p21-Activated Kinase 1 Is Required for Efficient Tumor Formation and Progression in a Ras-Mediated Skin Cancer Model

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Abstract

The RAS genes are the most commonly mutated oncogenes in human cancer and present a particular therapeutic dilemma, as direct targeting of Ras proteins by small molecules has proved difficult. Signaling pathways downstream of Ras, in particular Raf/Mek/Erk and PI3K/Akt/mTOR, are dominated by lipid and protein kinases that provide attractive alternate targets in Ras-driven tumors. As p21-activated kinase 1 (Pak1) has been shown to regulate both these signaling pathways and is itself upregulated in many human cancers, we assessed the role of Pak1 in Ras-driven skin cancer. In human squamous cell carcinoma (SCC), we found a strong positive correlation between advanced stage and grade and PAK1 expression. Using a mouse model of Kras-driven SCC, we showed that deletion of the mouse Pak1 gene led to markedly decreased tumorigenesis and progression, accompanied by near total loss of Erk and Akt activity. Treatment of KrasG12D mice with either of two distinct small molecule Pak inhibitors (PF3758309 and FRAX597) caused tumor regression and loss of Erk and Akt activity. Tumor regression was also seen in mice treated with a specific Mek inhibitor, but not with an Akt inhibitor. These findings establish Pak1 as a new target in KRAS-driven tumors and suggest a mechanism of action through the Erk, but not the Akt, signaling pathway. Cancer Res; 72(22); 5966–75. ©2012 AACR.

Introduction

The Ras genes—HRAS, KRAS, and NRAS—represent one of the most important oncogene families in human cancer, with activating mutations seen in approximately 30% of solid tumors (1). Ras proteins act as switch molecules by transmitting mitogenic signals in response to variety of extracellular stimuli by binding and hydrolyzing GTP, as well as regulating diverse cellular processes such as proliferation, migration, senescence, differentiation, and survival. In human cancer, activating mutations in RAS promote cell proliferation and result in tumorigenesis that generally correlates with poor prognosis and poor therapeutic response (2). As the oncogenic role of the Ras protein is well established, numerous attempts have been made to target this GTPase for the treatment of human cancers. Strategies for blocking activated Ras have included attempts to reduce its expression, interfere with its subcellular localization, and inhibit its downstream effectors (3, 4). With regard to the latter, more than 20 proteins have been reported as effectors of Ras, and many of these provide potentially suitable drug targets (5, 6).

The phosphoinositol-3 kinase (PI3K)/Akt/mTOR and Raf/Erk signaling modules are among the best-studied Ras effector pathways. A growing body of evidence indicates that members of the p21-activated kinase (Pak) family, in particular Pak1, are required for the activation of both these pathways. Paks are serine-threonine-specific protein kinases that act downstream of the small GTPases Cdc42 and Rac in a variety of signaling pathways (7–9). Mammalian cells encode 6 Pak isoforms—group A (Pak1, Pak2, and Pak3) and group B (Pak4, Pak5, and Pak6)—with partly overlapping but also clearly distinct signaling properties (10). In Erk signaling, Pak1 phosphorylates Raf at S338 and Mek1 at S298, sites that are required for full activation of the small GTPases Cdc42 and Rac in a variety of signaling pathways (7–9). Mammalian cells encode 6 Pak isoforms—group A (Pak1, Pak2, and Pak3) and group B (Pak4, Pak5, and Pak6)—with partly overlapping but also clearly distinct signaling properties (10). In Erk signaling, Pak1 phosphorylates c-Raf at S538 and Mek1 at S298, sites that are required for full activation of these proteins in some cell types (10, 11). In the Akt pathway, Pak1 is thought to act in a noncatalytic fashion, acting as a scaffold to bridge PDK1 to Akt (12, 13). Inhibition or loss of Pak1 might therefore be expected to interfere with the oncogenic potential of proteins such as Ras that induce transformation at least in part by activation of these pathways. A wealth of in vitro data support this view, as expression of...
dominant negative alleles of Pak1, reduction of Pak1 expression by RNAi and small molecule inhibitors of Pak1 have all been shown to interfere with Ras-driven transformation (11, 14, 15). However, the role of Pak1 in Ras-driven tumor genesis in vivo, and the particular signaling pathways affected, are not defined.

In this work, we asked if Pak1 plays a role in Ras-induced skin cancer. Using human skin cancer tissue microarrays, we found that Pak1 expression levels are associated with more aggressive grades and poorer differentiation of squamous cell carcinoma (SCC). Functional data were then obtained by crossing an inducible KrasG12D driven mouse model of skin cancer with Pak1 wild-type, heterozygous, or knockout mice. In such mice, we found that Pak1 gene dosage was positively correlated with tumor initiation and progression. KrasG12D mice lacking Pak1 showed marked reduction in both Erk and Akt activation, indicating that Pak1 function is required for activation of these signaling pathways by Kras in vivo. Tumor regression was also noted when KrasG12D/ Pak1 mice were treated with either of 2 distinct small molecule Pak inhibitors (PF3758309 or FRAX597) or a Mek inhibitor, but not with an Akt inhibitor. These findings establish Pak1 as a new target in Ras-driven tumors and define a mechanism of action primarily through the Erk, but not the Akt, signaling pathway.

Materials and Methods

Generation of transgenic mice and tumor measurement

K5-tTA and tet-KrasG12D transgenic mice (FVB-N; ref. 16) were crossed with Pak1 knockout mice (FVB-N; ref. 17) separately to generate K5-tTA/Pak1−/− and Pak1−/−/tet-KrasG12D colonies. Progeny from these colonies were subsequently bred to generate K5-tTA/tet-KrasG12D mice that were wild-type, heterozygous, or knockout for Pak1. Genotyping was carried out by PCR analysis of tail biopsy DNA. A doxycycline diet was given to a similar number of mice from each cohort as well as littermates from all 3 colonies not receiving doxycycline treatment as controls. All mice were examined daily to assess tumor onset. Tumor measurements were taken by calipers thrice weekly and tumor volume was calculated as width2 × length × 0.5. Mice were euthanized when the tumor size reached >1 cm or if mice exhibited signs of illness.

Doxycycline administration

Doxycycline-containing food was administered to mice at 3 months of age in a pelleted form with complete grain-based rodent diet and the control littermates were fed an identical diet, lacking doxycycline.

Cancer therapy using inhibitors

Mek inhibitor PD0325901 (18) was reconstituted and administered to mice receiving either a single dose of 20 mg/kg/day or an equivalent volume of vehicle (0.5% methylcellulose, 0.2% Tween-80) via oral gavage. Pak inhibitor PF3758309 was synthesized according to published protocols (19) and was formulated in saline and administered by intraperitoneal injection at 25 mg/kg/day or an equivalent volume of vehicle. Akt inhibitor GSK690693 (20) was dissolved in water and administered by intraperitoneal injection at dose of 30 mg/kg/day or equivalent volume of vehicle. FRAX-597 (6-(2-chloro-4-(thiazol-5-yl)phenyl)-8-ethyl-2-(4-(4-methylpiperazin-1-yl)phenylamino)pyrido[2,3-d]pyrimidin-7(8H)-one, Chemical Formula: C29H28ClN7OS, Exact Mass: 557.18, molecular weight: 558.10 (Fig. 4B; Afraxis) was formulated in 10% (PEG400:Tween 80:H2O:K30–90:5:5) 15% Vitamin E-TPGS and 75% of HPC (0.5%) in 50 mmol/L citrate buffer (pH 3) and administered by oral gavage at 90 mg/kg/day. All treatments were begun when tumors became visible (~20 mm3, ~5 day post doxycycline feedings) and were continued for 7 or 10 days, at which time the animals were sacrificed and SCC tissues taken for analysis.

Tissue preparation, histology, immunohistochemistry, and immunoblotting

All tumors, lesion controls and internal organs were fixed overnight in 4% paraformaldehyde, dehydrated and embedded in paraffin. Hematoxylin and eosin (H&E) stained sections were used for diagnostic purposes and unstained sections for immunohistochemical (IHC) studies. IHC was conducted with the following antibodies: rabbit polyclonal antibody for Pak1 (1:50), Pak2 (1:50), phospho-Erk1/2 (pThr202/pTyr204; 1:100), phospho-Akt (pThr308; 1:30), phospho-mTOR (pSer235/p236; 1:5,000), anticleaved caspase 3 (Cell Signaling Technology), and rat monoclonal antibody for K167 (Dako). The evaluation of the IHC was conducted blindly, without knowledge of the origin or genotype. The percentage of Ki67 positive cells was determined by scanning the slides using an Aperio CS Scanscope scanner and a nuclear detection software from the same manufacturer (2,000 to 5,000 cells were counted per mouse, 3 to 5 mice/group). Apoptosis was evaluated counting CC3 positively stained in 5 high magnification fields per mouse (×400, 3 to 5 mice per group, minimal number of cells counted/mouse was 500 per mouse).

Immunoblot analyses were carried out on lysates extracted from tumors. Protein concentration was determined, and equal amounts of total proteins were separated on SDS-PAGE. Antibodies used included Pak1, Mek, Erk, phospho-Erk1/2 (pThr202/pTyr204), Akt, phospho-Akt (pThr308), GSK3β, phospho-GSK3β (pSer9), mTOR, phospho-mTOR (pSer2448), p70S6K, phospho-p70S6K (pThr389), S6, phospho-S6 (pSer235/p236), and cyclin D1 from Cell Signaling Technology; K-ras, phospho-Pak (pSer141) and phospho-Mek (pSer298) were from Invitrogen. GAPDH was used as loading control.

Statistical analysis

Kaplan–Meier survival curves and statistical analysis were carried out using GraphPrism program. Tumor volumes of mice treated with different inhibitors were compared with control group using a 2-tailed Student t test with P < 0.05 considered statistically significant.

Results

Expression of Pak1 correlates with advanced grade of human squamous cell skin cancer

In normal human skin, Pak1 expression was largely restricted to the basal layer and scattered inflammatory cells (Fig. 1A,
Role of Pak1 in a conditional model of Kras-driven squamous cell skin cancer

To investigate the effect of Pak1 signaling on Ras-mediated oncogenesis in mice, we used a well-characterized mouse model in which transgenic animals express a tetracycline-inducible oncogenic KrasG12D mutant under the control of the K5 promoter, which is active in keratinocytes (16). Upon doxycycline induction, these mice develop a broad range of proliferative lesions and carcinomas in squamous epithelial cells such as skin and salivary gland (16, 22). Mice carrying K5::rTA or tet-KrasG12D transgenes were bred with Pak1 knockout mice (17) separately to generate Pak1−/−;K5::rTA and Pak1+/−;tet-KrasG12D animals. Intercrosses of these mice generated Pak1 wild-type, heterozygous, and knockout mice bearing the K5::rTA and tet-KrasG12D transgenes, in which KrasG12D activation is restricted to the basal stratified epithelial cells upon doxycycline induction.

In nontransgenic mice, loss of Pak1 did not affect skin development or cellular architecture (Supplementary Fig. S1). In wild-type and heterozygous animals, Pak1 IHC showed staining in the epidermis, hair follicles and sebaceous glands. Pak1−/− mice exhibited an almost total absence of Pak1 immunostain in the epidermis with mild immunostain still remaining in some sebaceous glands. Interestingly, Pak2 IHC showed that all cutaneous structures expressed Pak2 in the remaining in some sebaceous glands. Interestingly, Pak2 IHC showed that all cutaneous structures expressed Pak2 in the nontransgenic mice. However, Pak2 IHC in mice containing both the K5::rTA and tet-KrasG12D transgenes, the presence of doxycycline in the diet resulted in 2- to 3-fold elevated Kras expression whereas doxycycline did not affect Kras levels in animals that contained only a single transgene (i.e., K5::rTA or tet-KrasG12D) alone. Fig.
Interestingly, both Pak1 and Pak2 protein levels were 2- to 4-fold elevated in K5-rTA::tet-KrasG12D mice after doxycycline induction of KrasG12D expression. As early as 5 days after doxycycline administration, K5-rTA::tet-KrasG12D mice developed multiple dome-shaped lesions on the surface of the skin. Almost all such lesions rapidly transformed to frank SCC. Thus, activation of the Kras oncogene is tightly regulated in vivo and results in rapid formation of SCC. A control group of mice bearing only single transgenes (K5-rTA or tet-KrasG12D) that were treated with doxycycline did not develop any lesions or exhibit any signs of illness, irrespective of Pak1 genotype. No control mice died or developed skin lesions during this time period (data not shown).

We compared the incidence and latency of tumor appearance in Pak1+/+, Pak1+/−, and Pak1−/− mice in the presence of the KrasG12D transgene. After induction of Kras, Pak1+/+ mice rapidly developed skin tumors, with 50% of mice bearing visible lesions by 8 days, and all mice bearing visible lesions by 20 days (Fig. 2B and C). In contrast, the Pak1 heterozygotes took twice as long to develop visible lesions and a few mice remained tumor free by 40 days. Pak1−/− mice had an even greater latency period and about a third of the mice remained free of visible tumors at 40 days. Pak1+/+ mice showed much earlier tumor development, with a median of 8 days until tumors detection versus 17 days for Pak1+/− and 25 days for Pak1−/− mice (Fig. 2C). The significant delay in tumor initiation and progression in Pak1−/− mice was associated with increased...
survival, as all Kras\textsuperscript{G12D} expressing Pak\textsuperscript{1+/-} and Pak\textsuperscript{1-/-} mice had to be euthanized by 1 month, whereas 86% (6/7) of matched Pak\textsuperscript{1/-} mice were alive at 1 month. Although most of the K5\textsuperscript{-rTA:tet-Kras\textsuperscript{G12D}} mice fed with doxycycline-containing food eventually developed papillomas and SCC, irrespective of the Pak\textsuperscript{1} genotype, the total number of tumors (Fig. 2D) and the average total tumor volume was dramatically reduced in Pak\textsuperscript{1/-} mice (40 mm\textsuperscript{3}) compared with those in Pak\textsuperscript{1+/-} (90 mm\textsuperscript{3}) and Pak\textsuperscript{1-/-} (140 mm\textsuperscript{3}) mice (Fig. 2E).

Histologic analysis indicated that the tumors from Pak\textsuperscript{1+/-}::K5\textsuperscript{-rTA:tet-Kras\textsuperscript{G12D}} mice were hyperproliferative, as shown by an increased number of Ki67-positive cells (Fig. 3A and 3B). There was no statistically significant difference between Ki67 staining of tumors from these mice and their counterpart heterozygous Pak\textsuperscript{1/-} mice (23% vs. 21%). On the other hand, Ki67 staining was markedly reduced in Pak\textsuperscript{1/-}::K5\textsuperscript{-rTA:tet-Kras\textsuperscript{G12D}} mice (Fig. 3A and 3B). In addition, skin lesions were detected in 100%, 87%, and 71% of Pak\textsuperscript{1+/-} (n = 15), Pak\textsuperscript{1-/-} (n = 15), and Pak\textsuperscript{1-/-} (n = 7) mice, respectively (Fig. 3C). In Pak\textsuperscript{1-/-} mice, only 20% of tumors were papillomas whereas 80% were SCC, with some presenting a predominantly well-differentiated pattern of squamous differentiation but most featuring a poorly differentiated squamous histotype. The latter tumors frequently invaded the subjacent dermal connective tissue (Fig. 3A).

However, this advanced grade of carcinoma progression was much less common in Pak\textsuperscript{1/-} mice. In Pak\textsuperscript{1-/-} mice, about 60% had benign tumors (papillomas) and just 14% SCCs (Fig. 3C), and these few tumors tended to be smaller than those seen on wild-type mice (Fig. 2E). Taken together, the rapid tumor onset and progression of SCC in Pak\textsuperscript{1+/-} but not Pak\textsuperscript{1-/-} mice suggests that Pak1 is a key component in Kras-mediated oncogenesis. In this aggressive disease model system, deletion of the Pak1 gene is sufficient to slow tumor initiation and progression, alter the tumor spectrum, and prolong survival.

**Downregulation of multiple signaling pathways in Pak1\textsuperscript{-/-} tumors**

Pak1 has been implicated in regulating Erk signaling downstream of Ras, via phosphorylation of Mek1 and c-Raf (14, 23, 24) and also in activating Akt via a scaffolding interaction with PDK1 (12, 15). To investigate the molecular mechanisms underlying reduced cancerous growth in Pak1\textsuperscript{-/-} mice, we investigated the status of the Raf-Mek-Erk and PI3K-Akt pathways in the Kras\textsuperscript{G12D} model. We found that the Raf-Mek-Erk pathway is persistently activated in the epithelial lesions after onset of Kras mutant expression in transgenic mice. As shown in Fig. 4A and S2, activation of Erk signaling cascade was observed in tumors of Pak\textsuperscript{1+/-} and Pak\textsuperscript{1-/-} mice. Strikingly, we found a strong reduction in phosphorylation of Mek and Erk in tumors isolated in Pak1\textsuperscript{-/-} mice (Fig. 4A and Supplementary Fig. S2).

We next determined the status of the Akt pathway. Activation of Akt and GSK3\beta were markedly attenuated in Pak1\textsuperscript{-/-} mice, as assessed by immunoblot (Fig. 4B) and IHC (Supplementary Fig. S2). In addition, there was a decrease in the phosphorylation of Akt targets mTOR, p70 S6K, and S6 in Pak1\textsuperscript{-/-} tumors (Fig. 4C and Supplementary Fig. S2). Suppression of cyclin D1 expression was also noted in Pak1\textsuperscript{-/-} tumor tissues (Fig. 4C). Interestingly, in the few cases of large SCC

![Figure 3](https://cancerres.aacrjournals.org/content/72/22/5970/F3.large.jpg)
Inhibition of tumor growth and Ras pathway by small molecule signaling inhibitors

The long latency and slow growth of tumors in Pak1−/− mice could be related to defective activation of the Erk and/or Akt-mTOR signaling pathways that require Pak1 for full activity. To determine the relative contributions of these pathways, we used small-molecule inhibitors that target Pak directly, or that selectively target Erk or Akt activation. First, 2 Pak inhibitors—PF3758309, which potently suppresses both group A (Pak1, Pak2, and Pak3) and B (Pak4, Pak5, and Pak6) Paks (19), and FRAX-597, a new group A-specific Pak inhibitor (Fig. 5B)—were assessed for effects on tumor growth and signaling. It should be noted that both Pak inhibitors have certain off-target effects on other kinases, but that these off-target effects are largely nonoverlapping (Fig. 5A, Supplementary Fig. S3, and Supplementary Table S3).

To test the effects of these compounds, a cohort of K5-rTA::tet-KrasG12D mice were fed a doxycycline diet and, when tumors became visible (~5 days postonset of doxycycline), the mice were separated into 2 groups and treated daily with either inhibitor or vehicle, whereas remaining on a doxycycline diet. Mice were weighed weekly and tumor volume measured every 2 days for 2 weeks. The inhibitory effects of these compounds were evaluated at the end of the treatment course. Mice treated with either Pak inhibitor showed marked tumor regression. In the case of the pan-Pak inhibitor PF3758309, average tumor volume was reduced by 92% (from 64.80 ± 6.52 mm3 to 4.88 ± 0.89 mm3; Fig. 5C). Similar effects were noted in mice treated with FRAX-597, with reductions on average tumor volumes of 89% (Fig. 5D). In treated mice, tumor tissue, if any remained, was characterized by an increase in apoptosis without a notable change in proliferation, as assessed by staining with cleaved caspase-3 and Ki-67, respectively (Supplementary Fig. S4B).

For both cohorts, tumor tissues were examined by immunoblot analysis to determine the effects of the Pak inhibitors on signal transduction in vivo. As expected, both PF3758309 and FRAX-597 induced near complete abolition of Pak activation (Fig. 5G and H). Interestingly, FRAX-597, but not PF3758309 treatment was associated with marked loss of total Pak1 expression (Fig. 5G and H). Levels of Pak2, but not Pak4, were also reduced in tumor tissues from these animals (Fig. 5H). For either compound, Mek and Erk activity was reduced, comparable to levels seen in Pak1 knockout mice (Fig. 5G and H). Interestingly, Akt activity, as assessed by anti-phospho-Thr308 antibodies, was also severely reduced in mice treated with either anti-Pak agent. Analysis of tumor tissue from PF3758309-treated mice showed a marked increase in apoptosis, consistent with Akt inhibition (Supplementary Fig. S4B). It should be noted that SCC was not present in FRAX-597 treated mice, precluding evaluation of apoptosis in tumor tissue.

Treatment of mice with the Mek inhibitor PD0325901 (25) had similar beneficial effects on tumor regression (Fig. 5E), in the absence of any notable toxicity such as anemia. With this compound, activation of Mek and Erk, but not Pak or Akt, was reduced (Fig. 5I). In contrast, the Akt inhibitor GSK690693, which has activity in leukemia cell lines (26), in xenografts models (20), and in preclinical settings (27), had only a small effect on SCC tumor regression, even at a dose that markedly reduced Akt signaling activity (Fig. 5F and J) and induced apoptosis (Supplementary Fig. S4A).
did not notably affect the activity of Pak or Erk (Fig. 5J). These data show that Akt inhibition alone does not have a robust antitumor effect in this animal model.

**Discussion**

This study represents the first use of Pak-deficient animals and small molecule Pak inhibitors to study the role of these kinases in a genetically engineered mouse model of cancer. We report that loss of Pak1 gene function caused a marked reduction in the number, latency, and progression of Kras-driven squamous cell skin cancer. In cancer cells from such animals, Erk and Akt signaling activity were severely reduced. Moreover, loss of Pak function induced by either of 2 distinct small molecule Pak inhibitors was associated with regression of Ras-driven tumors of the skin, as well as reduction in Erk and Akt activity. These results could be replicated treating the mouse model with a small-molecule inhibitor of Mek, but not with an inhibitor of Akt signaling. These data suggest that Pak1, via activation of the Erk cascade, is required for efficient tumorigenesis and tumor maintenance in this aggressive and highly penetrant Kras cancer model.

Of the 6 Pak isoforms, Pak1 in particular has garnered much attention with respect to tumorigenesis, as amplification of the PAK1 gene, with concomitant overexpression of the Pak1 protein, is commonly observed in human cancers of the breast, ovary, and bladder (11). Unlike other oncogenic serine/threonine protein kinases such as B-Raf, activating point mutations or deletions of Pak1 have not been found in human cancers, despite the ability of activated Pak1 mutants to transform cells in vitro and in vivo. PAK1 gene amplification seems to be particularly relevant in human breast cancer, as it has been shown that such amplification is associated with resistance to tamoxifen treatment (28), but may well also play a role in Ras-driven tumors. For example, a recent study by Ong and colleagues reported that elevated Pak1 expression is prevalent in 61% of head and neck tumors and 64% of squamous non-small cell lung cancer (NSCLC; ref. 21). In both human head and neck and in human NSCLC cell lines with PAK1 gene amplification, Pak1 has been shown to activate the Erk cascade, leading to increased proliferation and survival of cancer cells. This suggests that Pak1, via activation of the Erk cascade, may be a key player in the development and progression of these cancers.

**Figure 5.** Tumor regression in mice treated with Pak inhibitors. A, specificity of Pak inhibitors. One micromol per liter FRAX597 or PF3758309 was tested for inhibitory activity against a panel of >300 protein kinases (Invitrogen). Inhibition of >50% is represented by yellow spheres, and >75% by red spheres, mapped onto a kinome tree diagram. Inset shows protein kinase family groups plotted on tree diagram. Unique and common targets of the 2 kinase inhibitors (>75% inhibition) are shown on a Venn diagram. B, structure of Pak inhibitor FRAX-597. C–F, volumetric changes in tumor size between untreated mice (vehicle) and mice treated with inhibitors for 7 (FRAX597) or 10 (PF3758309, PD0325901, and GSK690693) days. G–J, Immunoblot analysis of tumor lysates from animals treated with inhibitors or vehicle.
amplification, reduction of Pak1 expression by shRNA induced loss of Erk activity and gain of caspase activity, accompanied by increased cellular apoptosis. As both these cancers are associated with frequent KRAS mutation, these data suggest a general role for Pak1 in Ras-driven tumorigenesis. Such studies are also consistent with earlier work showing that active Pak1 is required for transformation by Kras in vitro (14, 15, 29).

Wang and colleagues recently reported that Rac1, a direct upstream activator of Pak1, is essential for DMBA/TPA-induced skin tumor formation in mice (30). In this model of skin cancer, Hras (and occasionally Kras) is frequently mutated and represents the essential initiating oncogenic event (31, 32). The effects of Rac1 gene deletion in this model were likely mediated at least in part by group A Paks, as loss of the Rac1 gene was accompanied by decreased keratinocyte hyperproliferation at least in part by group A Paks, as loss of the Rac1 gene was accompanied by decreased keratinocyte hyperproliferation and diminished activation of Mek and Akt, 2 pathways known to be linked to Pak. Interestingly, these signaling pathways were not altered in untreated Rac1-deficient skin, indicating a hyperproliferation-specific function of Rac1 in vivo. Our data are consistent with these findings, as Pak1-deficient mice do not have any notable defects in skin or hair development (Supplementary Fig. S1), yet resist Kras-driven carcinogenesis and show loss of Erk activation in this setting. We have also previously shown that Pak1 regulates Erk activation downstream of Ras in an NF1 mast cell model, in which Ras is activated (33). These findings support a model in which Pak1 is required for efficient activation of the Erk pathway by Ras in various cell types. Interestingly, our data also suggest that in some conditions, other group A Paks such as Pak3 may substitute for Pak1, as the rare large tumors that arose in Pak1−/−;K5-rtTA;KrasG12D mice showed restoration of an active Pak species of 67 kD, accompanied by reactivation of c-Raf, Erk and Akt (Fig. 4D).

The cell of origin in Ras-driven SCC is thought to reside in the hair follicle stem cell niche or from immediate progenitors (34, 35). We found that Pak1 is expressed in this compartment in both human and murine skin (Fig. 1A and Supplementary Fig. S1). Both the Erk and Akt pathways are known to be activated during Ras-induced tumorigenesis from hair follicle stem cells; thus the effect of Pak1 loss or inhibition might be mediated by reduced signaling through Erk and Akt in these cells.

Interestingly, we found that Akt activity was reduced in mice treated with either anti-Pak agent, PF3758309 or FRAX-597. Although these results are expected for FRAX-597, which induces loss of Pak1 protein and thus essentially phenocopies Pak1−/− mice, it is less clear why the pan-Pak inhibitor PF3758309 also inhibited Akt signaling, as the scaffold functions of Pak1 that are thought to link it to PDK and Akt would presumably be unaffected by this ATP-competitive compound. It is possible that Akt inhibition in this setting represents an off-target effect of PF3758309. Indeed, we found that PF3758309 has substantial inhibitory activity against the Akt activator PDK1 (Fig. 4A and Supplementary Table S5), and Murray and colleagues reported that 1 Akt isoform, Akt3, is strongly inhibited by this compound (19). Alternatively, PF3758309 might stabilize a particular conformation of Pak1 that shields its scaffolding elements.

Regarding the new group A Pak inhibitor, FRAX-597, this molecule has the interesting and unexpected property of reducing Pak1 and Pak2 expression levels in treated animals (Fig. 5H). Although the basis for these effects are not yet understood, we have found that similar effects are also seen in vitro when various growth-factor-stimulated cell lines are exposed to FRAX-597, and that loss of Pak expression, but not of Pak enzymatic inhibition, can be prevented by proteosome inhibitors such as MG-132 (data not shown). Combined with the animal data depicted in Fig. 5H and the biochemical data in Table S3, these data suggest that FRAX-597 has a dual inhibitory effect on group A Paks: it acts as a competitive inhibitor and also as a destabilizing agent, perhaps by binding to an "open" form of Pak.

In human skin cancer, we found that the expression of Pak1 is highly associated with advanced grade of SCC as illustrated in poorly differentiated carcinomas or undifferentiated tumors (Fig. 1C). In our mouse model of SCC, expression of Kras was associated with increased Pak1 expression (Fig. 2A). Whether Ras signaling augments Pak1 gene transcription or translation, or affects the stability of the Pak1 protein, is not known, though it is of interest that Reddy have reported that levels of the miRNA miR-7, which downregulates Pak1 expression, are reduced in highly invasive breast cancer cells compared with their noninvasive counterpart (36). In our Kras-driven mouse model of SCC, Pak1 levels correlated with histologic grade: 80% of tumors isolated in Pak1+/− mice were histologically SCC whereas only 14% of epidermal lesions in Pak1−/− mice displayed a malignant phenotype (Fig. 3C). Also of note, there was 25% reduction of SCC found between Pak1−/− and Pak1+/− mice (60% vs. 80%). However, loss of 1 Pak1 allele did not affect the activity of the Erk or Akt-mTOR pathways (Fig. 4), suggesting that signaling pathways in addition to Erk and Akt-mTOR are also regulated by Pak1.

Disruption of Ras-signaling pathways has been a major goal of anticancer drug development, with a particular emphasis on identifying key, targetable signaling proteins downstream of Ras. There is ample evidence supporting a key role for PI3K-Akt pathway in oncogenic Ras signaling, but our results suggest that, in the aggressive K5-Tet-Tet-Kras SCC model, Ras oncogenic signals are transmitted predominantly through a Pak1-Mek-Erk pathway (Supplementary Fig. S5). An inhibitor targeting Mek signaling (PD0325901) showed impressive tumor regression, consistent with other recent reports in Ras models (37–40). In particular, Scholl and colleagues have shown that genetic deletion of Mek1/2 in mouse epidermis abolishes transformation by Ras (41), and Ehrenreiter and colleagues have shown that c-Raf is also required for Ras-driven tumorigenesis in this setting (42). In contrast, we found only a small effect on tumor size with an Akt inhibitor (GSK690693), despite appropriate target pathway inhibition. This finding is consistent with the idea that inhibition of PI3K-Akt signaling alone is not adequate to diminish tumors driven by mutant Kras, once established, though such inhibition did add to the antitumor effects of a Mek inhibitor (43). Anti-Pak agents may offer a dual benefit by simultaneously inhibiting both Erk and Akt signaling, thus impeding proliferation and promoting apoptosis in cancer cells. Given that a Pak inhibitor (PF3758309)
recently entered clinical trials (http://clinicaltrials.gov/show/NCT00932126), it should soon be possible to determine the potential of Pak proteins as anticancer targets in human malignancies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Acknowledgments
We thank Fang Zhu of the FCCC Biostatistics Facility for statistical analyses, Joachim Rudolph of Genentech for chemical syntheses, C. Renner at the Histopathology Facility for tissue processing and immunohistochemistry, and Erica Golensis for commentary and reviewing the manuscript.

Grant Support
This work was supported by grants from the NIH to J. Chernoff (R01 CA142928 and R01 CA11784) and to the Fox Chase Cancer Center (F30 CA069627), as well as by an appropriation from the state of Pennsylvania.

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Received June 12, 2012; revised August 17, 2012; accepted August 27, 2012; published OnlineFirst September 14, 2012.

Erica Golemis for commentary and reviewing the manuscript.

Grant Support
This work was supported by grants from the NIH to J. Chernoff (R01 CA142928 and R01 CA11784) and to the Fox Chase Cancer Center (F30 CA069627), as well as by an appropriation from the state of Pennsylvania.

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Received June 12, 2012; revised August 17, 2012; accepted August 27, 2012; published OnlineFirst September 14, 2012.


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