

# Mouse models of PAK function

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**Abbreviations:** ANP, atrial natriuretic peptide; BMMC, bone marrow-derived mast cell; BNP, brain natriuretic peptide; cko, conditional gene knock out; CREB, cyclic AMP response element binding protein; CSF1, colony stimulated factor 1; ERK, extracellular regulated kinase; GDI, guanine-nucleotide disassociation inhibitor; GLUT4, glucose transporter 4; JNK, Jun kinase; LIMK, LIM kinase; LTD, long-term depression; LTP, long-term potentiation; MAPK, mitogen activated kinase; MKK, MAP kinase kinase; MRX, X-linked, nonsyndromic mental retardation; NF1, neurofibromatosis type 1; PAK, p21-activated kinase; TAC, transverse aortic constriction

p21-activated kinases are a family of highly conserved protein serine/threonine kinases that are increasingly recognized as playing essential roles in a variety of key signaling processes. Genetic analyses in mice, using constitutive or regulated gene disruption, have provided important new insights into PAK function. In this paper, we review the genetic analysis of all six PAK genes in mice. These data address the singular and redundant functions of the various PAK genes and suggest therapeutic possibilities for small molecule PAK inhibitors or activators.

is expressed mainly in the testis, prostate, brain, kidney and placenta.<sup>5</sup> The wide range of tissue distribution speaks to the expansive, and likely non-overlapping, biological functions of the PAK family members.

To understand the specific function of each member of the PAK family, individual and, on occasion, compound mutations have been constructed in the mouse. In the sections below, we will review what is known of the genetics of each of the six PAK genes in mice, and, where data are available, in man.

## Introduction

In mammals, the p21-activated kinase (PAK) family consists of two clearly defined subgroups, termed I and II.<sup>1,2</sup> Group I PAKs are closely related by structure and sequence, whereas the group II PAKs are more divergent, suggesting a more ancient evolutionary origin.<sup>3</sup> In man and mouse, both subgroups contain three members with distinct expression patterns (Table 1). The functions of these genes have been extensively probed in a variety of experimental settings, including gene knockdown and gene knockout in cells or organisms ranging from yeast to mice.<sup>4</sup> In this chapter, we will discuss the genetic analysis of group I and II PAKs in mammals, as much new has been learned over the last few years with the increasing availability of genetically engineered mice bearing constitutive or regulated loss of *PAK* alleles.

In general, both group I and group II PAK gene expression can be divided into one of two types: ubiquitous or restricted. *PAK2* and *4* are both ubiquitously expressed, and, as will be discussed, these genes are required for embryonic development in mice.<sup>4</sup> *PAK1* is highly expressed in the brain, muscle and spleen,<sup>5</sup> whereas *PAK3* is expressed predominantly in the brain. Expression of *PAK5* is also restricted to the brain, while *PAK6*

## Group I PAKs

**PAK1.** *PAK1*<sup>-/-</sup> mice are fertile, viable and have a normal life span.<sup>5</sup> However, these mice display subtle immune deficiencies, mainly manifesting in bone-marrow-derived granulated cells, including mast cells, eosinophils and basophils.<sup>6</sup> *PAK1*<sup>-/-</sup> mice have normal peripheral blood indices and, in culture, bone marrow-derived mast cells (BMMCs) from *PAK1* knockouts differentiate normally. However, *PAK1*<sup>-/-</sup> BMMCs fail to respond to stimulation with either IgE or Kit ligand.<sup>6,7</sup> When antigen-sensitized wild-type BMMCs are challenged with IgE, the activated Fc $\gamma$  receptor triggers rapid release of histamine containing granules. This response is greatly blunted in *PAK1*<sup>-/-</sup> BMMCs, which show a 3-fold reduction in release of granule contents as compared with control. Similarly, *PAK1*<sup>-/-</sup> BMMCs show an inability to disassemble F-actin after allergen stimulation, a function required for degranulation. These data place *PAK1* in a critical position for mast cell degranulation, through regulation of F-actin disassembly.<sup>6</sup> Importantly, the cellular defects also manifest in vivo, as Allen et al. showed that passive cutaneous anaphylaxis is greatly reduced in *PAK1* knockout mice, consistent with a defect in mast cell degranulation.

The signaling defects that underlie these phenomena are not completely understood, but are likely to include attenuated activation of the ERK, JNK and p38 pathways, as these pathways are known to affect mast cell function and PAK1 is required for their activation.<sup>6,7</sup> Mast cell motility is also affected by PAK1, as demonstrated in the context of heterozygosity of the *Nf1* gene. Such *Nf1*<sup>+/-</sup> mast cells are normally hyperresponsive to kit ligand due to dysregulated Ras activation, and this augmented response is

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**Table 1.** PAK tissue distribution and knockout phenotype

PAK isoform	Tissue expression	Knockout phenotype
PAK1	Brain, muscle and spleen	Immune defects, glucose homeostasis defects, neuronal defects, cardiac hypertrophy upon pressure overload
PAK2	Ubiquitous	Lethal E8.0
PAK3	Brain	Abnormalities in synaptic plasticity, defects in learning and memory
PAK4	Ubiquitous, high levels in prostate, testis and colon	Lethal E11.5, neuronal defects, improperly formed vessels
PAK5	Brain	Viable, healthy
PAK6	Testis, prostate, brain, kidney and placenta	Viable, healthy
PAK1/3		Loss of brain volume, impaired learning and memory, hyperactive behavior
PAK5/6		Deficits in learning and locomotion

thought to play a pathogenic role in NF1 syndrome.<sup>7</sup> Loss of *PAK1* in this setting inhibits the motility of mast cells, raising the possibility that PAK1-directed small molecule inhibitors might be of benefit in NF1-related pathologies.

Certain other immune cells are also known to be affected by loss of *PAK1*. For example, *PAK1*<sup>-/-</sup> macrophages display numerous, unstable lamellipodia upon adhesion, accompanied by attenuated ERK activation in response to CSF1.<sup>8</sup>

In addition to the immune defects, *PAK1*<sup>-/-</sup> mice have defects in glucose homeostasis.<sup>9</sup> Such mice display significant impairment of insulin secretion, in the absence of detectable defects in islet architecture or insulin content. These mice also display abnormal glucose clearance, with significantly higher glucose levels at 30 and 60 min in a glucose tolerance test, with fasting glucose levels comparable to those of wild-type mice. An insulin tolerance test also showed significantly higher levels of glucose at all time points suggestive of peripheral insulin resistance. Consistent with this idea, Wang et al. showed that, in skeletal muscle, GLUT4 does not translocate to the plasma membrane following insulin stimulation of *PAK1*<sup>-/-</sup> mice. Interestingly, loss of *PAK1* was found to be associated with distinct signaling defects in islet cells as compared with skeletal muscle cells. In islet cells, loss of *PAK1* did not affect phosphorylation of cofilin (a substrate of the PAK1 target, LIMK), but did lead to loss of ERK activation, whereas the opposite pattern was seen in skeletal muscle. In this latter case, defective cofilin activation, with resultant F-actin misregulation, might explain the observed failure of GLUT4 translocation in *PAK1*-null mice.<sup>9</sup> This hypothesis also is consistent with the previously noted role of *PAK1* in mast cell degranulation and F-actin depolymerization in mast cells,<sup>6</sup> and might indicate a general defect with vesicular traffic in *PAK1*-null cells.

Interestingly, in humans, *PAK1* expression levels in type 2 diabetic donor islets have been shown to be significantly reduced, while *Cdc42* and *RhoGDI* expression remain unchanged.<sup>9</sup> These data, together with the observed defects in insulin secretion and glucose tolerance in *PAK1*-null mice, suggest that PAK1 may play a role in glucose metabolism in man, and might represent a therapeutic target in certain forms of diabetes.

In the brain, the PAK1 activator Rac1 is known to be involved in dendritic spine formation, raising the possibility that PAK1 is involved in neuron structure and function. Asrar et al. noted that *PAK1*<sup>-/-</sup> gross brain structure is normal and, at the cellular level,

*PAK1*<sup>-/-</sup> cells show normal synaptic and spine structures, suggesting that PAK1 is not involved with the formation of synaptic structures.<sup>10</sup> However, despite their normal appearance, neuron function, or synaptic plasticity, which can be described by both long-term potentiation (LTP) and long-term depression (LTD), is notably altered in *PAK1*-null mice.<sup>10</sup> Such mice are deficient in LTP, but not LTD, suggesting a selective role for *PAK1* in synapse function. Further analysis showed that, compared with wild-type mice, whose dendritic spines are highly enriched in F-actin, polymerized actin is scarce in the spines of *PAK1*-null mice. In addition, phosphorylated cofilin is misregulated at stimulated synapses in *PAK1*<sup>-/-</sup> cells, potentially causing the deficits in F-actin at the spine.<sup>10</sup>

In contrast to an ablation of *PAK1* in the entire organism, Liu et al. used a conditional gene deletion system to specifically knock out *PAK1* in cardiomyocytes (*PAK1*<sup>cko</sup>).<sup>11</sup> These mice were also shown to be healthy and fertile, as expected, since constitutive *PAK1*-null mice show no gross defects in health or fertility. However, when *PAK1*<sup>cko</sup> mice were subjected to pressure overload by transverse aortic constriction (TAC), a significant increase in heart weight/tibia length ratio was noted.<sup>11</sup> Additionally, there was a significant increase in the cross-sectional area of cardiomyocytes in the *PAK1*<sup>cko</sup>-TAC mice compared with the control-TAC mice. It was also noted that more interstitial fibrosis developed in the *PAK1*<sup>cko</sup>-TAC myocardium. Importantly, atrial and brain natriuretic peptides (ANP and BNP), as well as fetal contractile protein isoforms such as  $\alpha$ -myosin heavy chain 7 mRNA, was found to be significantly elevated in hypertrophied *PAK1*<sup>cko</sup> myocardium. The increase in these genes suggests that the progression of ventricular hypertrophy to heart failure in this mouse model represents a re-activation of the fetal heart program in cardiomyocytes.<sup>12</sup> These results also suggest that PAK1 normally acts as an anti-hypertrophic agent. Indeed, when treated with FTY720, a sphingosine-like analog that activates PAK1, wild-type mice resisted developing pressure overload-induced hypertrophy. In addition to these studies, Liu et al. assessed the effects of prolonged TAC stress to determine if *PAK1*<sup>cko</sup> mice became more sensitized to heart failure. With extensive TAC treatment, they found that these mice displayed higher lung weight/tibia length ratio suggestive of pulmonary edema, a significant reduction in fractional shortening, indicating decreased contraction, and further enlargement of the heart compared with

the control group. These data show that extensive load stress renders *PAK1* knockouts more susceptible to heart failure as compared with controls. Similar data were obtained using a separate heart failure model, in which hypertrophy is induced by neuroendocrine stimuli. In terms of signaling, Liu et al. found that pressure overload induces an increase in MKK7, MKK4 and JNK in wild-type animals, but that this pathway is attenuated in cardiomyocytes from *PAK1*-null mice. These data implicate the JNK cascade, as opposed to the ERK cascade, as the major MAPK pathway linking *PAK1* to ventricular hypertrophy.<sup>11</sup>

**PAK2.** Though much is known about *PAK2* biochemistry and its role in cellular signaling pathways, much less is known about its function in the developing embryo. *PAK2*-null mice have been produced, but contrary to the other group I PAK members, loss of the *PAK2* gene leads to embryonic lethality at ~E8.0.<sup>4,5</sup> Embryogenesis fails due to multiple developmental abnormalities,<sup>5</sup> most prominently involving defective vascularization. *PAK2*<sup>ko</sup> mice have been produced by our laboratory and are currently under study.

**PAK3.** In man, lesions in *PAK3*, a gene whose expression is predominantly restricted to the brain, are associated with X-linked, nonsyndromic mental retardation (MRX).<sup>4,5</sup> To date, five separate MRX-30 kindreds have been reported, each with distinct missense or nonsense mutations in *PAK3* that are predicted to result in loss of function in the *PAK3* protein.<sup>13</sup> Interestingly, one of these mutations, R67C, does not affect kinase activity per se but does diminish the binding of *PAK3* to its activator, *Cdc42*.<sup>14</sup> It is also interesting to note that the various *PAK* mutants have distinct effects on neurons when expressed in cultured cells.<sup>14</sup>

To model MRX-30, Meng et al. produced *PAK3*-null mice.<sup>15</sup> These mice are healthy, fertile, have normal locomotor activities and a normal life span. Additionally, these mice display normal brain structure and no abnormalities in the CNS, including hippocampus and cerebellum, much like patients with nonsyndromic mental retardation. Furthermore, *PAK3*-null mice display normal dendrite and spine morphology, and normal basal synaptic strength, indicating that basal synaptic strength and presynaptic neurotransmitter release are not affected in these knockout mice. The group determined that while LTD and early phase LTP are normal between genotypes, late phase LTP (L-LTP) is significantly reduced in *PAK3*-null mice, a phenotype mimicking the *PAK1*-null mice. However, unlike *PAK1* knockout mice, phosphorylated CREB is reduced in the *PAK3* knockouts, whereas phosphorylated cofilin is unaffected.<sup>16</sup> Together with the molecular analysis, the group showed that these mice display deficient memory retention through taste aversion tests.<sup>15</sup> As CREB is known to be required for L-LTP and memory formation,<sup>15</sup> these findings provide a potential molecular pathway linking *PAK3* mutations to mental retardation. These findings also suggest that *PAK1* and *PAK3* are redundant in synapse function.

To test for such functional redundancy, Huang et al. crossed *PAK1*- and *PAK3*-null mice. The double-knockout mice were normal at birth but soon showed major loss of brain volume compared with that of wild-type mice, despite normal brain

organization.<sup>17</sup> *PAK1/PAK3* double-knockout mice also had deficits in learning and memory and displayed a hyperactive behavior, reminiscent of that of humans with *PAK3* mutations.<sup>17</sup> The double knockout mice also displayed a much less complex neuronal morphology, with reduced dendrite length and number of dendritic tips, suggesting that *PAK1* and *3* are involved in branch formation. As in *PAK1*-null mice, phosphorylated cofilin levels were significantly reduced in the double knockouts, suggesting a role for both *PAK1* and *3* in the regulation of cofilin and thereby in neuron structure and function.<sup>17</sup>

## Group II PAKs

**PAK4.** *PAK4*, as with *PAK2*, is expressed ubiquitously and its expression begins early in development.<sup>5</sup> In cells, *PAK4* is thought to mediate *Cdc42* induced filopodia formation,<sup>18</sup> induce changes in cytoskeletal organization and cell adhesion<sup>19</sup> and stimulate cell survival pathways.<sup>20</sup> The *PAK4* gene has been disrupted in vivo and causes embryonic lethality by day E11.5, most likely from a heart defect.<sup>5</sup> *PAK4*-null embryos show a thinning of the myocardial walls of the bulbous cortex and ventricle, likely leading to impaired ventricular functioning and pooling of blood within the heart, causing its distended appearance.<sup>21</sup> The *PAK4*-null mice also display other impairments, chiefly noted within the nervous system and in vessel formation.<sup>21,22</sup> *PAK4*-null embryos display a thin neuroepithelia in the hindbrain and forebrain, causing an unusual translucent head and neural tube in the intact embryo. These embryos have defects in neuronal differentiation and axonal outgrowth. While neuronal progenitors form properly, differentiation and migration of both motor neurons and ventral interneurons are defective.<sup>21</sup> These findings are consistent with in vitro data showing that *PAK4* is involved in the formation of filopodia, a structure known to play key role in the guidance of neuronal growth cones. Equally important, defects have been noted in the caudal portion of the neural tube of *PAK4*-null mice, consistent with misfolding. Such misfolding might cause the formation of two separate lumens, a process regulated by the cytoskeleton.<sup>21</sup>

In addition to neuronal and heart defects, *PAK4*-null mice also show defects in vascularization.<sup>22</sup> In a developing embryo, fetal blood vessels must integrate with maternal blood vessels in the placenta for proper nutrient delivery, specifically the labyrinth layer.<sup>22</sup> *PAK4*-null mice display defects within this layer including the lack of proper vessel branching. Additionally, the yolk sac of the developing *PAK4*-null embryo shows poorly developed vessel branching.<sup>21</sup> Since vessel formation is initiated in both the placenta and yolk sac, *PAK4* most likely is involved with the formation or extension of branching vessels and therefore angiogenesis. Both angiogenesis and the heart defect could be explained by a disruption in endothelial cell migration, a process known to be regulated by *PAK4*.<sup>21</sup> However, further evidence is needed to support this hypothesis. Correspondingly, it is likely that these defects contribute to the embryonic lethality caused by the genetic disruption of *PAK4*.

Due to embryonic lethality of *PAK4*-null mice and further interest in neuron development, conditional knockout *PAK4* mice have been developed, in which *PAK4* is deleted in the nervous

system.<sup>23</sup> Neuronal defects were seen in *PAK4<sup>cko</sup>* as early as day E16.5, with normal neuron development beginning around day E11; however, striking differences in brain anatomy were observed at birth. Gross morphology of the brain was normal, specifically all layers of the cortex were present, but significantly thinner than that of the controls, with the outer most layers being the thinnest. This thinning was attributed to a decrease in proliferation of neural progenitor cells. In addition, these mice displayed severe hydrocephalous, possibly contributing to their early demise between two and four weeks after birth. Furthermore, adherens junctions in the neuroepithelial were lost at the ventricular interface at day E18.5.<sup>23</sup> Since these junctions have been associated with cell proliferation, it is possible that their absence causes the observed decrease in cell proliferation. To further investigate the role of *PAK4* in the developing brain, *PAK4* substrates were examined for activity. No significant changes were detected in the phosphorylation or total expression levels of ERK, A-Raf, B-Raf, Raf-1, Stat3, c-Myc or p120-catenin. Interestingly, however, neuroepithelial adherens junctions were found to lack  $\beta$ -catenin, a known substrate of *PAK4*.<sup>23,24</sup> As disruption in  $\beta$ -catenin signaling has been shown to cause reduced levels of cell proliferation as well as hydrocephalous and post-natal death,<sup>25,26</sup> these data support a significant role for *PAK4* and  $\beta$ -catenin in the developing mammalian brain.

**PAK5 and PAK6.** Interestingly, *PAK5*-null mice are viable and healthy and through in depth examinations appear to be comparable to that of the wild-type.<sup>27</sup> The morphology of the brain, hippocampus, eyes, pancreas and other tissue such as the testes, prostate, epididymis and adrenal gland all appear to be normal.<sup>27</sup> Similarly, *PAK6*-null mice are viable, healthy and fertile.<sup>28</sup> The lack of abnormal phenotypes for these single knockouts could be due to redundancy between *PAK5* and *6*, due to their overlapping expression patterns.<sup>5</sup> To further examine this possibility, *PAK5* and *6* were knocked out in tandem. The

combined knockout mice were healthy and viable without any obvious signs of defects.<sup>28</sup> Unlike *PAK4*, *PAK5* and *6* have a restricted pattern of expression and are expressed later in development, perhaps explaining the lack of overt phenotype. The double knockouts did display deficits in learning as well as locomotor activity, consistent with the fact that *PAK5* and *6* are expressed in brain structures that are strongly involved in cognitive function. Further analysis in these mice showed normal motor neuron progenitor cells; however, they were reduced in numbers compared with that of wild-type mice. The authors also noted that motor neurons in these mice do not migrate to their proper location. In vitro data of cultured motor neurons suggest a lack in proper growth cone size and structure,<sup>28</sup> possibly linking *PAK5* and *6* to cytoskeletal changes in the brain, affecting neuronal plasticity.

## Summary

The use of genetic engineering, particularly constitutive and conditional gene knockouts in mice, has been essential in determining the specific functions of each *PAK* isoform in mammals. In the near future, such models should be useful in determining the role of *PAK* isoforms in various mouse tumor models as well as other disease models. For example, *PAK1* and *PAK4* have been implicated in a number of human malignancies; breeding *PAK*-deficient mice to the appropriate mouse cancer models could provide useful preclinical evidence for future therapeutic interventions. Also, knock-in models could help further define *PAK* function. For example, knock-in of “analog-sensitive” kinase alleles could be used to test the effects of small-molecule mediated *PAK* inhibition in vivo.<sup>29,30</sup> As genetic engineering methods are becoming increasingly facile, it is not unreasonable to expect that we will have a sophisticated understanding of the function of all six *PAKs* in the near future.

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