 (the fission yeast PAK regulator) and MAK11 (the budding yeast protein) (Xiao et al. 2002). Similarly, the neurofibromatosis type 2 (NF2) protein Merlin interacts with the N-terminal regulatory domain of PAK1 and inhibits Cdc42/Rac1-stimulated kinase activity (Xiao et al. 2002). The Cdc2-related kinase PITSLRE and the  $\alpha 5\beta 1$ -integrin binding partner Nischarin also inhibit the ability of PAK1 to phosphorylate substrates (Alahari et al. 2004; Chen et al. 2003).

## Therapeutic challenges

Due to the signal-dependent activation of group I PAKs, designing specific inhibitors to interfere with upstream regulators of PAK has been somewhat successful. Several small molecule inhibitors such as CEP-1347, SRC and ETK tyrosine kinase inhibitors, AG 879, and FK228 have been shown to effectively inhibit PAK activity in a variety of experimental systems (Nheu et al. 2004; He et al. 2004; Hirokawa et al. 2005). More recently, PAK1 activation has been also targeted by allosteric inhibitor such as IPA-3 (Deacon et al. 2008). However, it remains to be seen whether these PAK inhibitors are also effective and exhibits desired target selectivity in physiologic whole-animal setting. Given the diversity and overlapping nature of PAK regulators and effectors, it is likely that some of these inhibitors will have to overcome the expected problem of specificity and redundancy before these agents could move to clinical development. Targeting group II PAKs is a bigger challenge because they do not require external stimuli for activation and are constitutively active. While our present understanding of PAK signaling points to interesting possibilities for PAK therapy, further studies are required to fully elucidate PAK functions in normal and cancer cells. It is expected that the development of inhibitors to this family of enzymes will further unravel the role of PAKs in tumorigenesis and help to establish the PAK family as therapeutic targets.

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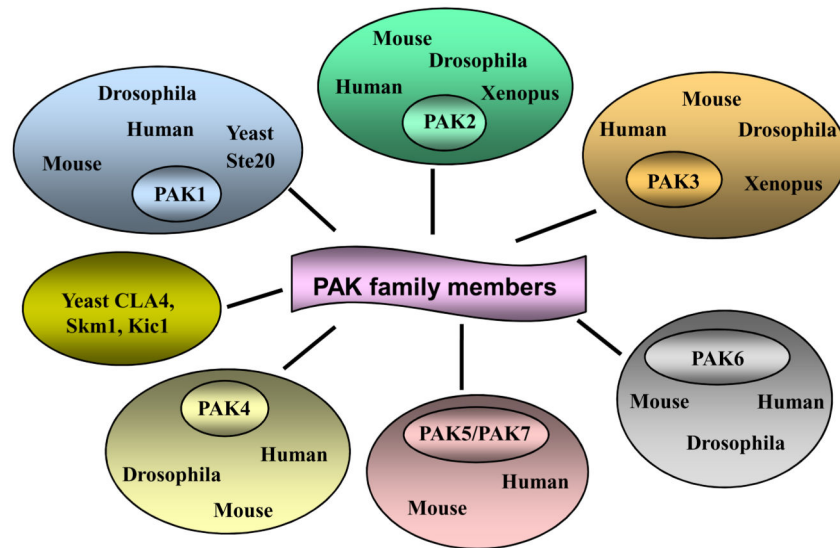
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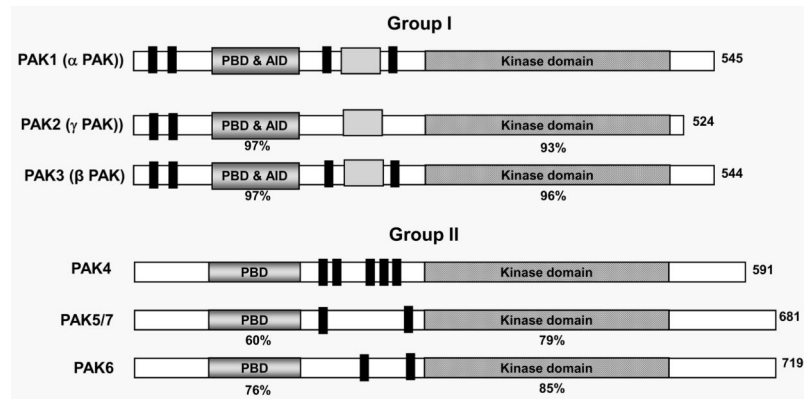
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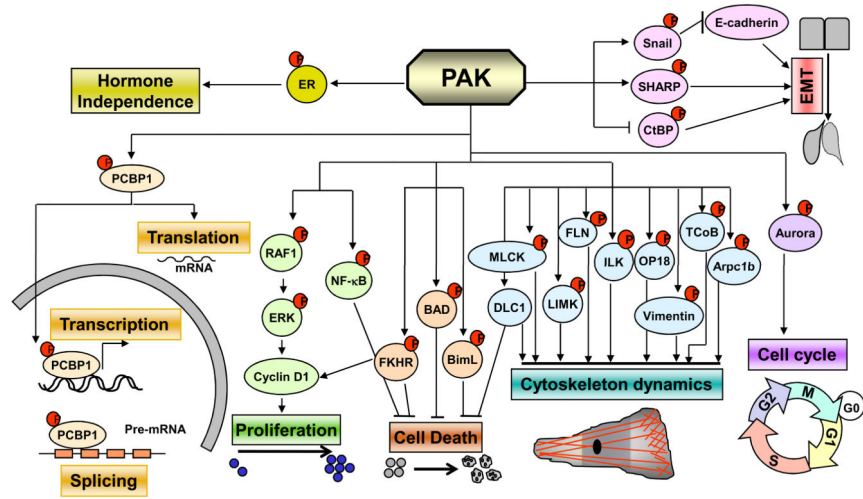
**Figure 1. PAK family members**

PAKs are evolutionarily conserved. On the basis of structural and functional similarities, the six members of human PAK family can be categorized into two subgroups- group I consisting of PAK1, PAK2, PAK3 and group II consisting of PAK4, PAK5/PAK7 and PAK6. The PAK homologues in lower organism with functional similarities are included in each of the subgroups.



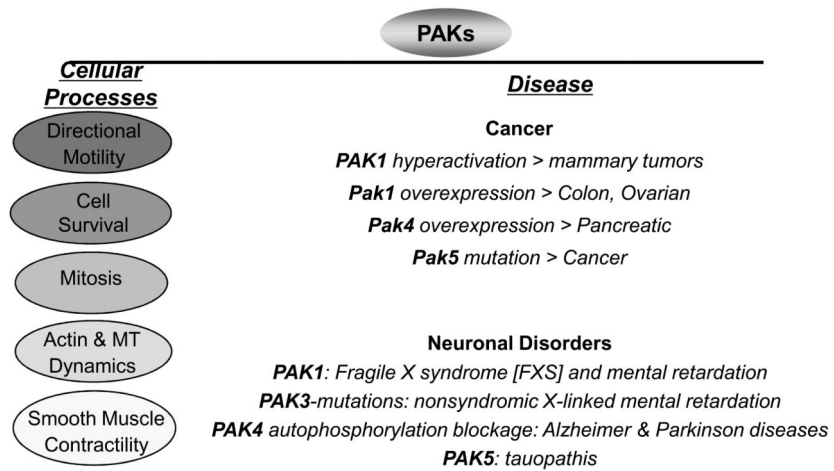
**Figure 2. Structural domains of group I and group II PAKs**

The group I PAKs contain a conserved PBD/AID. Binding of GTPases- Cdc42 and rac to the PBD releases it from the kinase domain. The group II PAKs contains a PBD but lacks AID. Only group I PAKs have PIX binding region but all PAKs have conserved proline-rich motifs. Percentage similarities within the group for PBDs and kinase domains are indicated. PBD, p21 binding domain; AID, autoinhibitory domain; ■ proline rich region; □ PIX binding domain.



**Figure 3. PAK stimulation of signaling cascades**

PAKs activated by extracellular signals participate in various signaling networks. PAK activate the mitogen-activated protein kinase (MAPK) pathway by phosphorylating Raf1 in addition to nuclear factor  $\kappa$ B (NF $\kappa$ B). PAKs also phosphorylates a number of regulators of the cytoskeleton such as myosin light chain kinase (MLCK), LIM domain kinase (LIMK), filamin A, integrin linked kinase (ILK), merlin, Arpc1b, tubulin co-factor B (TCoB) and stathmin (OP18). In addition, PAKs regulate survival and apoptotic pathways through phosphorylation of its effectors such as dynein light chain 1 (DLC1), BimL and the pro-apoptotic protein BAD. On translocation to the nucleus, PAK directly affect gene transcription. Several transcription factors and transcriptional co-regulators such as FKHR, SHARP, C-terminal binding protein 1 (CTBP1) and Snail homologue 1 (SNAI1) are substrates to PAK1. PAK1 co-ordinates transcription, splicing and translation by phosphorylating Poly C-binding protein 1 (PCBP 1). PAKs also regulate cell cycle progression through phosphorylation of histone H3, Aurora A and polo like kinase 1(PIK1).



**Figure 4. Altered expression of the PAK family members**

Cell proliferation, differentiation and migration are tightly regulated process and perturbation of this balance is associated with tumorigenesis. A role for PAKs has been established in both controlling normal cellular processes and in cancer progression. PAKs are either overexpressed or hyperactivated in various cancers and neurological disorders.