Genomic alterations link Rho family of GTPases to the highly invasive phenotype of pancreas cancer

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Communicated by David M. Livingston, Dana–Farber Harvard Cancer Center, Boston, MA, October 10, 2008 (received for review May 7, 2008)

Pancreas ductal adenocarcinoma (PDAC) is a highly lethal cancer that typically presents as advanced, unresectable disease. This invasive tendency, coupled with intrinsic resistance to standard therapies and genome instability, are major contributors to poor long-term survival. The genetic elements governing the invasive propensity of PDAC have not been well elucidated. Here, in the course of validating resident genes in highly recurrent and focal amplifications in PDAC, we have identified Rio Kinase 3 (RIOK3) as an amplified gene that alters cytoskeletal architecture as well as promotes pancreatic ductal cell migration and invasion. We determined that RIOK3 promotes its invasive activities through activation of the small G protein, Rac. This genomic and functional link to Rac signaling prompted a genome wide survey of other components of the Rho family network, revealing p21 Activated Kinase 4 (PAK4) as another amplified gene in PDAC tumors and cell lines. Like RIOK3, PAK4 promotes pancreas ductal cell motility and invasion. Together, the genomic and functional profiles establish the Rho family GTP-binding proteins as integral to the hallmark invasive nature of this lethal disease.

Pak4 | pancreatic cancer | Rac | Rio Kinase3

Pancreas ductal adenocarcinoma (PDAC) is a leading cause of cancer death with a dismal 5 year survival (1). The rapid demise of PDAC patients relates to detection of disease at advanced inoperable stages and intense resistance to conventional and targeted therapies (1, 2). Defining and validating the genetic aberrations underlying PDAC genesis and progression, particularly those governing its robust invasive and metastatic tendencies, remains a critical area for active investigation. Molecular genetic studies have defined an expanding atlas of highly recurrent genetic events in PDAC including activating mutations in the KRAS oncogene and epigenetic and/or mutational extinction of the INK4a, p53, and SMAD4 tumor suppressor genes. That these events contribute to PDAC pathogenesis is evident from genetically engineered mouse models, demonstrating key roles of activated KRAS in disease genesis and loss of the aforementioned tumor suppressors in malignant progression (reviewed in ref. 3). In addition, array-comparative genomic hybridization (aCGH) as well as other whole genome analysis techniques has revealed many amplifications and deletions in PDAC (4–12). Many of these copy number aberrations (CNAs) appear to be relevant to the pathogenesis of PDAC as inferred from their highly recurrent nature and presence of known cancer genes in some loci. However, for the majority of loci, there remains the need to identify and validate the relevant cancer gene(s), to define their linked signaling pathways, and to determine how these genetic lesions govern the characteristic tumor biological properties of this lethal disease. In this study, we performed high-resolution aCGH to better define the atlas of recurrent CNAs in PDAC, particularly those that might contribute to the highly invasive and metastatic nature of this disease. These genomic and functional validation studies support a role for the Rho family of GTP-binding proteins in PDAC invasiveness.

Results

Atypical Kinase, RIOK3, Is a Target of Recurrent Amplification and Overexpression in PDAC. High resolution aCGH and bioinformatic analysis identified recurrent copy number alterations (CNAs) in a collection of 14 human PDAC tumors and 15 cell lines [Fig. 1*A* and supporting information (SI) Table S1] (42). Among the most notable CNAs in our dataset is a highly recurrent, focal and high amplitude amplicon targeting 18q11. Using a combination of tumors, the minimal common region (MCR) of amplification was delimited to a 2-Mb span harboring eight characterized genes, and two conserved ORFs (Fig. 1 *A* and *B*). Tumor microarray fluorescence in situ hybridization (TMA-FISH) was performed to assess recurrence in a larger panel of PDAC tumor samples. Using a probe within the 18q amplicon, six of 38 (16%) informative tumor cores showed definitive gain/amplification (Fig. 1*C*). The 18q11 amplicon was also prioritized for in-depth analysis based on its recurrence across multiple tumor types. Specifically, array-CGH profiles showed recurrent gains in nonsmall cell lung cancer (18/63, 28.6%), colorectal adenocarcinoma (8/74, 10.8%), melanoma (9/123, 7.3%), and pancreatic cancer (7/30, 23.3%). Gene expression levels can correlate with gene copy number (13), prompting examination of MCR resident gene expression in relation to amplification status. Two of the eight MCR resident genes were excluded upfront, as Cables1 is a tumor suppressor (14, 15) and CTAGE1 showed minimal or absent mRNA expression in the PDAC cell lines tested (data not shown). The remaining eight genes were analyzed in our panel of 20 PDAC cell lines and their expression was compared with that of an immortalized human pancreatic ductal cell line. Quantitative real-time reverse transcriptase PCR (qRT-PCR) revealed

Author contributions: A.C.K., A.F.H., A.J.A., H.Z., J.-h.P., and R.A.D. designed research; A.C.K., A.F.H., A.J.A., H.Z., J.-h.P., H.Y., G.C.C., J.X.Z., E.S., G.Y., A. Ponugoti, R.N., S.D., S.Y., X.W., J.P.M., M.P., E.I., J.Z., A. Protopopov, and P.A.F. performed research; M.S.T., M.R., W.C.H., D.S.K., and L.C. contributed new reagents/analytic tools; A.C.K., A.F.H., A.J.A., H.Z., J.-h.P., H.Y., G.C.C., J.X.Z., E.S., G.Y., A. Ponugoti, R.N., S.Y., J.P.M., M.P., E.I., J.Z., B.F., M.R., A. Protopopov, Y.X., P.A.F., L.C., and R.A.D. analyzed data; and A.C.K. and R.A.D. wrote the paper.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0809966105/DCSupplemental.

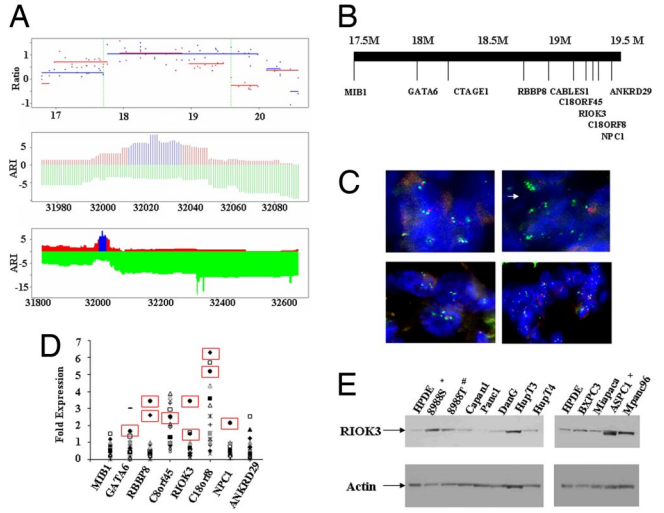

Fig. 1. Analysis of the 18q11 minimal common region. (*A*) (*Top*) Minimum common region (within green dotted lines) of the RIOK3 amplicon in two tumors, in blue and red. Dots are normalized log2 ratio for each probe; lines are segmented log2 ratios in the region. (*Middle*) expanded region on chromosome 18 showing ARI (Abberation Recurrence Index, a summation of log2 ratios from all samples for each probe) for both amplification/gain and deletion/loss with the probe index on the *x*-axis. (*Bottom*) ARI for whole chromosome 18. (*B*) Schematic of the minimal 18q11.2 amplicon (*C*) Tumor microarray fluorescence in situ hybridization (TMA-FISH) using a probe within the amplified genomic region labeled with FITC (green) and centromere reference probe with Cy3 (red). Amplification is indicated by an increase in ratio of green to red signals. Shown are four different tumor cores with amplification. White arrow shows a nucleus with increased signal. (*D*) quantitative real time PCR (qRT-PCR) was performed with 20 PDAC cell lines and a human pancreatic ductal cell line as a reference. The *x*-axis shows the resident genes and relative expression is on the *y*-axis. The two lines that harbor the 18q amplicon (8988T and 8988S) are boxed in cases of overexpression. (*E*) Western blot confirming RIOK3 overexpression in pancreatic tumor lines. (HPDE, human pancreatic ductal cells).

that several known genes as well as uncharacterized ORFs showed varying levels of overexpression in samples with amplification (Fig. 1*D*). We were initially interested in RIOK3 as it is a kinase that has not been characterized. This, along with the fact that it appears to be both amplified and overexpressed, made RIOK3 an attractive candidate on which to focus further characterization and mechanistic analyses. The overexpression of RIOK3 seen by qPCR was confirmed by western blot analyses (Fig. 1*E*). Given the presence of this amplification in a significant proportion of tumors, we assessed whether any potential somatic mutations were present in RIOK3 that could lead to its activation. Sequence analysis of the coding region of RIOK3 in a collection of 16 primary tumors and 21 cell lines detected one homozygous, nonsynonymous mutation in a PDAC cell line, HPAFII. This variant, resulted in the change of a serine residue to a leucine (S447L) in the conserved RIO domain. In summary, these multidimensional data implicated RIOK3 as a potential target of the 18q11 amplification in PDAC and multiple other tumor types.

Functional Analysis of RIOK3. Loss-of-function studies were performed to determine the oncogenic relevance of those genes that showed overexpression using 8898T cells that harbor the 18q amplification: GATA6, C18ORF45, RIOK3 and C18ORF8. Interestingly, knockdown of each of these genes showed varying levels of inhibition of colony formation (data not shown, Fig. 2*A*), implicating them as putative PDAC oncogenes. In particular, RIOK3 suppression resulted in a significant decrease in the ability to form colonies in soft-agar as did siRNA-mediated knockdown of KRAS and MAPK (Fig. 2*A*). Confirmatory RIOK3 shRNA knockdown studies showed decreased soft agar growth that correlated with the robustness of gene suppression (Fig. S1 *A* and *B*). Furthermore, xenotransplants of 8988T cells with stable RIOK3 knockdown resulted in reduced tumor growth, mirroring the soft agar findings (Fig. 2*B*). In contrast to the decreased tumorigenicity in the loss-of-function experiments, gain-of-function studies showed only minimal activity for wild-type RIOK3 as well as its S447L variant allele. Ectopic RIOK3 expression did not affect malignant transformation of primary Ink4a/Arf null MEFs (16; data not shown) and enforced RIOK3 expression in an immortalized human pancreatic ductal cell line did not alter the growth rate (Fig. S2*A*). Finally, RIOK3 alone, or together with oncogenic KRAS, failed to confer anchorage independence or tumorigenic potential on these and other cell types (Fig. S2*B*; data not shown). Although RIOK3 showed minimal growth and transformation potential, enforced expression produced a distinct morphological change characterized by readily visible membrane alterations. As shown in Fig. 2*C* whereas the control human pancreatic ductal cells were round and displayed characteristic epithelial cell morphology (*Upper Left*), RIOK3 expression promoted membrane architecture changes in both pancreatic ductal cells (*Upper*) and immortalized mouse embryo fibroblasts (Fig. 2*C Lower*) consisting of membrane ruffling, cell flattening, and increased size. Correspondingly, RIOK3 knockdown strikingly altered the appearance of the 8988T cell line morphology from small rounded cells with multiple protruding edges (Fig. 2*D Upper*) to larger flattened cells with altered membrane protrusions (Fig. 2*D Lower*). These morphological changes and alteration of membrane architecture prompted assessment of enforced RIOK3 expression on the motility of pancreatic ductal cells. In the standard scratch assay, RIOK3-expressing pancreas ductal lines exhibited accelerated migration into the cleared area relative to controls (Fig. 2*E*). Additionally, in the modified Boyden Chamber assay, RIOK3-expressing pancreatic ductal cells showed increased migratory activity relative to controls (Fig. 2*F*). These cells also exhibited an increased capacity to invade through Matrigel-coated membranes (Fig. 2*G*).

RIOK3-Induced Migration Involves the Small GTPase Rac. The small GTPase, Rac, is a key regulator of lamellipodia formation and cell motility (reviewed in refs. 17 and 18). The impact of RIOK3 on these processes prompted us to assess whether Rac is a critical downstream effector. In standard Rac activation pull-down assays, RIOK3 expression led to accumulation of GTP-bound activated endogenous Rac (Fig. 3*A*). As a first step to elucidate the connection to its cell morphology and motility phenotypes, we attempted to determine whether RIOK3 was in complex with any known proteins that play a role in these processes. Our initial focus was on three proteins, PAK1, betaPIX, and GIT1 as RIOK1 was previously found in complex with these proteins (19). Whereas, RIOK3 interaction was not detectable with betaPix and GIT1, it was found in complex with PAK1, which is a key downstream effector of Rac (17, 18) (Fig. 3*B*; data not shown). Consistent with a RIOK3 link to Rac signaling, treatment of pancreas ductal cells with a Rac inhibitor, NSC23766 (20), was able to reverse the RIOK3-induced morphological changes to a more rounded morphology characteristic of control cells (Fig. 3*C*). Furthermore, Rac inhibition by pharmacological means (Fig. 3*D*) or by dominant-negative interference (Fig. 3*E*) decreased the RIOK3-dependent pancreas ductal cell migration through transwell chambers. Together, these data support the view that RIOK3 promotes cell migration through the activation of Rac. To determine whether the RIOK3 directed activities are dependent on its kinase activity, we engineered two different mutations that would be expected to abrogate its kinase activity based on the structure/function analysis of bacterial and yeast RIOK1 and 2 (21, 22). Interestingly, neither the D406N nor the K290R mutant appeared to diminish RIOK3-induced activation of Rac (Fig. 3*A*) and these mutants appeared to retain promigratory activity in pancreatic ductal cells (data not shown).

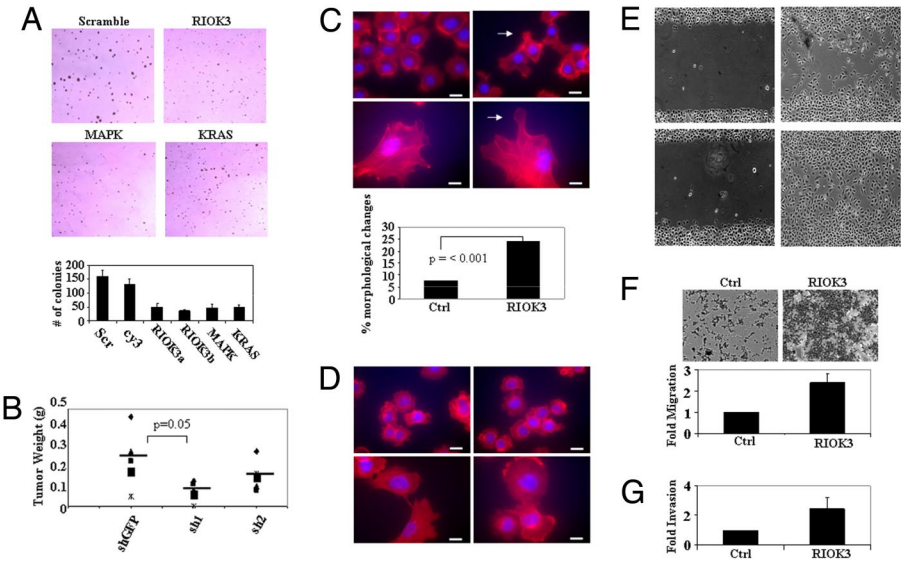

Fig. 2. Functional analysis of RIOK3 in PDAC and immortalized human pancreatic ductal cells. (*A*) 8988T cells were transfected with two different siRNAs for RIOK3 (*a* and *b*) and controls (scrambled or a cy3 labeled control) and seeded into semisolid agar. Note the significant reduction of colonies of significant size as well as that of the KRAS and MAPK siRNAs when compared with the control scrambled siRNA (quantitation below). (*B*) 8988T cells transduced with RIOK3 shRNAs were injected s.c. into the flanks of nude mice. Tumor weights were plotted for each cohort. Horizontal lines represent the mean weight for the cohort. Xenografts derived from cells with highly efficient knockdown of RIOK3 (sh1) demonstrated a greater reduction in weight as compared with that with a less robust knockdown (sh2) and that of control tumors (shGFP) ($P = 0.05$; *t* test). (*C*) Top right image depicts immortalized human pancreatic ductal cells that have enforced expression of RIOK3 and are stained with rhodamine conjugated phalloidin to visualize the actin cytoskeleton. Note the change in cell morphology as compared with control cells (top left), particularly in the larger flattened morphology and development of membrane protrusions (white arrow). The lower images show a similar phenomenon in immortalized MEFs (right image, with enforced RIOK3 expression). White bar, 5 μm. Histogram shows the quantitation of the number of pancreatic ductal cells with morphological changes with an increase as compared with control ($P < 0.001$; Fisher Exact Test). (*D*) 8988T cells were transduced with a lentiviral shRNA for RIOK3. The top 2 images show the normal morphology of control (shGFP) infected cells. The bottom two images depict cells with RIOK3 knock down. As shown, these cells have a more flattened, enlarged shape with a decrease in cytoplasmic protrusions. White bar, 5 μm. (*E*) A scratch assay using pancreatic ductal cells either expressing a control vector (*Top*) or RIOK3 (*Bottom*) showing increased closure of the scratched area in the RIOK3 expressing cells. (*F*) These same cells were used in transwell migration assays, showing increased cell migration in ductal cells with enforced RIOK3 expression, which has been quantified in the graph below and expressed as fold of control cells. (*G*) Data from a representative experiment, showing RIOK3 increases cell invasion in Matrigel coated wells compared with control ductal cells.

Furthermore, the RIOK3 S447L PDAC variant allele, which alters the RIO domain (containing the kinase signature motif) did not significantly have an impact on Rac activation or cell migration above that of the wild-type RIOK3 protein (Fig. 3 *A*, *D*, and *E*). Together, these data indicate that RIOK3-induced activation of Rac and enhanced cell migration may not be dependent on an intact kinase domain. Finally, we assessed whether enforced RIOK3 would alter the invasive properties of Panc1 cells, which typically generate poorly invasive xenograft tumors and express low levels of endogenous RIOK3 (Fig. 1*E*). RIOK3- and vector-transduced tumors were allowed to reach ≈1 cm in greatest dimension and harvested to assess the extent of invasion into the muscle. RIOK3-transduced tumors exhibited greater invasive potential relative to vector-transduced controls (Fig. 3*F*). This *in vivo* activity, together with enhanced invasion and migration profile in cell culture, supports a role for RIOK3 in PDAC tumor cell invasion.

Genomic Alterations of Rho Family Pathway Components in PDAC. The identification of RIOK3 as a mediator of pancreatic ductal cell migration and invasion and its link to the small GTPase, Rac, prompted investigation of the genomic status of other genes in this family in PDAC. Examination of downstream kinases of the Rho family members Rac, Rho, and Cdc42 (Table S2) revealed amplification of PAK4 in primary tumors and cell lines. (Fig. 4*A*). Confirmatory TMA-FISH showed evidence of gain/amplification in 14 of 63 (22%) of PDAC specimens (Fig. 4*B*) as well as 14/50 (28%) prostate, 20/59 (34%) lung, 20/60 (33.3%) ovary, 4/18 (22%) melanoma, 9/76 (11.8%) colon, and 4/27 (14.8%) liver carcinomas (data not shown). RT-qPCR showed elevated PAK4 expression in multiple lines including those with and without amplification, suggesting multiple mechanisms, in addition to copy number, driving increased PAK4 expression (Fig. 4*C*). Western blot analysis confirmed over expression of PAK4 in multiple cell lines (Fig. 4*C*). To assess PAK4 in PDAC biology, PAK4 knockdown was performed in 8988T cells that show PAK4 amplification and robust expression. shRNA-mediated knockdown resulted in a significant reduction in anchorage independent growth relative to controls and ineffective PAK4 shRNAs (Fig. 5 *A* and *B*). Additionally, expression of an activated PAK4 mutant (25) in pancreatic ductal cells caused an elongated morphology in a significant fraction cells consistent with altered cytoskeletal dynamics as has been reported previously in rodent fibroblasts (23) (Fig. 5*C*). These cytoskeletal changes prompted gain-of-function studies to assess the impact of wild-type and activated mutant forms of PAK4 on migration and invasion of pancreatic ductal cells. While wild-type PAK4 expressing ductal cells showed only minimal migratory enhancement, activated PAK4 produced an increase in migratory capacity through transwell chambers as well as increased invasion (Fig. 5 *D* and *E*). Reciprocally, PAK4 siRNA-transfected 8988T cells showed significantly diminished invasion compared with control siRNA-transfectants (Fig. 5*F*). Similar observations were generated with PAK4 shRNAs (data not shown). Together, the genomic and functional data support a role for PAK4 in PDAC cellular migration and invasion.

Discussion

In this study, aCGH analysis of human PDAC led to the identification of two genes, RIOK3 and PAK4, that are genomically amplified in PDAC and other human tumors and appear to have roles in tumor cell motility and invasion. Importantly, our findings serve to link the Rho family of GTPases to PDAC pathobiology, particularly its hallmark invasive activity. Although our data support RIOK3 and PAK4 as targets of their respective amplicons, the complexity of the genome certainly allows for the possibility of additional genes on these loci that contribute to PDAC tumorigenesis. In this regard, there are two microRNAs on the 18q11 amplicon (MIRN1–2 and MIRN133A1) that could potentially play a role in PDAC biology. In further support of the idea that multiple genes on an amplicon may be functionally important, a report published during the preparation of this manuscript demonstrated that GATA6, present on the 18q11 amplicon, may also be a PDAC oncogene (26). Our data are consistent with these findings and

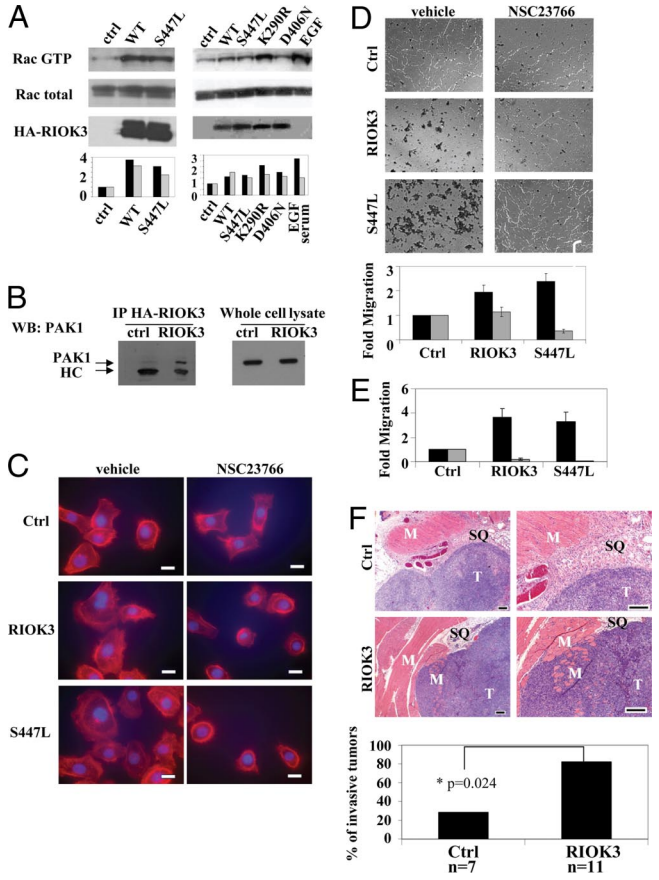

Fig. 3. RIOK3 activates the small GTPase, Rac1. (*A*) 293T cells were transfected with the indicated plasmids and Rac activation assays were performed. The top panel shows the active (GTP-bound) fraction of Rac1 with increased amounts in cells transfected with various RIOK3 constructs compared with the control. Cells were triggered with serum or EGF as a positive control (last lane). Total Rac1 expression (*Middle*) and RIOK3 (*Lower*) are shown as well. Histograms are shown below showing GTP-Rac normalized to total Rac expression by densitometric analysis and expressed as fold of control. The results from separate experiments are depicted (*Left* and *Right*). Black and gray bars show results from two representative assays. (*B*) Immunoprecipitation (IP) for HA-RIOK3 in 293T cells either transfected with HA-RIOK3 or a control plasmid. Western blotting was performed for endogenous PAK1. The blot on the left shows the IP and the right shows whole cell lysate demonstrating the expression of endogenous PAK1. Note the enrichment of PAK1 that is brought down in the RIOK3 transfected cells. HC, Ig heavy chain. (*C*) RIOK3-expressing human pancreatic ductal cells were stained with phalloidin showing RIOK3 induced membrane changes. Cells were treated with the Rac1 inhibitor NSC23766 (100 μM) before staining (right column). These cells showed reversion of morphology to that of the parental cells, with decreased membrane ruffling and decrease in cell size. White bar, 5 μm. (*D*) Transwell migration assays were performed with the ductal cells as described previously. Cells were pretreated with 50 μM NSC23766 and the assay was performed in the presence of the inhibitor. Inhibition of Rac1 decreased the RIOK3 induced migration of the cells (right column). This is quantified below with black bars (untreated cells) and gray bars (NSC23766) and expressed as fold of control. (*E*) Similar results were obtained in cells coexpressing a dominant negative mutant of Rac1 (RacN17). (*F*) Panc1 cells, which express low levels of RIOK3 were transduced with RIOK3 expression plasmids and injected into the flanks of immunocompromised mice. Tumors were dissected *en bloc* when they reached $\approx$1 cm in greatest dimension. The ability of the tumors (T) to invade from the s.c. tissue (SQ) into the underlying muscle (M) was assessed histologically on H&E slides. The top panels show a tumor derived from control Panc1 cells that remains confined in the s.c. tissues at lower and higher magnification (scale bar, 200 μm). The bottom panels show a tumor from RIOK3 overexpressing cells that has invaded into the surrounding muscle. The graph below shows that there is a statistically significant increase in the number of tumors that show muscle invasion in the RIOK3 over expressing tumors ($P = 0.024$, Fisher Exact Test) as compared with tumors from control cells.

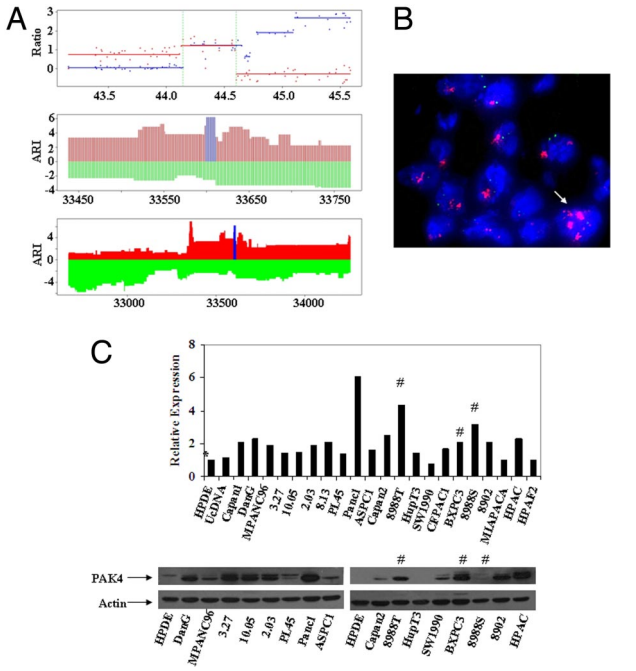

Fig. 4. PAK4 is amplified and overexpressed in PDAC. (*A*) (*Top*) Minimum common region (within green dotted lines) of the PAK4 amplicon in one tumor and one cell line, in blue and red. Dots are normalized log2 ratio for each probes; lines are segmented log2 ratios in the region. (*Middle*) Expanded region on chromosome 19 showing ARI (as above) for both amplification/gain and deletion/loss. (*Bottom*) ARI for whole chromosome 19. (*B*) TMA-FISH using a probe within the PAK4 amplicon labeled with Cy3 (red) and a centromere reference probe labeled with FITC (green). Amplification is indicated by an increase in ratio of red to green signals. Shown is a representative tumor core with amplification. White arrow shows a nucleus with increased signal. (*C*) qRT-PCR was performed with a panel PDAC cell lines and human pancreatic ductal cell line as a reference to determine the relative expression of PAK4 across the lines. Results are expressed as fold over control (ductal cells marked by the asterisks). Those with genomic amplification of this region (depicted by pound symbols) all show marked overexpression. Western blot for PAK4 expression is shown below.

additionally demonstrates that other genes such as C18ORF8 and C18ORF45 show evidence of overexpression in lines harboring the 18q amplicon. Moreover, suppression of each of these genes, including GATA6 as well as the 2 ORFs, causes a decrease in soft agar colony growth. Further efforts to characterize these genes either singly or in combination is certainly warranted given the prevalence of this amplification in human cancers. Another point of interest is that some tumor lines, such as 8988T contain the 18q as well as the 19q amplicon (PAK4 locus) and further study of the interaction of genes on multiple amplicons is of great interest.

The RIO proteins are members of the atypical protein kinase family, given their lack of similarity to typical eukaryotic kinases. RIO 1 and 2 proteins are conserved from archea to humans; however, the RIO3 family is only seen in multicellular eukaryotes (27, reviewed in refs. 28 and 29). RIO1 and RIO2 have been shown to be essential genes in yeast as well as play a role in ribosome biogenesis (30) and RIO1 is critical for proper cell cycle progression (21). To this point, the function of RIOK3 has not been studied, nor have its biochemical properties. RIOK3 expression has been observed to be increased in metastatic Head and Neck cancers compared with nonrecurrent tumors (31). RIOK3 is assumed to be an active kinase, based on its sequence as well its relation to RIO1 and RIO2, which have been studied in yeast and bacteria and have been crystallized (32, 33). To date, there are no known physiological substrates of any of the RIO kinases, however RIO1 and 2 are capable of autophosphorylation and can phosphorylate serine

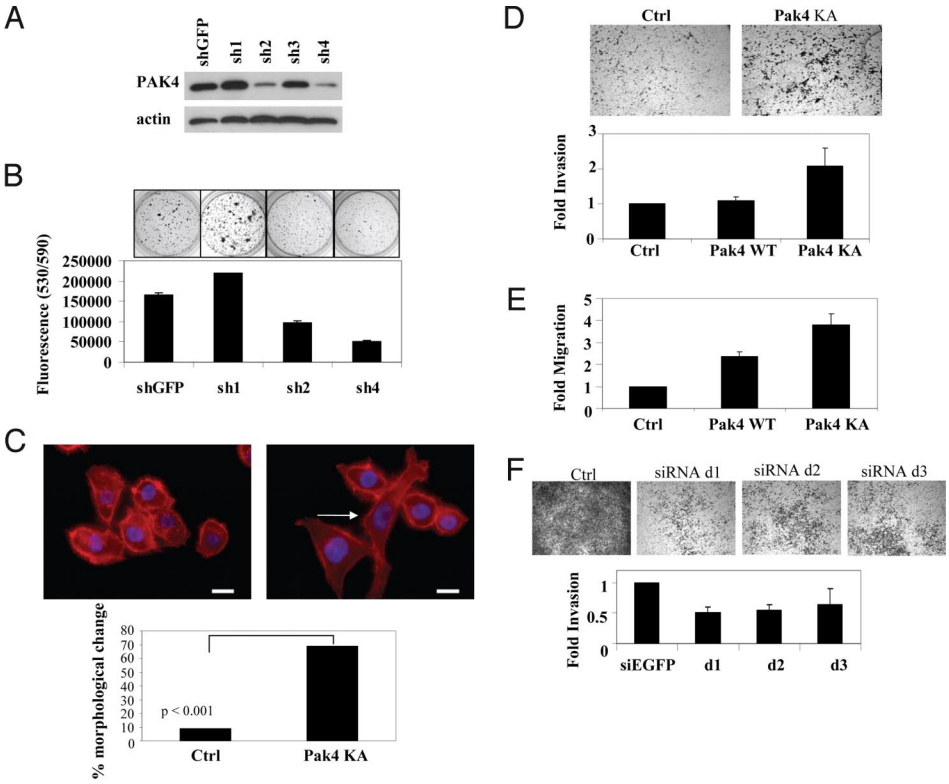

Fig. 5. Knockdown and overexpression of PAK4 reveals a potential role in PDAC biology. (*A*) Western blot analysis showing knockdown of PAK4 expression with various shRNAs (top) with corresponding actin loading control (bottom). (*B*) Results from soft agar assays showing significant reduction in number of colonies with PAK4 suppression by shRNAs in 8988T cells. Results are quantified below, showing reduction in colony number as determined by Alamar blue staining and fluorescence intensity. (*C*) Human pancreatic ductal cells expressing activated Pak4 were fixed and stained with phalloidin, as described previously. The left image depicts control cells, whereas the right image shows an example of an elongated cell (white arrow) in cells expressing the activated PAK4. White bar, 5 μm. The histogram shows a statistically significant difference (Fisher Exact Test) in the number of elongated cells in the activated PAK4 cells compared with control cells. (*D*) Ductal cell lines expressing activated or wild-type PAK4 were used for transwell migration assays. Depicted here is an increase in cell invasion with activated PAK4 cells (KA) shown on the right, compared with control cells (left). Results are quantified below and expressed as fold of control. (*E*) Similar experiments performed in uncoated wells show an increase in cell migration. (*F*) 8988T cells were transfected with siRNAs directed to PAK4 and invasion assays were performed. All three duplexes show significant reduction in invasion compared with control cells transfected with a control siRNA (GFP) and are quantified below (expressed as fold of control).

residues of artificial substrates (28, 29, 34). Importantly, RIO kinases lack the "activation loop", which is thought to be essential in all eukaryotic kinases to function (28, 29). Given this, it is possible that these proteins may not function as kinases in eukaryotes. Indeed, this idea is consistent with our data using kinase inactivating mutants, which show no abrogation of function in our assays. The fact that we identified a sequence variant in the RIO domain of RIOK3 that does not appear to alter its function further supports the concept that it is not acting as a kinase. Interestingly, this variant has recently been reported to be a putative SNP that provides another possible explanation as to why there are no obvious differences between the wild-type and the S447L variant in our assays. It is tempting to speculate that this gene, although structurally related to RIO1 and 2, may have evolved in eukaryotes to function more as a scaffolding protein and its kinase-related sequences are a vestigial remnant.

Linking RIOK3 to Rac activation provides mechanistic insight into its role in cytoskeletal and cell motility phenotypes. Interestingly, whereas mutations analogous to that of activating Ras mutations can also activate Rac, such mutations have not been found in human tumors (35). Thus, amplification of RIOK3 appears to provide one mechanism through which Rac is activated in multiple human tumors including PDAC. Interestingly, RIOK3 knockdown in the overexpressing 8988T PDAC line did not abrogate the ability of these cells to migrate or invade (data not shown), suggesting possible redundancy in the signaling maintaining the invasive phenotype. Given the well known complexities of Rac and Rho signaling, this is certainly a possibility. The Rho family of GTP-binding proteins consists of at least 20 members (reviewed in ref. 17), which generally function as regulators of the actin cytoskeleton with complex interactions between family members and a multitude of downstream effectors. Indeed, the finding that PAK4, a downstream effector of CDC42 (36), was amplified extends these findings to other members of this important gene family. Importantly, it has been demonstrated to be essential for Ras transformation and when activated by phosphomimetic point mutations, can transform cells and cause alterations in the actin cytoskeleton (23, 24). Recent data shows that wild-type PAK4 is capable of transforming cells as well (41). The fact that knockdown of PAK4 expression in PDAC cell lines attenuates the invasive phenotype, further underscores its importance in this disease and makes the study of its role in PDAC highly relevant.

In conclusion, we have identified RIOK3 and PAK4 as proinvasion genes in PDAC, further validating the use of tumor genome profiling as a means for indentifying genetic elements governing tumor pathobiology. Given the remarkable resistance to therapy and the dismal prognosis of PDAC, these two genes point to the Rho signaling pathway as a focus for future cancer drug discovery efforts.

Methods

Cell Lines and Primary Tumors. Tumor cell lines were obtained from the American Type Culture Collection (ATCC) or the German Collection of Microorganisms and Cell Cultures (DSMZ). The establishment and characterization of the near normal immortalized pancreatic duct epithelial (HPDE) cells were reported previously (37, 38). F-Actin was visualized by staining with rhodamine-conjugated phalloidin (Sigma). Ten fields under the 20× objective were counted and the number of cells with morphological changes scored. The Rac inhibitor, NSC23766, (Calbiochem) was used at 50–100 μM. Primary tumor samples were obtained from Memorial Sloan Kettering Cancer Center and Brigham and Women's Hospital tumor banks. All were verified by H&E staining by an expert pathologist (G.C.C) and only those with >70% tumor cells were included.

Plasmids. The construction of RIOK3 expression plasmids was performed by standard PCR based cloning approaches (see *SI Methods*). siRNAs were purchased from IDT and transfected with Hiperfect (Qiagen) at a final concentration of 10–20 nM. Lentiviral shRNAs were obtained from the RNAi Consortium at the Broad Institute (sequences available upon request). The PAK4 expression constructs (WT and the S445N activated mutant) were amplified from expression

plasmids obtained from Dr. S. Gutkind (NIH/NIDCR, Bethesda, MD) and cloned in frame with an N-terminal HA-tag into pBabe puro.

Expression Analysis. RNA was prepared from cell lines by standard methods and reverse transcription was performed with SuperScript II (Invitrogen) and oligo(dT) priming. PCR primers were designed to amplify products of 100–150 bp within target and control sequences (available upon request). Quantitative PCR was performed on an Mx3000P cycler (Stratagene) using QuantiTect SYBR green (Qiagen). Western blot analysis was performed via standard protocols. Antibodies against RIOK3 (mouse monoclonal antibody: Abnova), HA-HRP (Sigma), Actin (Santa Cruz), Rac1 (Millipore), PAK1 (Chemicon), β-Pix (Millipore), and Git1 (Cell Signaling).

Invasion and Migration Assays. Modified Boyden chamber assays were performed by using standard protocols with 24-well inserts—Matrigel coated for invasion and uncoated for migration (Biocoat). Cells (200,000–250,000) were washed and then seeded in Opti-MEM (Invitrogen). The chemoattractant was keratinocyte serum-free media + EGF and bovine pituitary extract (Invitrogen). For inhibition of Rac1, cells were pretreated overnight with 50 μM NSC23766 and then seeded in the chambers in the presence of the inhibitor. Scratch assays were performed in 6-well plates (see *SI Methods*).

Rac Activation Assay. To determine the activation state of Rac, pull down assays were performed as per the manufacturer's instructions (Upstate) using 293T cells (see *SI Methods*).

Tumorigenicity Assays. Soft agar assays were performed in duplicate or triplicate in 6 well plates per standard protocols (see *SI Methods*). For *in vivo* tumorigenicity assays, 10^6 PDAC cells were injected s.c. into flanks of NCr nude mice (Taconic). Tumor size was measured by caliper. For muscle invasion studies of xenografts, tumors were harvested when they reached approximately 1 cm in the greatest dimension with a wide enbloc resection to preserve tissue architecture. Muscle invasion was scored by a pathologist who was blinded to the identity of the samples (G.C.C.). All animal experiments were approved by IACUC under Protocol No. 04–114.

aCGH. Genomic DNAs from cell lines and primary tumors were extracted according to the manufacturer's instructions (Gentra Systems). Genomic DNA was fragmented, random-prime labeled and hybridized to human oligonucleotide microarrays as described (39). Raw aCGH profiles were processed to identify statistically significant transitions in copy number by using a segmentation algorithm (40). Significant copy-number changes were determined on the basis of segmented profiles only (*SI Methods*).

Fluorescence *in Situ* Hybridization. Tissue microarrays were purchased from Cybrdi or US Biomax. Fluorescence *in situ* hybridization was performed following standard protocols. BAC RP11–606B16 was used as a marker for the 18q11 amplicon (chr.18, 18M) and RP11–208H9 as a marker of the 19q13 amplicon (chr.19, 44M). Centromere-specific probes (Abbott Labs) served as ploidy references (*SI Methods*).

ACKNOWLEDGMENTS. A.C.K. is a recipient of the Leonard B. Holman Research Pathway Fellowship. A.F.H. is supported by National Institutes of Health K08 and an ASCO/PanCan young investigator award. H.Z. was supported by the Helen Hay Whitney Foundation. J.-H.P is a Damon Runyon Fellow, supported by the Damon Runyon Cancer Research Foundation. H.Y. is supported by the American Brain Tumor Association. P.A.F. is supported by the Wellcome Trust. Funding was also provided by National Institutes of Health grants U01 CA84313, R01CA84628 and P01CA117969–01 (to R.A.D) and RO1 CA099041 and RO1 CA93947 (to L.C.) and National Cancer Institute Grants P50 CA127003–01 (to R.A.D. and L.C.) and R33 CA128625 and U54 CA112962 (to W.C.H.). The Rac dominant negative plasmid was a gift from Dr. Andrew Chan (Mount Sinai School of Medicine, New York, NY). The PAK4 expression plasmids were a gift from Dr. J. Silvio Gutkind (NIH/NIDCR, Bethesda, MD).

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