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## PAK1 AS A THERAPEUTIC TARGET

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### Abstract

**Importance of the field**—P21-activated kinases (PAKs) are involved in multiple signal transduction pathways in mammalian cells. PAKs, and PAK1 in particular, play a role in such disorders as cancer, mental retardation and allergy. Cell motility, survival and proliferation, the organization and function of cytoskeleton and extracellular matrix, transcription and translation are among the processes affected by PAK1.

**Areas covered in this review**—We discuss the mechanisms that control PAK1 activity; its involvement in physiological and pathophysiological processes; the benefits and the drawbacks of the current tools to regulate PAK1 activity; the evidences that point to PAK1 as a therapeutic target; and the likely directions of future research.

**What the reader will gain**—The reader will gain a better knowledge and understanding of the areas covered in this review.

**Take-home message**—PAK1 is a promising therapeutic target in cancer and allergen-induced disorders. Its suitability as a target in vascular, neurological and infectious diseases remains ambiguous. Further advancement of this field requires progress on such issues as the development of specific and clinically acceptable inhibitors, the choice between targeting one or multiple PAK isoforms, elucidation of the individual roles of PAK1 targets and the mechanisms that may circumvent inhibition of PAK1.

### Keywords

angiogenesis; cancer; p21-activated kinases; Rho GTPases; signal transduction; allergy

## 1. Introduction

In the early-1990s, Manser and co-workers made the seminal observation that specific downstream effects of a group of Rho GTPases (P21) in rat brain cytosol is mediated through a p21-activated kinase (PAK)[1]. In a gel-overlay assay, these researchers identified

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3 proteins of 68, 65 and 62 kDa in a specific screen designed for identifying the binding partners of Rho-GTPases. From subsequent studies, these three proteins were identified to be the members of group I PAK family; PAK1, PAK2 and PAK3 that are activated by GTP bound, but not GDP bound, Rac and cdc42[2-4]. With this discovery, a new area of research on serine-threonine kinase PAK was originated, that later found to be important for many physiological function and as a major underlying cause of many pathological conditions. The structure, substrate specificity and functional role of group I PAKs have been evolutionarily conserved right from the protozoan to the yeast and mammals<sup>5</sup>. PAKs are important for a variety of cellular functions such as cytoskeletal remodeling, focal adhesion assembly, cell migration[6], survival[7], mitosis[8] as well as transcriptional regulation and protein synthesis involving ERK and NFκB pathways[9, 10]. Currently, PAKs are among the best characterized downstream effectors of the Rho family GTPases Rac and cdc42 in the regulation of lamellipodia and filopodia formation, respectively[6, 11]. Furthermore, deregulation of PAK activity has been linked to a variety of cancers[12]. In addition to Rac and cdc42, many other signaling pathways have also been implicated in the regulation of PAK activity. These include activating and inactivating phosphorylations of PAK, inactivation by phosphatases, direct activation through protein-protein interactions, and inactivation via interaction with inhibiting proteins (see below).

In addition to the group I PAKs, recent studies have uncovered a new family of kinases, whose kinase domains have similarities to that of group I PAKs. These group II PAKs include isoforms PAK4, PAK5 (also known as PAK7) and PAK6[13]. Although termed as PAKs, these proteins display some important differences from group I PAKs[14]. The most prominent difference is the lack of activation of group II enzymes by a Rho-GTPase mediated mechanism[14]. Despite the similarities in the kinase domains, structural and biochemical differences between the two groups of PAKs suggest a different intra-cellular substrate specificity and cellular function for these two groups[13]. However, most of the few currently known substrates of group II PAKs such as Raf, BAD, LIMK, GEF1, Par-1 and estrogen receptor are also phosphorylated by group I PAKs[14-16]. A recent study using position scanning peptide library approach suggested that group I PAKs phosphorylate substrates that have consensus sequence RRRRRSWYFS, whereas the group II PAKs phosphorylates RRRRRSWASP[17]. It is important to note that despite the high degree of similarity group I PAKs may perform distinct functions even when present in the same cell[18, 19]. Furthermore, individual PAKs may be represented by different splice variants (the information is accessible at [www.uniprot.org](http://www.uniprot.org)), which may possess distinct biological properties. For example, in human PAK1B, an alternatively spliced variant of PAK1, the canonical sequence of amino acids 518-545 (HQFLKIAKPLSSLTPLIAAAKEATKNNH) is replaced by VRKLRFQVFSNFSMIAASIPEDCQAPLQPHSTDCCS.

PAK1 is a participant in a complex network of molecular interactions[9, 15, 20-22], as is evidenced by a large list of its regulators, co-factors and downstream targets (see Tables 1 and 2 for examples). It is important to note that depicting any of the individual interactions as inhibitory or stimulatory is an oversimplification: at least in some cases, the end result is a qualitative change in the behavior of the protein, such as caused by intracellular re-localization. In the current review, we will focus on normal and pathological functions of PAK1, its structural and functional similarities with other isoforms, the modes of its natural and artificial regulation, as well the prospects of therapeutic targeting of this enzyme.

## 2. Tissue distribution of PAKs

Tissue distribution of a potential therapeutic target is an important parameter that may offer clues about the likely sites affected by such a therapy. Tissue distribution of individual PAK isoforms has been studied using a variety of RNA- and protein-based detection techniques.

A serial analysis of gene expression (SAGE) indicates that mammalian brain is a unique organ that expresses significant levels of mRNAs for all the 6 different isoforms of PAK abundantly[15]. This may indicate the importance of PAK isoforms in neurological function[23]. Microarray analyses of mRNA expression[24] (accessible at [www.BioGPS.gnf.org](http://www.BioGPS.gnf.org)) show that, among the various isoforms, group I PAKs are expressed in most of the tissues (Figure 1). PAK1 and PAK2 proteins as well as PAK1 mRNA[25] are reported to be highly expressed in brain. While heart tissue expresses high levels of PAK1 protein[26], high levels of PAK1 mRNA levels has been reported in neutrophils[27]. Presence of abundant PAK3 mRNA has been indicated in brain[25] and the pituitary[28]. PAK4, the best studied member among the group II PAKs[29, 30] has been reported to be highly expressed in tissues such as prostate, testis, lung, heart, brain and liver. While PAK4 knockout mice are embryonically lethal[31], PAK5 knockout[32] and PAK5/PAK6 double knockout mice[33] are viable. The lack of gross phenotypic defects in PAK5 and PAK6 knockout animals is consistent with the SAGE data, which suggests that these isoforms have the most restricted expression pattern of all the PAKs. While PAK5 RNA is abundant in brain[34], PAK6 is expressed in testis, prostate, brain, kidney and placenta[13, 35].

It is important to note that the reports on tissue distribution of various PAK isoforms are not always perfectly concordant. Barring possible artifacts in individual studies, the discrepancies could be attributed to imperfect correlation between the protein and RNA levels, to inability of some techniques to discriminate between the individual isoforms or splice variants of such isoforms, and to a different degree of detail in analyzing a “tissue” or an “organ” (e.g. total brain vs separate brain structures; whole blood vs distinct sets of blood cells). Of note, the lack of correlation between the mRNA and protein levels may be a sign of translational regulation by miRNAs, as has been already documented for PAK1[36], and warrants further investigation.

### 3. Structure and mechanism of action of group I PAKs

PAKs in general have similar structural organization (Figure 2), including an N-terminal regulatory domain and a C-terminal catalytic domain. The conserved N-terminal non-catalytic domain contains a p21 binding domain (PBD) (amino acids 67-113 in human PAK1), which binds the GTP bound members of the Rac and cdc42 family[37-39]. One characteristic feature of group I PAKs is that its regulatory domain consists of an auto-inhibitory domain (AID) (amino acids 83-149 in human PAK1) that overlaps partially with the PBD[38]. Additionally, the proline-rich region in the regulatory domain that bind to adapter proteins Nck and Grb2 as well as guanine exchange nucleotide factor PIX/COOL are also highly conserved among group I PAKs[40, 41]. A region of amino acid diversity between the proline-rich domains such as T212 in human PAK1 and D212 in human PAK2 confers selective specificity to the different PAK isoforms for its caspase cleavage[42]. Catalytic domain of human group I PAKs have highly conserved regions in amino acids 255-559 in PAK1, 235-509 in PAK2 and 254-258 in PAK3. In between the N-terminal domain and catalytic domain, there is an acidic region rich in Glu/E and Asp/D whose functional significance is yet to be determined[43]. In contrast to group I PAKs[38], group II PAKs do not possess an auto-inhibitory domain, proline-rich regions to interact with Nck and Grb2, and the acidic regions (Figure 2)[14] (Information is available at [www.pymol.org](http://www.pymol.org) and <http://www.sgc.ox.ac.uk/structures/KIN.html>). Many recent reports indicate that regulation of PAK expression and activation has implications for various physiological and pathological processes. Up-regulation of PAK1 expression has been reported in many types of cancers (see below) and ischemic conditions such as stroke[44]. Similarly, phosphorylation and subsequent activation of PAK1 is important for a variety of cellular functions, including actin cytoskeleton remodeling, cell migration and extracellular matrix (ECM) assembly (see below). The structural[38], genetic[43] as well as biochemical[45, 46]

analyses has provided necessary insights into the mechanisms of GTPase-mediated activation of group I PAKs. In the current model, PAK activity within a resting homodimer is inhibited via a trans-inhibitory switch through the interaction of its auto-inhibitory domain (AID) with the kinase domain of the dimer partner. The interaction of GTP-bound (activated) Rac1 or Cdc42 with the CRIB of PAK1 results in a structural re-arrangement that disrupts the mutual interaction and removes the trans-inhibitory switch[47, 48,49]. While GTPases bind weakly to the so called CRIB (cdc42 and Rac interactive binding) region in the regulatory domain (amino acids 75-90 in human Pak1), interaction of GTPases with the adjacent PBD region further strengthen this binding[38].

Studies *in vitro* have demonstrated that upon release of AID, PAK1 undergoes autophosphorylation at Thr-423 in the catalytic domain[50]. However, studies *in vivo* suggest that phosphorylation of Thr-423 require an additional kinase such as PDK1[50]. It has been reported that autophosphorylation of PAK1 at Ser-144 and PAK2 at Ser-139 also plays a role in maximal activation of these kinases[1, 45, 46].

Regulation of PAK1 by extracellular stimuli typically involves redistribution of this protein within a cell, as illustrated in Figure 3. The figure illustrates that, in resting NIH 3T3 fibroblasts, PAK1 is mostly located intracellularly in the cytosol. Upon stimulation with basic fibroblast growth factor, PAK1 was observed to translocate to the cell periphery, in accordance with the proposed role for PAK1 in cytoskeletal re-modeling in fibroblasts (Figure 3). This is also in agreement with the published literature on PAK1 localization in cells[51]. Although group II PAKs can interact with the GTPases under certain circumstances, this interaction does not result in their activation, but is reported to be necessary for their translocation to a different cellular compartment[52].

Among the GTPases, isoforms of Rac such as Rac1, Rac2 and Rac3[1, 37], cdc42[1] as well as CHP/Wrch2/RhoV[53], TC10/RhoQ[54] and Wrch1/RhoU[55] are known to activate group I PAKs. Interestingly, a mutant of cdc42 (Y40C), which cannot interact with group I PAKs, retains its ability to interact with group II PAKs [29]. A short lysine-rich region in human PAK1 (amino acids 66-68) upstream of the CRIB domain confers some specificity to certain GTPases to bind to the CRIB region[37, 39]. Activating mutations (Q61L) in Rac and cdc42 that convert them into GTP-hydrolysis deficient variants increase the affinity for effector binding[47]. Subsequent studies revealed that interaction between PBD of PAK and activated GTPase may also act as an allosteric mechanism promoting its activation[38,46]. Once PAK is activated, it is not necessary to have the GTPase bound to the kinase for its prolonged activity[1].

Many recent studies have identified a family of guanine exchange nucleotide factors (GEFs) that are specifically involved in the GTPase-mediated activation of PAK. This family of GEFs termed PIX (PAK Interacting Exchangers) or COOL (Cloned Out Of a Library) interact with Rac and cdc42 and form a complex with PAK thereby resulting in its activation[40, 41]. Interaction of  $\alpha$ PIX with PAK1 was shown to induce PAK1 activation involving Rac or cdc42[56, 57]. However, the molecular mechanism of  $\alpha$ PIX-mediated PAK activation remains elusive. In addition to  $\alpha$ PIX, a second member of PIX family,  $\beta$ PIX is involved in the modulation of PAK activity[41, 57]. However, there are conflicting reports on whether interaction of  $\beta$ PIX with PAK enhance or inhibit its activity[58, 59].

#### 4. Alternative mechanisms of group I PAK activation

Although PAK is a major downstream candidate of Rho family of GTPases, additional mechanisms have been implicated in the regulation of PAK activity. Sphingolipids and their derivatives have been shown to activate PAK[60]. Amino-terminal domain of PAK has PXXP-motifs that are binding sites for SH-3 containing adapter proteins such as Nck and

Grb2[61, 62]. Interaction with these proteins recruits PAK to the plasma membrane resulting in its activation[61, 62]. This apparently GTPase-independent activation of PAK1 may be due to its localization in proximity to 3-phospho-inositide dependent kinase-1 (PDK1) that phosphorylates the conserved Thr423 of the PAK1 activation loop[9, 60]. Membrane recruitment of PAK has also been shown to modulate receptor tyrosine kinase mediated responses[61, 62]. Serine-threonine kinase Akt, which enhances PAK1 activity, has been proposed as one of the kinases for Ser21 of PAK1[63], although the context of this serine is distinct from the classical Akt recognition motif. Phosphorylation of Ser21 decreases association of PAK1 with Nck and is likely to affect cell polarity and migration[63]. Plasma membrane-recruited PAK1 is also subjected to tyrosine phosphorylation in a GTPase dependent manner, as demonstrated by McManus and co-workers in a study involving transformed cells expressing constitutively active v-ErbB receptor[64]. Etk/Bmx, a Tec family member of non-receptor tyrosine kinase, has been shown to directly bind and phosphorylate PAK1[65].

Importance of multiple phosphorylations for activation of PAK also suggests a prominent role for phosphatases in the regulation of PAK activity. Serine-threonine phosphatase PP2A has been shown to interact with PAK[66]. Two additional phosphatases that are known to de-activate PAK are POPX1 and POPX2 (Partner of Pix1 and Pix2)[67]. These phosphatases interact with the Pix family of PAK-specific guanine exchange nucleotide factors and become part of a multi-protein complex that also involves PAK. These phosphatases are shown to dephosphorylate Thr423 in the activation loop of PAK1 [67].

## 5. PAK signaling in cytoskeletal remodeling and cell motility

Small Rho GTPases such as Rac and cdc42 have been projected as the major regulators of cytoskeletal dynamics[9]. Both these GTPases mediate their effects through PAK, and PAK utilizes its catalytic activity as well as interaction with other proteins in the regulation of GTPase dependent cytoskeletal remodeling [16, 56]. Many cytoskeletal and adaptor proteins, guanine exchange nucleotide factors, intermediate filaments, microtubules, integrins, kinases as well as phosphatases have been shown to interact with or get phosphorylated by PAK[9, 20]. Myosins, a large family of actin-based molecular motor proteins, are targets of phosphorylation by PAK in the regulation of cell spreading, motility and cell division[68]. Ste20 and Cla4, two PAK-related kinases in *Saccharomyces cerevisiae* that are highly homologous to group I PAKs, are among the first identified Rac/cdc42 effectors regulating myosin light chain (MLC) phosphorylation both *in vitro* and *in vivo*[69, 70]. Mammalian PAK phosphorylates MLC on Ser19 in neuronal cells, resulting in the stabilization of the localized actin network through formation of a GIT1/PIX/Rac/PAK complex[71]. Alternatively, PAK can also regulate activity of myosin light chain kinase (MLCK) and indirectly modulate MLC phosphorylation[72]. In addition to MLC, PAK can also phosphorylate LIM Kinase (LIMK), a serine-threonine kinase involved in the regulation of assembly of actin cytoskeleton and dis-assembly microtubules[73]. Phosphorylation of LIMK at Thr508 triggers phosphorylation of cofilin, a cytoskeletal protein that acts as an actin capping and severing protein[74]. LIMK is also involved in mediating the formation of lamellipodia, membrane ruffles, filopodia and cell motility via Rac/cdc42-PAK signaling[9]. PAK1 is necessary for the phosphorylation of actin binding protein filamin A at Ser2152, which is necessary for the cross-linking of actin networks. In turn, filamin A interact with the CRIB region of PAK stimulating its activation, thus operating a local loop activation of PAK[75]. In addition, PAK can also phosphorylate p41-Arc (Arp2/3; a 41 kD subunit actin-related 2/3 complex) at Ser21[76], cortactin, an F-actin binding protein involved in actin polymerization at Ser113[77] and caldesmon, an actin filament regulatory protein at Ser657 and Ser687[78] in the regulation of actin polymerization.

A study performed by Rakesh Kumar's group suggests that phosphorylation of integrin linked kinase (ILK) by PAK1 at Thr 173 and Ser 246 *in vitro* and *in vivo* plays a role in nuclear export of ILK[79]. Although functional significance of this PAK1-mediated nuclear function of ILK is not very clear, this study indicates that changes in the nuclear activity of ILK are directly linked to the changes in nuclear lamins, which are critical proteins necessary for the nuclear integrity and function.

PAK is also involved in the phosphorylation of proteins that control microtubule dynamics. Stathmin, also known as oncoprotein 18 (op18), destabilizes microtubules by binding to tubulin dimers and inhibits tubulin polymerization to promote microtubule dis-assembly[80]. Phosphorylation of op18 by PAK at Ser16 inactivates this protein, resulting in stabilization of microtubules at the leading edges of migrating cells. Tubulin cofactor B (TCoB) augments the heterodimerization of  $\alpha/\beta$ -tubulins and is phosphorylated by PAK at Ser65 and Ser128[81]. PAK can also phosphorylate dynein light chain 1 (DLC1), a component of cytoplasmic dynein complex, which moves along the microtubules. DLC1 is phosphorylated by PAK1 at Ser88, which affects vesicle formation and trafficking[76].

Apart from its prominent role in actin and microtubule remodeling, PAK is also known to phosphorylate a variety of other substrates in the regulation of cell motility. PAK has been reported to phosphorylate the armadillo adaptor protein, a drosophila counterpart of mammalian  $\beta$ -catenin, at Ser561 and Ser688, in the regulation of cadherin-mediated adhesion[82]. Paxillin, an adaptor protein abundant in integrin dependent focal and fibrillar adhesions, is phosphorylated by PAK at a serine residue[83]. A more detailed account of the signaling partners of PAK1 in the regulation of cytoskeletal assembly and cell motility is provided in Tables 1 and 2 as well as in Figure 4.

## 6. PAK signaling in vascular biology and angiogenesis

Migration of endothelial cells and fibroblasts is essential for angiogenesis and tissue remodeling[84]. In order to migrate in a specific direction, cells must adhere and detach to the substratum (extracellular matrix proteins) in a coordinated manner. Coordination of events such as formation of filopodia, lamellipodia and focal adhesions in the leading edge and detachment of focal adhesions at the lagging edge of the cell is necessary for the directional migration. Rho family of GTPases (Rho, Rac and cdc42) is well known as regulators of directional migration in vascular cells[85]. Importance of PAK in the regulation of lamellipodia formation and migration of vascular cells is known for a long time[86]. Many mouse gene knockout studies have also revealed the importance of PAK in the regulation of cell migration by other kinases such as Rac GTPase[87] and Akt[6, 88]. Impaired cytoskeletal assembly and cell migration observed in *Rac1*<sup>-/-</sup> and *Akt1*<sup>-/-</sup> fibroblasts was rescued by over-expression of constitutively active form of PAK1 (T423E mutant)[6, 87]. In addition to fibroblasts, PAK is also important for the directional migration of endothelial cells[89]. Inactivation of Rac1 and PAK1 *in vitro* in endothelial cells and fibroblasts results in impaired cytoskeletal assembly, formation of lamellipodia and migration[6, 90]. In contrast, constitutively active mutants of Rac1 and PAK1 results in enhanced cell motility in these cell types.

In addition to its role in migration, Rac/Pak signaling has also been implicated in the regulation of cell survival, cell cycle and proliferation[91]. While VEGF-mediated activation of PAK in endothelial cells is primarily focused on cytoskeletal remodeling, PAK activation by bFGF is necessary for the anchorage-dependent cell survival in endothelial cells[92]. However, specific role of PAK and the mechanism by which PAK regulates endothelial cell survival has not yet been fully addressed. The first in-depth study on the role of PAK in endothelial cells was performed by Kiosses and co-workers[86]. In this study, 14

different mutants, truncated and wild-type variants of PAK were used to determine the function of PAK in endothelial cell function *in vitro*. Expression in endothelial cells of either an inactive mutant or the auto-inhibitory domain (PAK 83-149) of PAK1 significantly inhibited endothelial motility[93]. Interaction of PAK with Nck adaptor protein is also shown to stimulate PAK kinase activity in endothelial cells. In a study involving dominant-negative p65 PAK peptide that disrupts the interaction between PAK and Nck, endothelial migration and tube formation in 3D Matrigel environment *in vitro* was blunted[94]. This finding was corroborated *in vivo* using a chick allantoic membrane (CAM) angiogenesis assay[94]. Nck-mediated activation of PAK is also necessary for integrin activation, focal adhesion turn-over and maintenance of endothelial-barrier integrity[95].

Formation of a lumen in blood vessels is an essential step in neo-vascularization. However, due to the difficulty in developing proper *in vivo* models, events leading to lumen formation are poorly understood. A number of attempts were made to unveil this process using 3D matrix gel models *in vitro*[96]. Formation of endothelial lumens (endothelial tubes) in 3D collagen gel matrices is regulated by the coalescence of newly formed intracellular vacuoles that was dependent on integrin-mediated outside-in activation of Rac-PAK signaling[97]. These observations along with another study performed in a zebrafish model *in vivo*[98] suggests that Rac-PAK signaling is a key regulatory pathway mediating vascular lumen formation *in vivo*.

Another important event in vascular biology that is directly under the control of PAK is the regulation of endothelial-barrier function and vascular permeability. Rac-PAK signaling is expected to play a dual-role in the process since it is known to regulate both assembly and dis-assembly of VE-cadherin-based cell-cell junctions. VE-cadherin mediated activation of Rac-PAK signaling stabilizes the endothelial-barrier junctions [99]. VE-cadherin null endothelial cells exhibit impaired Rac activity and impaired localization of its guanine exchange nucleotide factor Tiam1[100]. In contrast, permeability stimulating agents such as thrombin disrupts endothelial-barrier junctions via activation of Rac-PAK signaling[101]. At the same time, VEGF mediated activation of endothelial cells disrupts endothelial-barrier via Rac-mediated internalization of VE-cadherins[102]. In response to stimuli from serum, VEGF, TNF, bFGF, histamine and thrombin, endothelial cells exhibit Rac-mediated activation of PAK via phosphorylation at Ser141 resulting in its translocation to endothelial cell-barrier junctions[103]. PAK can also directly phosphorylate VE-cadherin on a highly conserved serine residue which controls its stability at the cell-cell junctions[102]. Apart from this, atherogenic flow profiles, oxidized LDLs and pro-atherogenic cytokines stimulate PAK in endothelial cells and its translocation to barrier junctions; whereas blocking PAK activity reverses endothelial-barrier leakage in atherosclerosis prone regions[89]. Inhibition of PAK activity and function by disrupting the protein complex of PAK/Pix/GIT using a cell permeable peptide that prevents interaction between PAK and reduces vascular leakage in a mouse model of acute lung injury caused by lipopolysaccharide treatment[58]. Inactive mutations in PAK in zebrafish exhibits cerebral hemorrhage without imparting any obvious alterations in the vasculature[104]. While it is evident that Rac-PAK signaling is important for the regulation of endothelial cell and fibroblast function, extracellular matrix remodeling, vascular permeability, lumen formation as well as angiogenesis, these studies suggest that PAK may emerge as an important therapeutic target for many of the integrin, angiogenesis, matrix and vascular permeability related clinical conditions. A recent report indicating that interference with PAK function prevents smooth muscle cell proliferation and pathological vascular remodeling[105] also suggests PAK1 as a potential target in such conditions as hypertension, atherosclerosis, and vascular stenosis.

## 7. PAK1 in human cancer

Changes in the levels and activity of PAKs, and PAK1 in particular, are frequently described in human malignancies. A few examples listed in Table 3 demonstrate that this phenomenon is seen in tissues of various origins and using a variety of techniques. The reported abnormalities include amplification of the corresponding gene, elevated levels of the mRNA and protein, and increased accumulation of phosphorylated and, presumably, activated form of this enzyme. In addition to malignant tumors, benign schwannomas that arise in patients with neurofibromatosis type 2 display the elevated levels of phosphorylated and, presumably, active PAK1[106]. There are also intriguing observations about accumulation of phosphorylated PAK1 specifically in the nuclei of malignant cells[107], which parallel the changes observed during progression of breast cancer in a mouse model of the disease[108]. Importantly, elevated level of PAK1 was identified as an independent prognostic predictor of poor survival in ovarian cancer[107]. In breast cancer, nuclear expression of PAK1 in conjunction with phosphorylation of estrogen receptor on the PAK1 site (serine 305) predicts resistance to tamoxifen therapy, while the cytoplasmic levels of PAK1 correlate with recurrence rate and mortality[109, 110]. Similarly, in patients with gastric cancer higher levels of PAK1 were associated with advanced tumor stages, metastasis and reduced survival[111]. There are also numerous reports of elevated PAK1 activity in cell lines, although in most cases such reports cannot rule out that the changes had been selected or caused by in vitro cultivation of the cells.

Overall, there is little doubt that the levels and activity of PAK1 are frequently increased in various cancers. The essential question is what contribution this protein makes to cancer progression and how critical this contribution is.

Cancer progression is a multistage process during which an evolving population of malignant cells has to overcome numerous hurdles of natural tumor suppressive mechanisms. For example, for a solid tumor to form and to progress to a metastatic state, the diseased cell has to proliferate despite the inhibitory effects of its neighbors and the lack of exogenous mitogens, to attract the flow of nutrients and oxygen in order to sustain the tumor growth, to escape its normal physical niche, to survive in an environment that may lack its normal survival factors, and to maintain a higher rate of metabolism and protein synthesis in order to support its more active “lifestyle”. When the involvement of some of the better known oncogenes in these processes was elucidated, PAKs came into the spotlight as potential components of the relevant molecular mechanisms. Remarkably, PAKs, and PAK1 in particular, have been linked in one way or another to all of these phenomena. Such a comprehensive involvement makes PAK1 an attractive therapeutic target.

Kinases from Akt family are frequently activated in malignant cells, where they contribute to multiple oncogenic traits[112]. One of the best known phenomena induced by activated transforming oncogenes in cells of solid tissue origin is the lack of contact inhibition[113]. In confluent cultures of cells this is typically manifested by characteristic foci of multi-layer growth. The activated form of Akt relieves rodent fibroblasts from contact inhibition and this process requires the activity of PAK1 [6]. Reduction of PAK1 activity in Rat1 cells dissociates the activation of Akt from that of the ERKs, and reduced to ability of Akt to induce focus formation and tumor growth in a xenograft model[6]. Interestingly, activation of Akt facilitates redistribution of PAK1 to the plasma membrane in a pattern similar to that assumed by cRaf in the same conditions. Such co-localization may provide a mechanistic basis for the role that PAK1 plays as a liaison between the PI3K/Akt and the Raf/MEK/ERK pathways. Importantly, the decrease in PAK1 function is well tolerated by non-transformed Rat1a cells[6].



Another common oncogenic event is the activation of the oncogenes from Ras family. Ras is known to require the function of Akt for efficient transformation. Accordingly, at least some transformed features of Ras-expressing cells are also PAK1- dependent. In particular, focus formation and another characteristic of oncogenic transformation, growth in semi-solid medium, were abolished when PAK1 function was suppressed in Ras-transformed rat fibroblasts[114]. Growth enhancement by Ras involves the activation of cyclin-dependent kinases (CDKs), which is achieved by reducing the production of CDK inhibitor p27 and by enhancing the production of CDK activator cyclin D1. While both of these phenomena depend on phosphoinositol-3-kinase, the latter specifically depends on PAK1 and on PAK1 -dependent activation of ERKs[115]. Interestingly, it appears that in Ras-transformed cells, in comparison to their non-transformed counterparts, there is a heightened dependence on PAK1 for keeping the MAP kinase cascade active and, ultimately, for cell growth. It also appears that constitutive activation of Raf may be able to supplant the requirement for PAK1. These observations are very important for future clinical development of PAK inhibitors: they point both to potential utility and specificity of such an approach, as well as to a possible mechanism of resistance to such a therapy.

The molecular pathology of neurofibromatosis type 2, which arises due to the lack of merlin, a putative negative regulator of PAK1, suggests that reducing activity of the latter may have a therapeutic value in this disease[106]. Indeed, rat schwannoma cells appear sensitive to inhibition of PAK1, while inhibition of all three type I PAKs suppressed transformation of NIH3T3 cells by dominant-negative mutant of merlin[106]. Although, the conduct of the critical experiments in rodent cells and the lack of some technical controls leave the significance and relevance of this study in need of further confirmation, it is important to note that merlin-deficient human mesothelioma cell lines are also hypersensitive to the treatments aimed at PAK1.[116]

Of note, oncogenic viruses often rely on the same mechanisms that are associated with spontaneous disease. For example, Kaposi sarcoma virus, a herpesvirus with particular significance to the malignancies in AIDS patients, requires a PAK1-dependent pathway for efficient transformation of cultured cells[117], while hepatitis B virus induces PAK1-dependent relocalization of Raf-1 to mitochondria[118]. In both of these cases, the clinical relevance of these phenomena is yet to be confirmed.

If PAK1 is indeed the main component of the major oncogenic signaling pathways, one may expect that direct activation of PAK1 would recapitulate at least some features of oncogenic transformation. Indeed, overexpression of the activated mutant form of PAK1 (PAK1 T423E) in tissue culture models stimulates anchorage-independent growth in breast cancer cell lines[119], and yields hyperplasia of mammary epithelium[120] and, eventually, breast carcinomas[108] in transgenic mice with tissue-specific expression of the transgene. Certainly, overexpression of an artificially mutated protein, especially, when achieved through a promoter with a very complex hormonal control, on its own would not suffice to draw clinically-relevant conclusions. Nevertheless, these results are concordant with the observations on PAK1 in human malignancies and on the consequences of PAK1 inhibition in transformed cells (see above).

It is important to note that the ability to grow in semi-solid medium requires simultaneous enhancement of proliferative and survival signals, the latter to avert anoikis. It is uncertain whether the pro-survival signaling of Ras was affected in the experiments that observed suppression of Ras-mediated transformation by dominant-negative PAK1[114]. There are many examples of studies that describe the involvement of group I PAKs in protection from cell death, including anoikis and killing by chemotherapeutic agents [7, 121-123]. The specific mechanism of anti-apoptotic protection was originally reported to involve direct

inactivation of pro-apoptotic Bcl-2-family member Bad[124]. Serines 112 and 136 of Bad were originally reported as relevant targets [124]. However, a later report, which examined multiple PAK1 and Raf-1 mutants and combinations thereof *in vivo* and *in vitro* assays, indicates that these positions are poor targets for PAK1 itself, but, instead, are targets of PAK1 – direct activity of Raf-1[125]. Interestingly, anti-apoptotic function of PAK1 is well established in cells that express very low levels of Bad[126], suggesting that additional mechanisms may be also involved in this phenomenon. In fact, at least in 3D cultures of normal mammary epithelium cells, the protective function of  $\alpha 6\beta 4$  integrin is mediated through Rac1 and PAK1, which in turn activate NF $\kappa$ B. In these cells the sensitization rendered by inactivation of PAK1 was overcome by activation of NF $\kappa$ B via an alternative mechanism [127]. Interestingly, an earlier report by the same group suggests that this protection is independent of ERKs and PI3K[128]. However, the prior study of anti-apoptotic function of  $\alpha 6\beta 4$  integrin in transformed cells has implicated PI3K/Akt pathway in this process[129]. Barring experimental artifacts, these data imply that in different cell environments different modes of PAK1 operation contribute to cell survival. This may indicate an attractive opportunity to selectively abolish protective functions of this protein in tumor, but not in most normal cells.

Another possible involvement of PAK1 in resistance to programmed cell death may be through maintaining expression of anti-apoptotic protein survivin [130]. Although the original observation was made in osteoclasts, survivin is a major pro-survival protein in cells from a variety of cancers[131], and it is worth further investigation whether its expression in those cells is PAK1-dependent as well.

In order for a cancerous cell to escape from its normal environment, the heightened mobility, as rendered by elevated activity of PAK1, has to be matched by the ability to destroy and restructure extracellular matrix[132]. Not surprisingly, matrix metalloproteinases, which normally restructure tissues during embryogenesis and inflammation, are commonly found expressed in advanced tumors, where they also contribute to the processing of certain bioactive peptides[133]. In this regard, it is noteworthy that the increase in pericellular proteolysis observed in a model of pre-malignant progression of breast cancer corresponded to and was dependent on the increase in the activity of PAK1[134].

It has been reported that inflammatory cytokines induce the expression of MMP-9 through activation and stabilization of PAK1, which activates JNK and MMP-9 expression [135]. Specific relevance of this phenomenon to cancer has yet to be demonstrated, but one may consider a scenario in which MMP-9 is contributed to the tumor by appropriately activated stroma cells[136]. It is worth noting that mutational activation of some signaling cascades (e.g. that of NF $\kappa$ B) known to be active in cancer cells leads to increased secretion of potentially inflammatory cytokines (e.g.[137]). It is conceivable that one may attempt to target PAK1 function in the normal cells of stroma to suppress the invasiveness and metastasis of the tumor.

Finally, it is important to note that the gene encoding PAK1 is located in relative proximity to the one encoding cyclin D1. These genes are often co-amplified and, consequently, co-expressed. Therefore, one has to be cautious in attributing the consequences of amplification of human 11q13 to either one of these genes individually. Furthermore, it is likely that the properties of a sizeable number of tumors are determined specifically by the interplay between these proteins, rather than by either one of them alone, and the issue of their functional interaction merits further investigation.

## 8. PAK1 in neurological and mental disorders

PAK3 is clearly involved in some neurodegenerative disorders and variants of mental retardation[138-140] (reviewed in 141) and plays a special role in synapse formation and plasticity in hippocampus[142]. However, the involvement of PAK1 in these processes is less clear-cut. For example, both PAK1 and PAK3 were reduced in the hippocampus affected by Alzheimer disease, yet only PAK3 was affected in some other areas of the diseased brain[140]. However, this reported loss of the PAKs from the cytosol appears to be accompanied by re-localization of PAKs to the membrano-cytoskeletal fractions, where they appear to be active[143]. Using staining for drebnin and reduction in dendrites as indicators, Dr. Cole's group has observed that a dominant-negative form of PAK1 sensitizes, while the wild type form protects from some effects of beta-amyloid oligomers in cultured primary neurons[140, 143]. However, in both cases it is hard to rule out that ectopically expressed PAK1 in some of these experiments acted as a surrogate for the highly homologous PAK3.

Dominant-negative PAK1, which, potentially, inhibits other PAK isoforms as well, upon expression in mouse forebrain affected synapse morphology and consolidation of long-term memory[144], but rescued some defects of a mouse model of Fragile X syndrome[145].

In case of Huntington's disease, PAK1 specifically co-localizes with huntingtin inclusions in the affected brain[146]. In tissue culture models, interference with PAK1 function modestly decrease the formation of aggregates by mutant huntingtin, while the constitutively active PAK1 enhances the aggregation[146]. Accordingly, similar activity was reported for PAK1 regulator  $\alpha$ -PIX[147]. The matter is complicated, however, by the observation that kinase activity of PAK1 is dispensable for this phenomenon[146]. Overall, it appears that pathological changes in the brain could be associated both with elevated and reduced function of PAKs and the specific role of PAK1 in these processes may be variable as well.

## 9. PAK1 in infection and immunity

The PAK-dependent cellular events are significant for the biology and pathogenicity of various viruses (recently reviewed elsewhere[21, 148]). It is interesting to note that historically the nucleocapsid protein of Rous sarcoma virus was one of the first targets of the kinase later identified as PAK2, and this phosphorylation appears important for the packaging of viral RNA[149, 150]. However, we are not aware of any evidence that PAK1 is critically important for in vivo propagation of this virus. In the pathogenicity of another group of retroviruses, the lentiviruses, the roles of the two PAKs are subjects of an ongoing controversy. It is clear that lentiviral Nef protein interacts with at least one of these kinases[151, 152], but contradictory data have been reported on which one it is[153-155]. Although Nef is an important player in lentiviral pathogenesis[156], it appears that its interaction with PAK is not absolutely indispensable for proliferation of HIV-1 and development of AIDS[156-158], reducing the value of PAKs as therapeutic targets. Since PAK1 is important for macropinocytosis[159], it plays a role in infection by various viruses that depend on this process, such as adenoviruses[160] and vaccinia virus[161]. PAK1 and PAK2 have been shown to play important, but distinct roles in alphaherpesvirus infection[162].

The fundamental cellular processes affected by PAK1 are important for the function of the cells of immune system as well. As mentioned above, the activation of ERKs, as well as formation of broad stable lamellipodia in macrophages during spreading depend on the presence of PAK1 in the cells[163]. While PAK1 was reported to play a role in directional migration of several types of myeloid cells towards a chemoattractant[164], chemotaxis of PAK1<sup>-/-</sup> macrophages towards CSF-1 appears normal[163]. Although it remains to be seen whether these effects of PAK1 remain critical in the context of in vivo response to

pathogens, it would be prudent to consider the immune system as a potential site of side effects of any future anti-PAK1 therapy. This may be further exacerbated by the protective role that PAK1 may play in the resistance of microvascular endothelial cells towards invading bacteria[165].

Fortunately, the effects of PAK1-deficiency on the immune system have a very encouraging up-side. As demonstrated by otherwise relatively healthy PAK1<sup>-/-</sup> mice, Pak1 is critical for disassembly of cortical F-actin upon allergen stimulation, and PAK1 deficiency prevents the release of pro-inflammatory molecules from the granules of mast cells during the IgE-associated allergic responses[166].

## 10. Current approaches to inhibition of PAK1

The position of PAK1 in the middle of multiple signal transduction pathways (Figure 4) makes it very likely that any intervention affecting the upstream elements of those pathways will affect PAK1 among other molecules. In these cases the actual target of intervention is clearly distinct from PAK1 and, typically, the consequences of such an intervention cannot be attributed solely to the changes in PAK1 status. Hence, a detailed discussion of such targeting strategies falls outside of the scope of the present review. Functional inhibition of PAK1 itself has been achieved experimentally using several forms of dominant-negative mutants, RNA interference and a number of chemical inhibitors with various degrees of specificity.

PAK1 participates in a multitude of protein-protein interactions, which creates opportunities to disrupt all or some of these functions by expressing various fragments of mutant forms of this kinase that could compete with the endogenous protein. PAK1 exists as a dimer prior to activation and functions within a large multi-protein complex. Therefore, if a non-functional (e.g. devoid of kinase activity) PAK1 is introduced into a cell it may successfully compete with the endogenous form for inclusion into such complexes. Conceivably, a dimer of the wild type and mutant proteins may be less capable of activation, which, presumably, involves trans-autophosphorylation[49]. Indeed, a “kinase-dead” mutant (e.g. PAK1 K299R) is an effective dominant-negative reagent, which is commonly used to document PAK-dependence of various phenomena (e.g.6) . Also, the autoinhibitory domain of PAK, when expressed without the rest of the protein, binds to all group I PAKs and is a potent suppressor of their function [167]. This protein was expressed in a tissue-specific manner to investigate the PAK-dependent processes in mouse forebrain, where it affected synaptic morphology and impaired memory consolidation, but also relieved some symptoms in the model of fragile X syndrome [144, 145].

It is important to note, that some functions of PAK1 may be carried out independently of its kinase activity. For example, it has been proposed that in such a manner PAK1 may play a role of a scaffold for Akt-PDK-1 interaction [168]. This concern could be somewhat alleviated by the use of very short fragments of PAK1. For example, a cell-permeable small peptide called WR-PAK18, which includes a PIX-binding site of PAK1 (PPVIAPRPEHTKSVYTRS) added to a transmembrane transport peptide (RRWRRWRRWRRWRR), selectively blocks the PAK-PIX interaction and can suppresses RAS transformation in vitro[169, 170]. Remarkably, the peptide was selective in its growth suppression, inhibiting Ras-transformed, but not the parental non-transformed cells. Similarly, addition to endothelial cells of a peptide, which combines the Nck-binding motif (KPPAPPMRNTSTM) from PAK1 and the transmembrane transport motif from HIV-1 Tat (YGRKKRRQRRRG), effectively disrupted the interaction between PAK1 and Nck and reduced motility, contractility and ability to form blood vessels [94]. The fact that the short peptides may be active in vivo[94, 140] suggests that this route could be explored

for the development of therapeutic interventions. In this case, a reduction in cost and further protection from proteolysis may become welcome improvements.

The use of dominant-negative forms or fragments of PAK1 carries an inevitable question about the specificity of their effects. Since those molecules take up the place of PAK1 in complexes with other proteins, one may be reasonably concerned that the observed effects ensue from sequestration of the latter, which might affect both PAK1 - dependent and PAK1- independent functions. It is also difficult, if not impossible, to separate the functions of PAK1, -2 and -3, which share many common interactions. Furthermore, expression of the kinase inhibitory domain of PAK1 alone was reported to inhibit cell growth even when the mutations were introduced to prevent the interaction of this peptide with PAK1 [171]. This casts a shadow of doubt over a large body of work conducted using this tool. Therefore, it is critical that the conclusions reached using the dominant-negative proteins be confirmed by alternative means.

An obvious complementary approach to the use of dominant-negative peptides is to suppress PAK1 via RNA interference. Successful suppression of PAK1 expression by RNAi is readily achievable [6, 107], and provides a better discrimination between the individual PAK isoforms than the use of dominant-negative proteins. It is important to note that RNA interference is associated with a well-recognized danger of artifacts and off-target effects and necessitates appropriate controls (discussed in 172).

Typically of a kinase, PAK1 is sensitive to the molecules that occupy its ATP-binding site. For example, a rather promiscuous kinase inhibitor staurosporin binds PAK1 with high affinity [173]. A celecoxib derivative OSU-03012, which has been investigated primarily because of its effect on PDK1, inhibits PAK1 as well [174], but cannot be considered as a specific inhibitor. Considerably more specific are the bulky ATP antagonists CEP-1347 and KT D606, which do not fit in the ATP pockets of most kinases, but inhibit both PAKs and Mixed Lineage Kinases (MLKs), which possess an unusually large ATP pocket [175]. Although the transformation-suppressive activity of these compounds has been attributed to PAK inhibition, they are much more potent against MLKs, their specificity against individual PAKs has yet to be studied in detail, and their affinity towards PAKs is too low for clinical considerations. Similarly, the large ATP pocket of PAK1 has been targeted by bulky octahedral ruthenium complexes [176], but the ability of these compounds to target other PAKs and other proteins in general still remains to be explored. For research application, the conclusions based on the use of these compounds inevitably have to be corroborated by complementary techniques to exclude the contribution of alternative targets to the phenomena in question.

An alternative route towards developing small molecule inhibitors of PAK1 follows the example of dominant-negative fragments in disrupting specific protein interactions, rather than the kinase activity per se. The proof of principle has been achieved in a high throughput screen that discovered a molecule (named IPA-3) that prevents the functional interaction between PAK1 and Cdc42 [177]. The original screening had in vitro PAK1 activity as a read-out, but the use of Cdc42 as an activator of PAK1 in the reaction mixture enabled the discovery of IPA-3. IPA-3 inhibits group I PAKs, although the effect is the strongest against PAK1 [177]. A broad analysis using Invitrogen's Z-Lyte assay revealed that at least 9 other kinases (out of a collection of 214 purified wild type or mutant enzymes) lose 50% or more of their activity in vitro in the presence of IPA-3 [177]. Notable on this list are Akt2 (68% inhibition), GSK $\alpha$  (66.8%), GSK $\beta$  (53.9%), p38 $\alpha$  (70.1%), and PLK3 (88.1%). Among the kinases that showed lower, but still substantial inhibition are BRAF (46.4%), Aurora A (41.5%), SGK-1 (46.4%) and IKK $\beta$  (30%). This estimate of the breadth of off-target effects is a conservative one and has to be taken very cautiously. In fact, the expected mechanism of

IPA-3 activity (prevention of PAK1-Cdc42 interaction) could not be detected in this assay, which tested purified enzymes without their activators. IPA-3 does not inhibit PAK1 molecules, which already have been activated, but appears to disrupt the effect of Cdc42 by binding covalently to the autoregulatory domain of PAK1 [178]. It is not clear how it affects the other sensitive kinases. Furthermore, efficient inhibition of PAK1 by IPA-3 required relatively high doses of the latter, which could be explained, at least in part, by inactivation of this compound by reducing molecules. Even though IPA-3 is unlikely to become a therapeutic compound, it should serve as an inspiration for the further screens targeted at the activating mechanisms, rather than the enzymatic activity of this and other kinases.

## 11. Expert opinion: Is PAK1 a suitable target for therapy?

As discussed above, there is growing evidence that PAKs are involved in the phenomena that are clinically significant for various cardio-vascular disorders, but the specificity of PAK1 involvement is still uncertain. Studies indicate that even closely related PAKs (e.g. PAK1 and PAK2) have non-identical sets of substrates. The issue is further complicated because of the multiple and sometimes opposing roles of PAKs in these processes and certainly merits further investigation.

The reports on the involvement of PAK1 in various diseases of the brain indicate that both up- and down-regulation of this enzyme may be associated with pathological changes. This, along with the uncertainty about the relative contribution of other isoforms, clouds the prospect of targeting PAK1 for therapeutic intervention in these conditions. Furthermore, these observations necessitate a close attention to the affects that any anti-PAK therapy targeted at other organs might have on the nervous system, including the cognitive functions and the memory. In this regard, failure of an anti-PAK1 agent to penetrate the blood-brain barrier may not be a detriment to its therapeutic utility. Similarly complicated is the question of PAK1 targeting in infections: while it may partially attenuate certain viruses, it would also negatively impact some functions of the immune system. In fact, the recent report of PAK1-deficient animals having IgE-mediated responses to allergens[166] may indicate that, at least, for such acute life-threatening conditions as anaphylaxis the benefits of suppressing PAK1 may outweigh the risks.

Overall, the main effort of PAK-oriented therapy comes from the field of oncology. In oncology, an ideal candidate for therapeutic targeting should frequently play a critical role in progression and persistence of the disease, while being less essential for the survival of the whole organism. In addition, there should be efficient strategies to target it, while the cancer cells should be unlikely to develop resistance to it, or there should be a strategy to overt or to overcome the development of such resistance. Does PAK1 satisfy these criteria?

The currently available data point to PAK1 as an important intermediate in the signaling pathways that are engaged through various mechanisms in the majority of cancers. Moreover, there is a growing body of evidence to suggest that the role of PAK1 in these cases is essential for survival and progression of the tumor. It has to be noted that these evidence were obtained either using inhibitors with imperfect specificity, or by more specific means, but in in vitro or xenograft models. A direct experiment with specific suppression of PAK1 in the context of a natural tumor is yet to be reported, and some of the earlier conclusions may have to be re-evaluated or refined using inhibition of individual PAK isoforms.

The reports on specific vulnerability of transformed cells to suppression of PAK1 and the fact that PAK1-deficient animals retain normal life span, growth, or fertility[166], suggest that a therapy targeted at PAK1 is likely to be well tolerated by a patient. However, a much more detailed evaluation of the PAK1 knockout mice is warranted before a conclusion could

be made about severity of their phenotype. Also, we cannot rule out the possibility that compensatory mechanisms were engaged during the development of these animals, and the consequences of acute inhibition of PAK1 may be more severe.

RNA interference provides an efficient way of suppressing PAK1 in model systems, but the clinical applications of this technology, however promising, still await successful resolution of the issues of efficient and targeted delivery, in vivo stability and safety (reviewed elsewhere[179]). Unfortunately, short of RNA interference, currently there are no tools to target PAK1 specifically. Although a compound suitable for clinical use has not been described in the literature yet, targeting of the unusually large ATP-binding pocket and specific targeting of the interaction of PAK1 with its interactor proteins have yielded very promising results. It is likely that both approaches will be pursued further. In addition to targeting the cdc42-dependent mechanism of activation, inhibition of the functional interaction between PAK1 and Akt may emerge as another strategy for selective targeting of the cancer-specific functions of PAK1.

The issue of isoform-specificity in targeting PAKs is very complex. On the one hand, the consequences of combined knockouts of all group I PAKs in an organism have not been reported yet, and it is certain that targeting a single isoform would incur fewer side effects than targeting all of them. On the other hand, with a few notable exceptions, the bulk of the published data on PAK inhibition was generated with techniques that poorly discriminate between the isoforms. In oncology, this issue goes beyond the question of whether PAK1 is essential for growth and survival of an individual tumor: if another isoform can fully compensate for the loss of PAK1 function, a treated tumor is more likely to acquire resistance to therapy. Therefore, inhibition of more than one isoform may be required for effective treatment, increasing the risk of side effects. In general, the studies on the possible mechanisms of resistance to PAK-directed therapy are conspicuously missing today, and this gap in knowledge would have to be filled before such an intervention proceeds to clinical trials. Furthermore, delineation of specific mechanisms of PAK involvement in oncogenesis may point to common downstream steps that are required for the oncogenic function of all the isoforms. Exclusive targeting of these steps may have fewer side effects, but the same anti-cancer efficacy as targeting PAKs.

Overall, in our opinion there is sufficient evidence to implicate PAKs, and PAK1 in particular, as attractive targets for the therapy of cancer and certain other diseases. Development of compounds with improved pharmacological properties, clarification of the roles of individual PAK isoforms, and an understanding of the mechanisms that could circumvent inactivation of PAK1 are the research area of critical importance for the further clinical advancement of PAK-oriented therapy.

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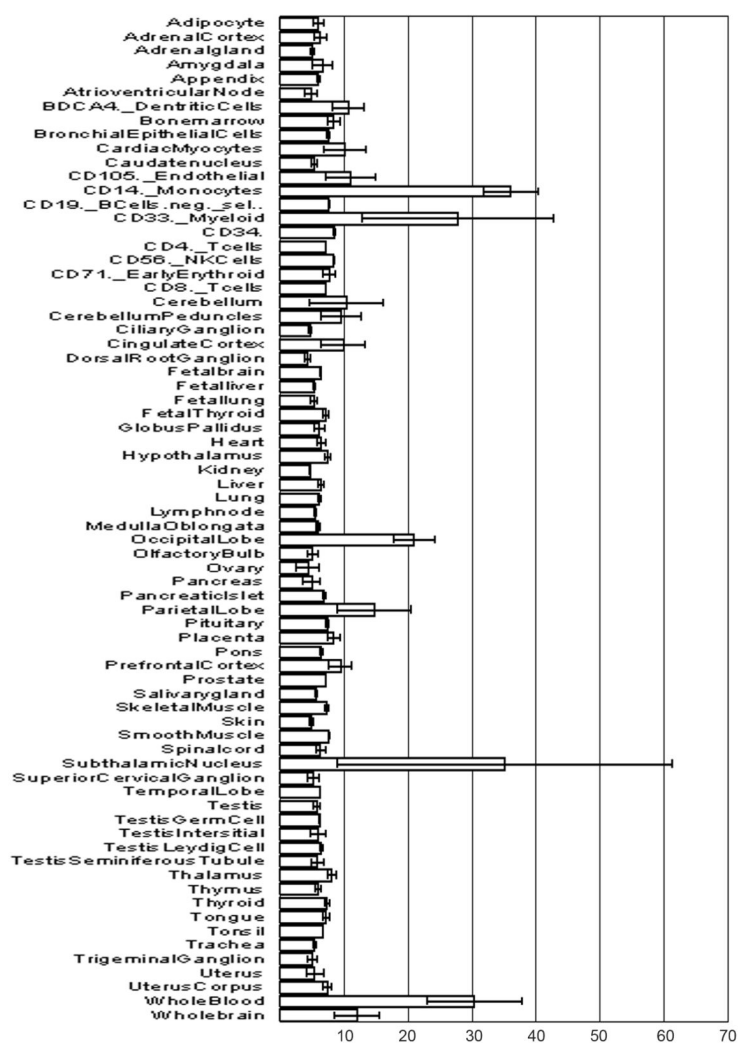


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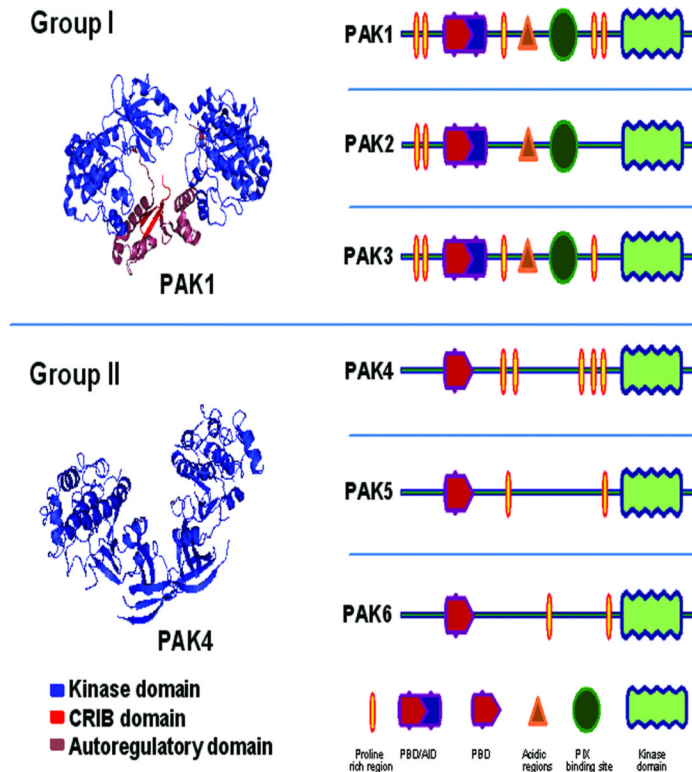
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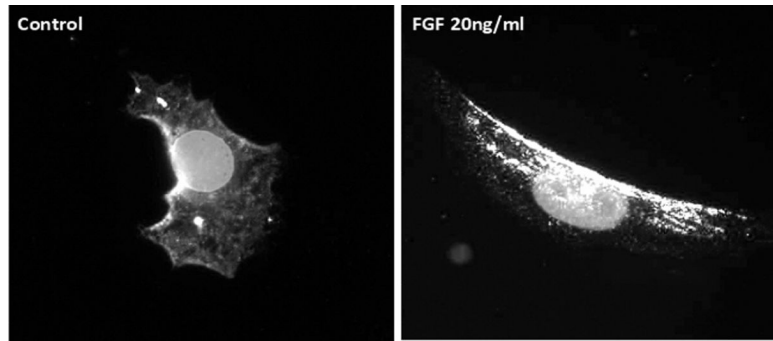
**Figure 1. Relative expression of PAK-1 mRNA in human tissue**

Oligonucleotide microarray hybridization data has been obtained from [www.biogps.com](http://www.biogps.com). Relative signal intensity values are depicted in arbitrary units with ranges.



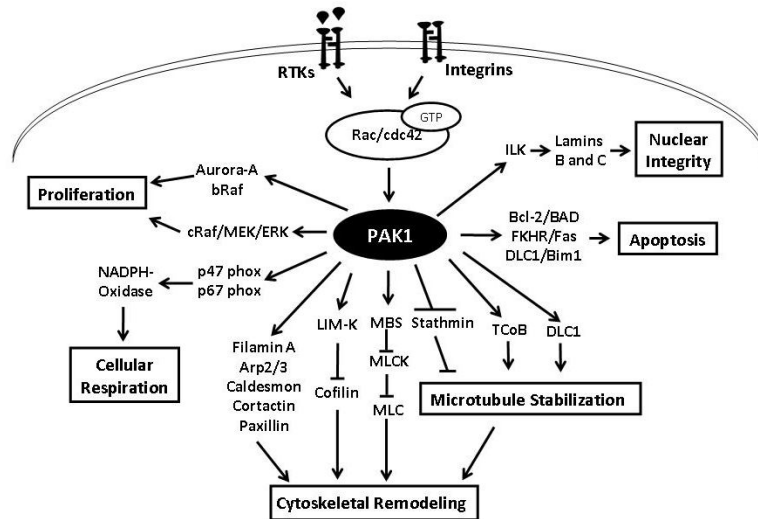
**Figure 2. Schematic representation of human group I and group II PAKs indicating the structural similarities and differences**

Left upper panel shows a 2.3 Å resolution structure of human PAK1 (Group I) kinase domain from the available information on their crystal structures (retrieved from [www.PyMOL.org](http://www.PyMOL.org)). The asymmetric unit of the crystals contains two complexes, an autoregulatory domain (aa 7-149) and a Kinase domain (aa 249-545) with a mutation in the kinase domain (K299R) that ablates its catalytic activity. These domains are linked into a dimer by the N-terminal segments (aa 78-88) of the auto-regulatory fragments. Right upper panel represents linear structure of Group I PAK isoforms. Group I PAKs contain a conserved overlapping PBD/CRIB/AID region. Interaction of Rac and cdc42 with the CRIB domain has been shown to release the group I PAKs from auto-inhibition. Left lower panel shows 1.6 Å resolution structure of enzymatically active human PAK-4 catalytic domain (aa 291-591) when it is phosphorylated at the activating loop positions corresponding to Ser-474. It can be noted that the overlapping CRIB (aa 75-90) and AID (aa 83-149) regions that are present in group I PAKs is missing in PAK4. Right lower panel is a linear representation of group II PAKs. Group II PAKs lack AID, CRIB, acidic and PIX binding regions that are present in Group I PAKs.



**Figure 3. Intracellular localization of PAK1**

Fluorescent microscopic images of NIH 3T3 fibroblasts treated with control (PBS) and fibroblast growth factor (FGF; 20ng/ml) showing intracellular distribution of PAK1. In a resting cell, PAK1 is localized mostly in the cytoplasm. In order to study the PAK1 translocation in activated cells, NIH 3T3 fibroblasts were treated with 20ng/ml of FGF and incubated for 30 minutes in a CO<sub>2</sub> incubator at 37°C. Cells were fixed with 2% para-formaldehyde and stained with PAK1 antibody (Santa Cruz biotechnology). Upon treatment with FGF, PAK1 is seen to be translocated to the plasma membrane according to its well-characterized role in the regulation of cytoskeletal dynamics and cell motility.



**Figure 4. Schematic representation of PAK1 and some of its substrates regulating various cellular functions**

Various stimuli, such as growth factors and integrins, activates PAK1 via GTP-bound Rac and Cdc42. Enhanced expression of Lamins B and C via phosphorylation and activation of Integrin Linked Kinase (ILK) has been implicated in the regulation of nuclear integrity. PAK1 is known to protect cells from apoptosis through at least three different pathways involving FKHR, Bcl-2 and DLC1. In the regulation of cellular proliferation, PAK1 regulates activity of Raf and Aurora kinases. PAK1 is also known to regulate cellular respiration via phosphorylations of p47- and p67- phox proteins. The most studied and well-characterized function of PAK1 is in the regulation of cytoskeletal remodeling. While PAK1 regulates microtubule dynamics through inhibition of stathmin and activation of TCoB and DLC1, it also regulates actin assembly and disassembly through phosphorylations of LIM Kinase and cytoskeletal proteins such as Filamin A, Arp2/3, Caldesmon, Cortactin and Paxillin. Apart from this, PAK1 also phosphorylates myosin binding unit (MBS) that in turn, inhibits phosphorylation of myosin light chain (MLC).



**Table 1**

Examples of interacting partners of PAK1

FUNCTION	INTERACTING PARTNER	Activation (+) /Inhibition (-)	REF
CYTOSKELETAL PROTEINS	Paxillin	N/A	83, 180
	Akt	+/-	126
KINASES	Cdk5	-	181
	Cdk2	-	182
	Heregulin- $\beta$ 1	+	183
	PDK1	+	50
	PI3-K	+	184
PHOSPHATASES	PP2A	-	66
	POPX1	-	67
	POPX2	-	67
ADAPTER PROTEINS	Grb-2	+	61
	Nck	+	62
GTP-ases	cdc42	+	1
	CHP	+	53, 185
	Rac1	+	2
	Rac2	+	39
	Rac3	+	186
	TC10	+	54
	Wrch-1	+	55
	GEFs	$\alpha$ -PIX	+
	$\beta$ -PIX	-	41
OTHERS	CIB1	+	187
	G $\alpha$ $\beta$	-	188
	SphingolipidS	+	46
	RILPL2	+	189
	CRIPak	-	190
	hPIP1	-	191
	LC8	N/A	192
	GIT1/PKL	N/A	193, 194
	FMR1	+	145
	Huntingtin	N/A	146
	HIV-1/Nef	+	153

Table 2

Examples of substrates of PAK1

FUNCTION	CELL TYPE/ANIMAL	SUBSTRATE	PHOSPHORYLATION SITE	REF	
APOPTOSIS	MCF-7	DLC1	S88	81, 195	
	MCF-7	FKHR	S256	195	
	293T, Rat-1, BALB3T3	BAD	S111	125	
PROLIFERATION	HeLa, NIH-3T3 variant S2-6, Cos-7, 293T	C-Raf1	S338, S339	10	
	HeLa, NIH-3T3 variant S2-6, 293, REF52	MEK1	S298	196	
	HeLa	Plk1	S49	81	
	HeLa, MCF-7, ZR75, Ishikawa, MDA-MB-231	ER $\alpha$	S305	97	
	MCF-7	Histone H3	S10	198	
	NIH3T3, RT4 Schwann cells	Merlin	S518	199	
	NIH3T3, Cos-7	AuroraA	T288, S342	200	
	293	B-Raf	S638, S446	201	
	293	MEKK1	S67	202	
	CYTOSKELETAL ASSEMBLY/CELL MOTILITY	Baby hamster kidney-21, HeLa	MLCK	S439, S991	72
		Cos-1, HeLa, Jurkat T cells	GEF-H1	S885	203
		Cos-7, NIH3T3	GIT1	S517	200
		Cos-7	Vimentin	S25, S38, S50, S56, S65, S72	204
HEp-2		Op18/Stathmin	S16	205	
NIH3T3, 293		NET1	S152, S153	206	
MCF-7		P41-ARC	T21	76	
Smooth muscle		Caldesmon	S657, S687	207	
-		CPI17	T38	208	
Smooth muscle		Desmin	-	209	
MCF-7		Filamin A	S2152	210	
293		LIM kinase	T508	211	
-		PP1-MBS	T641	208	
293		$\alpha$ -PIX	S488	17	
293		$\beta$ -PIX	S340, S525	212	
293T, HeLa		Rho-GDI	S101, S174	213	
MCF-7		TCoB	S65, S128	214	
A431 cells		CtBP1	S158	215	
Breast cancer cells		SNAIL	S246	216	
Cardiac muscle cells		TroponinI	S149	210	
Endothelial Cells	Paxillin	S273	180		
OTHERS	K562 leukemia cells	PGM	T466	217	
	Human neutrophils	P47 phox	S303, S304, S320, S338	32	
	MCF-7, ZR75, MCF12A	ESE1	S207	18	
	Mouse mammary epithelial cells	STAT5a	S779	219	
	PC12	Synapsin I	S603	220	
	Swiss 3T3	P67 phox	T233	221	

FUNCTION	CELL TYPE/ANIMAL	SUBSTRATE	PHOSPHORYLATION SITE	REF
	293	Gαz	S16	43
	293T	PGAM-B	S23, S118	222
	293	SHARP	S3486, T3568	214
	MCF-7	ILK	T173, S246	79

**Table 3**

Examples of alterations in PAK1 status in human cancers

Cancer type	Reported events	Detection method	References
Bladder carcinoma	elevated levels of RNA and protein	oligo-microarray; qPCR, IHC	223
Breast carcinoma	elevated levels of RNA, total and phosphorylated protein; nuclear accumulation; gene amplification; increased kinase activity	WB; qPCR; IHC; aCGH; in vitro kinase assay.	110, 119, 224-228
Colorectal carcinoma	elevated levels of protein; gene amplification	mCGH; aCGH; FISH; IHC.	229, 230
Gastric carcinoma	elevated levels of protein	WB	111
Glioblastoma	elevated levels of phosphorylated protein	IHC	231
Hepatocellular carcinoma	elevated levels of RNA and protein	qPCR, WB, IHC	232
NK and NK-like T cell lymphomas	gene amplification	mCGH; aCGH	233
Ovarian carcinoma	elevated levels of RNA, total and phosphorylated protein; gene amplification.	qPCR; WB; FISH	107, 234
Renal cell carcinoma	elevated levels of RNA and protein	qPCR; WB; IHC	235
Shwannoma	elevated levels of phosphorylated protein	WB	106

qPCR – quantitative RT-PCR; IHC – immunohistochemistry; WB – Western blotting; aCGH – array comparative genomic hybridization; mCGH - metaphase comparative genomic hybridization; FISH- fluorescence *in situ* hybridization.