KRas Induces a Src/PEAK1/ErbB2 Kinase Amplification Loop That Drives Metastatic Growth and Therapy Resistance in Pancreatic Cancer


KRas Induces a Src/PEAK1/ErbB2 Kinase Amplification Loop That Drives Metastatic Growth and Therapy Resistance in Pancreatic Cancer

Jonathan A. Kelber1,3, Theresa Reno1,3, Sharmeela Kaushal3, Cristina Metildi3,4, Tracy Wright1,3, Konstantin Stoletov1,3, Jessica M. Weems1,3, Frederick D. Park1,2,3, Evangeline Mose3, Yingchun Wang5, Robert M. Hoffman4,6, Andrew M. Lowy3,4, Michael Bouvet3,4, and Richard L. Klemke1,3

Abstract
Early biomarkers and effective therapeutic strategies are desperately needed to treat pancreatic ductal adenocarcinoma (PDAC), which has a dismal 5-year patient survival rate. Here, we report that the novel tyrosine kinase PEAK1 is upregulated in human malignancies, including human PDACs and pancreatic intraepithelial neoplasia (PanIN). Oncogenic KRas induced a PEAK1-dependent kinase amplification loop between Src, PEAK1, and ErbB2 to drive PDAC tumor growth and metastasis in vivo. Surprisingly, blockade of ErbB2 expression increased Src-dependent PEAK1 expression, PEAK1-dependent Src activation, and tumor growth in vivo, suggesting a mechanism for the observed resistance of patients with PDACs to therapeutic intervention. Importantly, PEAK1 inactivation sensitized PDAC cells to trastuzumab and gemcitabine therapy. Our findings, therefore, suggest that PEAK1 is a novel biomarker, critical signaling hub, and new therapeutic target in PDACs.

Cancer Res; 72(10); 2554-64. ©2012 AACR.

Introduction
Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer-related death. Newly diagnosed patients with PDACs have a median survival of less than 1 year and a 5-year survival rate of 3% to 5% (1, 2). While oncogenic KRas is the initiating mutation in nearly all PDACs, the development of effective therapies targeting this GTPase or downstream effectors has shown little promise in the development of effective therapies targeting this GTPase. Oncogenic KRas induced a PEAK1-dependent kinase amplification loop between Src, PEAK1, and ErbB2 to drive PDAC tumor growth and metastasis in vivo. Surprisingly, blockade of ErbB2 expression increased Src-dependent PEAK1 expression, PEAK1-dependent Src activation, and tumor growth in vivo, suggesting a mechanism for the observed resistance of patients with PDACs to therapeutic intervention. Importantly, PEAK1 inactivation sensitized PDAC cells to trastuzumab and gemcitabine therapy. Our findings, therefore, suggest that PEAK1 is a novel biomarker, critical signaling hub, and new therapeutic target in PDACs.

Materials and Methods

Cell lines, DNA/ lentiviral constructs, and reagents
Details about cell lines and culture conditions are given in the Supplementary Materials and Methods. shCntrl and shPEAK1 (FG12 vector) constructs have been previously published (12). The shPEAK1 [3′ untranslated region (UTR)] and shErbB2 (pLK0.1 vector) constructs were purchased from Open Biosystems/The RNAi Consortium (Thermo Scientific). Control, PEAK1, and ErbB2 shRNA sequences and details about lentiviral production are given in the Supplementary Materials and Methods. Short hairpin RNA (shRNA)/siRNA pools (containing at least 4 unique siRNA sequences each)

Authors' Affiliations:
1Departments of Pathology and Medicine, 2Moores Cancer Center, 3Department of Surgery, Division of Surgical Oncology, UCSD, La Jolla, California; 4Institute of Genetics and Developmental Biology, Chinese Academy of Science, Beijing, China; and 5AntiCancer, Inc., San Diego, California

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Corresponding Author: Richard L. Klemke, School of Medicine, UCSD 9500 Gilman Drive #0612, La Jolla, CA 92033. Phone: 858-822-5610; Fax: 858-822-4566; E-mail: rklemk@ucsd.edu

doi: 10.1158/0008-5472.CAN-11-3552

©2012 American Association for Cancer Research.

2554
Cancer Res; 72(10) May 15, 2012

American Association for Cancer Research
against KRas (Santa Cruz Biotechnology, shRNA lentivirus particles) and Src (Dharmacon, siRNA transient transfection) were purchased along with the appropriate nontargeting control siRNAs, GFP-vector, GFP-PEAK1, and GFP-PEAK1 mutants have been previously described (12). For stable overexpression of PEAK1 and 3’UTR shRNA rescue experiments, human PEAK1 was subcloned into the pCSC-SP-PW-GFP lentiviral vector (Addgene) with EcoRI and KpnI sites and transduced cells were sorted for GFP. Empty vector and NeuT constructs (pBabe-puro vector) have been previously described and were used with the appropriate packaging DNAs to generate retroviral particles for cell transduction (14). Details about antibodies, inhibitors, and other reagents are given in the Supplementary Materials and Methods. Trastuzumab was provided by Genentech under MTA agreement #210708.

**Oncomine analysis**
Normalized PEAK1, Src, or ErbB2 expression data and P values were downloaded from Oncomine (Compendia Bioscience). Citations for individual studies are included in the Supplementary Data. Heatmaps were generated by Excel. Gene expression levels were plotted with Tukey or average ± SEM analyses for each patient group.

**Immunohistochemistry**
Human pancreatic cancer tissue array was purchased from US Biomax, Inc. PDX-1-CreLSL-KRASG12D mouse pancreas sections were kindly provided by A.M. Lowy. Samples from patients BK-13 and BK-14 were collected and processed as described later in accordance with UCSD (La Jolla, CA) Institutional Review Board (IRB) #071136X. PEAK1 protein expression was determined with the standard avidin-biotin immunoperoxidase procedures, sodium citrate antigen retrieval methods, and the VECTASTAIN Elite ABC Kit from Vector Laboratories according to the manufacturer’s protocol. Mouse monoclonal anti-SGK269 and rabbit polyclonal anti-PEAK1 were purchased from Santa Cruz Biotechnology and Millipore, respectively, and used at a 1:200 dilution. Anti-ErbB2 (2D98) and anti-Src (3D610) antibodies were purchased from Cell Signaling and used according to manufacturer’s recommendations. Images were collected with a Leica DM2500 microscope.

**Mouse strains**
The PDX-1-CreLSL-KRASG12D transgenic murine model was previously described (15).

**Patient sample collection and preparation**
In accordance with IRB #071136X (M. Bouvet), fresh tumor specimens from patients undergoing routine cancer surgery were collected in cold RPMI for subsequent PCR and immunohistochemical preparation and analysis. All patient identification information was removed and all samples were made anonymous. Patients signed an informed consent document before their operative procedure, which acknowledged that a portion of the resected tissue would be used for research.

**RT-PCR and quantitative PCR**
mRNA extraction, purification, DNA synthesis, and reverse transcription (RT) or quantitative (qPCR) primers used are described in the Supplementary Materials and Methods. All qPCR data were processed with the relative quantification method and plotted as average fold change (±SEM, unless otherwise indicated) in relation to normal tissue, appropriate untreated, vehicle-treated, or shRNA controls.

**CyQUANT direct cell proliferation assay**
Analysis of cell growth was conducted according to manufacturer’s protocol (Invitrogen). Briefly, cells were plated in triplicate into 96-well plates at a density of 200 to 500 cells per well in complete media. Twenty-four hours later, cell media were changed to 2% serum-containing media and left untreated or treated with indicated reagents. Viable cell number was measured on days 3 or 8 based upon CyQuant Green fluorescence emission at 525 nm.

**Three-dimensional spheroid assay**
Analysis of three-dimensional (3D) tumorsphere growth was conducted as previously described (16). Briefly, cells were plated into Dulbecco’s Modified Eagle’s Medium (DMEM): F12 (1:1) containing B27 supplement, 10 μg/mL basic fibroblast growth factor, 10 μg/mL EGF, and 4 μg/mL heparin sulfate. Cells were grown for 14 to 21 days and spheroids were imaged with a wide-field light microscope.

**Soft agar transformation experiments**
Analysis of anchorage-independent cell growth was conducted according to the manufacturers’ protocol (Cell Biolabs). Briefly, 96-well plates were prepared with 50 μL per well surface layers consisting of 0.6% agar resuspended in cell culture media. An additional 75 μL per well of 0.33% agar cell culture media containing 5,000 cells was then added to each well, followed by the addition of 50 μL of cell culture media. Brightfield microscope images were collected at ×10 magnification with a Nikon TE instrument and colonies were resuspended and quantified with Cell Biolabs’ CyQuant Green fluorescent dye at 525 nm.

**Orthotopic implantation experiments**
Protocols were followed as previously described (17). Details are given in the Supplementary Materials and Methods section. For survival analysis, animal health was assessed 3 times per week and animals were sacrificed when they reached a predefined state of premorbidity (i.e., >3 cm tumor diameter and one of the following: impaired movement, cachexia, or observable ascites accumulation; or impaired movement, cachexia, and observable ascites accumulation). Kaplan–Meier curves were plotted for each animal group, and statistical analysis was conducted with Prism software (GraphPad). Details of sample processing are given in the Supplementary Materials and Methods.

**Chicken embryo metastasis assay**
Fertilized chicken eggs were purchased from McIntire Poultry and incubated for 14 days (37°C, 100% humidity). A 1-cm²
square window was created in the eggshell to expose the underlying vasculature. Tumor cells were suspended in sterile PBS at a concentration of $3 \times 10^5$ to $3 \times 10^6$ cells/mL and $3 \times 10^5$ tumor cells were injected into one of the chorioallantoic membrane (CAM) veins. After incubating embryos for another 48 hours, Rhodamine-lectin (Vector Biolabs, 100 μL per chick, 0.5mg/mL) was injected into the CAM vein. Following appropriate euthanizing procedures, the chicken brain and liver were removed and imaged with either a Leica MZFLIII microscope (×1.25) or a Nikon c1-si confocal microscope (×10–60).

### Immunoprecipitation and Western blotting
All immunoprecipitations and Western blotting were conducted as previously described (12).

### Confocal microscopy
Imaging and processing were conducted as described in Supplementary Materials and Methods.

### Statistical analysis
All quantified data were plotted and analyzed in GraphPad Prism 5.0 with ANOVA, Student t test, nonlinear regression analysis, or Kaplan–Meier curve analysis. Data are representative of at least 3 independent experiments and are reported as replicate averages ± SEM, unless otherwise indicated. * and ** represent $P$ values of $<0.05$, 0.01, and 0.001, respectively, as determined by a one-way ANOVA. IHC, immunohistochemical.

#### Results
**PEAK1 is overexpressed in multiple human malignancies and is an early biomarker for PDAC development and progression**

Because it is not known whether oncogenic transformation regulates PEAK1 expression or whether PEAK1 contributes widely to human malignancies, we first sought to determine the expression profile of PEAK1 in various human cancers in comparison with normal tissues. PEAK1 expression is significantly upregulated in multiple human tumors in comparison with normal tissues (Fig. 1A) and shows a strong correlation with various subcategories of these cancers that are associated with poor disease prognosis (Supplementary Table S1). No significant changes in PEAK1 mRNA levels were observed between normal and malignant tissues for bladder, head and neck, lung, sarcoma, and melanoma tumors. Because the

---

Figure 1. PEAK1 is overexpressed in multiple human malignancies and is an early biomarker for PDAC development and progression. A, heatmap of fold change in PEAK1 expression in tumor samples. Data are publicly available on Oncomine, and citations are included in Supplementary Data. B, immunohistochemistry of PEAK1 in human PDACs or normal pancreatic tissue sections (left). Arrowheads indicate normal ductal epithelium. Scale bar, 50 μm. Quantitative analysis of immunohistochemical staining for 7 normal, 9 grade I, 15 grade II, and 16 grade III human samples by blind scoring on a scale of 0–3 (right). C, immunohistochemistry of PEAK1 in normal PDX-1-Cre control mice (ac, acinar cells; is, islet) and PanIN 1A/B and 2 from 6 month-old PDX-1-Cre:LSL-KRASG12D mice. Arrowheads indicate normal ductal epithelium or PanIN lesions. Scale bar, 50 μm. * and ** represent $P$ values of $<0.05$, 0.01, and 0.001, respectively, as determined by a one-way ANOVA. IHC, immunohistochemical.
mortality and incidence for PDAC are almost equal due to few diagnostic biomarkers and therapeutic targets, we focused our subsequent PEAK1 expression analyses on this disease. We examined changes in PEAK1 protein levels in human PDAC patient tissue arrays and a murine model of KRas-induced PanIN (PDX-1-Cre:LSL-KRASG12D; ref. 15). PEAK1 protein expression was increased in PDAC tissues in comparison with normal tissues and correlated with grade (Fig. 1B). Furthermore, we observed that PEAK1 expression was elevated in PanIN lesions (Fig. 1C and Supplementary Table S1), showing that PEAK1 represents an early biomarker for pancreatic ductal neoplasia (1, 18).

KRas induces Src-dependent PEAK1 expression in PDAC and other human malignancies

While the above findings suggest that PEAK1 is deregulated during oncogenic reprogramming, the mechanism driving PEAK1 upregulation remains unclear. Because PanIN and PDAC progression are nearly always associated with activating mutations in the KRas oncogene and because PEAK1 is upregulated in PanIN tissues from PDX-1-Cre:LSL-KRASG12D mice, we tested whether PEAK1 expression correlated with oncogenic Ras mutations in human cancers. RT-PCR, qPCR, and immunohistochemical analyses of normal pancreas and oncogenic KRas-positive PDAC tissues collected from patients BK-13 and BK-14 after surgical resection showed that PEAK1 is overexpressed in PDAC tissue in conjunction with the presence of the G12D oncogenic KRas mutant (Fig. 2A and B and Supplementary Fig. S1A). Furthermore, human cancers positive for oncogenic KRas or NRas mutations (Fig. 2C) or HRas-transformed breast epithelial cells (Supplementary Table S1) showed a significant increase in PEAK1 expression. In agreement with these data, introduction of constitutively active KRasG12D into human pancreatic nestin-expressing (HPNE; refs. 11, 19) cells or KRas knockdown in established PDAC cell lines robustly increased or decreased, respectively, PEAK1 protein and mRNA levels (Fig. 2D and Supplementary Fig. S1B). We subsequently sought to identify the pathway downstream of KRas that induces PEAK1 expression. Pharmacologic inhibition of phosphoinositide 3-kinase (PI3K), mitogen-activated protein (MAP)/ERK kinase (MEK), or Src in HPNE-KRas and PANC1 cells revealed that PEAK1 expression is dependent upon Src-mediated transcriptional regulation (Fig. 2E and Supplementary Fig. S1E). Importantly, suppression of Src kinase in these cells via RNA interference (RNAi) also reduced PEAK1 expression (Supplementary Fig. S1E). Taken together, these findings show that PEAK1 protein expression is positively regulated by KRas/Src signaling in human malignancies and human PDAC primary samples and cell lines.
PEAK1 is necessary for KRas-induced anchorage-dependent and -independent expansion of pancreatic cancer cells

Consistent with the role of KRas in driving PEAK1 expression, PEAK1 was necessary and sufficient for KRas-induced anchorage-dependent expansion in HPNE cells (Fig. 3A and B and Supplementary Fig. S2A and S2B). Importantly, overexpression of wild-type PEAK1 or a C-terminal truncation mutant (C1) containing the kinase domain was sufficient to induce HPNE cell expansion under anchorage-dependent conditions. Mutational inactivation of the ATP-binding site abrogated this effect, showing that PEAK1 kinase activity (12) is critical for its effect in these assays (Fig. 3C and Supplementary Fig. S2C). Conversely, depletion of KRas or PEAK1 from FG and PANC1 PDAC cell lines (Fig. 2D and Supplementary Fig. S2A) inhibited anchorage-dependent cell expansion (Fig. 3D). In addition, we discovered that PEAK1 is essential for KRas-induced formation and expansion of 3D tumorspheres (Fig. 3E and Supplementary Fig. S2D), which is an in vitro measure of tumor-initiating potential (16). We further analyzed the ability of PEAK1 overexpression and knockdown to modulate HPNE cell transformation, as analyzed by anchorage-independent growth. As shown in Fig. 3F, HPNE-KRas cells overexpressing PEAK1 formed more colonies in soft agar, and these colonies were also larger. PEAK1 knockdown significantly reduced the ability of HPNE-KRas cells to grow under these conditions. Importantly, this effect was rescued by overexpressing PEAK1.

PEAK1 is necessary for tumor formation, progression, and metastasis in vivo

The above results strongly suggested that PEAK1 might regulate pancreatic cancer growth in vivo. Therefore, we next investigated whether altering PEAK1 expression could interfere with PDAC tumor formation and metastasis in vivo. In these studies, FG cells were measured for their ability to form tumors and metastasize with a preclinical orthotopic implantation mouse model of PDACs (Fig. 4A; refs. 17, 20). PEAK1 knockdown significantly reduced tumor formation (Fig. 4B and Supplementary Fig. S3A and S3B), whereas PEAK1 overexpression caused a significant increase in tumorigenesis in vivo (Supplementary Fig. S3C–S3E). Furthermore, PEAK1 knockdown suppressed metastasis to multiple sites and increased animal survival compared with animals implanted with control cells expressing PEAK1 (Fig. 4C and D and Supplementary Fig. S3F). Notably, the median survival of control animals was 64 days, whereas median survival of the PEAK1-depleted group was 97 days (P = 0.0022; Fig. 4D). In support of these data, depletion of PEAK1 in HPNE-KRasG12D cells abrogated liver and brain metastasis (Fig. 4E and Supplementary Fig. S3G) in the chicken embryo (21). These data are also consistent with our observations that...
PEAK1 levels are significantly increased in the highly metastatic FGM cell line derived from FG cells (Supplementary Fig. S3H) and in metastatic foci originating from human PDAC and other cancers in comparison with the primary tumor of origin (Fig. 4F, Supplementary Fig. S3I and Table S1; ref. 22). Together, these data show an essential role for PEAK1 during PDAC growth and metastasis in vivo.

Suppression of ErbB2 in PDAC cells drives PEAK1-dependent tumor formation in vivo

Similar to PEAK1, both ErbB2 and Src are upregulated in human PDAC downstream of oncogenic KRas (Fig. 5A and B and Supplementary Fig. S4A–S4E; refs. 23–25). While Src is known to cooperate with KRas during pancreatic neoplasia (4), the role of ErbB2 in PDAC remains unclear (1, 2). For these reasons, we initially sought to determine whether there was a functional relationship between the coinduction of PEAK1 and ErbB2 in PDAC. Surprisingly, silencing ErbB2 (Supplementary Fig. S4F) in FG cells significantly increased orthotopic tumor formation in mice (Fig. 5C and Supplementary Fig. S4G). Notably, this response was associated with increased PEAK1 expression in vivo (Fig. 5D) and in vitro (Fig. 5E and Supplementary Fig. S4H), suggesting that PEAK1 upregulation compensated for the loss of ErbB2 to drive
tumor formation. Indeed, silencing PEAK1 in ErbB2-depleted FG cells fully abrogated tumor formation beyond that of PEAK1 knockdown alone (Fig. 5C–E and Supplementary Fig. S4G and S4H). These findings are also consistent with our data showing that PEAK1 overexpression in FG cells increases tumor formation (Supplementary Fig. S3C–S3E). These data show that ErbB2 suppression in PDAC cells potentiates tumor formation by upregulating PEAK1 protein levels. Interestingly, overexpression of a constitutively active form of ErbB2 (NeuT) in FG cells did not alter PEAK1 expression or the tumorigenic potential of these cells in vivo (Supplementary Fig. S4I–S4L). This result suggests that ErbB2 is a permissive factor in PDACs and that oncogenic programs active in this disease are selectively sensitive to decreased ErbB2 levels.

PEAK1 modulates Src and ErbB2 kinase activities and complex assembly

PEAK1 knockdown inhibited ErbB2 kinase activity as indicated by decreased phosphorylation of Y1248 and Y877 (Fig. 5E and Supplementary Fig. S4H; refs. 26, 27). ErbB2-Y877 is a reported Src substrate site that potentiates ErbB2 autophosphorylation on other tyrosine residues such as Y1248 and enhances its transforming potential (26). Therefore, these findings suggest that PEAK1 can regulate Src activity toward ErbB2 to modulate its kinase activity. In this regard, PEAK1 knockdown potently inhibited Src kinase and the assembly of an active Src/ErbB2 signaling complex (Fig. 5E and F and Supplementary Fig. S4H). Furthermore, active Src and ErbB2 co-precipitated with PEAK1 from PDAC cell lysates indicating that all 3 proteins participate in a molecular...
complex (Fig. 5F and Supplementary Fig. 5H). Because KRas induces PEAK1 expression in a Src-dependent manner (Fig. 2E and Supplementary Fig. S1C–S1F), we further hypothesized that PEAK1 upregulation following ErbB2 depletion may also occur via an Src-dependent mechanism. Pharmacologic or RNAi inhibition of Src in FG cells with and without ErbB2 suppressed PEAK1 expression at both the transcript and protein levels (Fig. 5G and H). Taken together, our findings show that KRas activation induces the upregulation of Src, PEAK1, and ErbB2 to form a feed-forward, self-sustaining tyrosine kinase amplification loop that promotes PDAC growth and metastasis.

**Src/PEAK1/ErbB2 signaling drives trastuzumab and gemcitabine resistance in pancreatic cancer cells**

Because Src and ErbB2 inhibitors are currently in clinical trials for pancreatic cancer and we have shown that inhibition of Src activity and ErbB2 expression modulate PEAK1 protein levels, we sought to determine whether Src (dasatinib) or ErbB2 (trastuzumab) inhibition might elicit compensatory expression of PEAK1, Src, and/or ErbB2 genes. Importantly, inhibition of PEAK1 expression did not induce compensatory transcription of Src or ErbB2 (Fig. 6A). In contrast, whereas dasatinib inhibited PEAK1 expression, it significantly increased transcription of both ErbB2 and Src (Fig. 6A, middle and right). This compensatory upregulation of ErbB2 in response to dasatinib may have important clinical implications. However, we show here that increased ErbB2 activity above endogenous levels in PDAC cells does not promote increased tumor growth (Supplementary Fig. S4I–S4L). Therefore, as dasatinib potently blocks both Src activity and PEAK1 expression, even in the context of upregulated ErbB2, it is likely that this therapeutic approach will provide some benefit to patients (4, 9). On the other hand, directly inhibiting ErbB2 expression and function with trastuzumab alone may not be a viable therapeutic option because it induced PEAK1 expression (Fig. 6A, left, and 6B), which in turn, can drive Src activity and PDAC growth (Figs. 5C–E and 6B, and Supplementary Fig. S4G and S4H). Taken together, these data suggest that targeting PEAK1 may be a preferred method for treating PDAC as it minimizes compensatory signaling through Src and ErbB2 kinases.

The above results do suggest that dual inhibition of both ErbB2 and PEAK1 may further overcome trastuzumab resistance in PDAC preclinical and clinical studies. Trastuzumab (Herceptin, Genentech Inc.) acts to cause internalization, degradation, and inactivation of the ErbB2 receptor (28) and is currently being evaluated in combination with gemcitabine, which is the standard chemotherapy given to patients with PDAC. However, studies to date suggest that trastuzumab offers little benefit alone or in combination with gemcitabine (2, 6, 7, 29). These data, combined with the fact that PEAK1 is upregulated in trastuzumab-resistant PDAC cell lines and patients with amplified ErbB2 levels (Supplementary Fig. S5A and S5B), led us to investigate whether the ineffectiveness of trastuzumab-based therapies could be because of a PEAK1/Src compensation mechanism following ErbB2 suppression. Consistent with shRNA knockdown of ErbB2 in FG cells, trastuzumab treatment of PANC1 cells (a trastuzumab-resistant PDAC line; ref. 6) reduced ErbB2 protein levels (Supplementary Fig. S1F), we further hypothesized that PEAK1 upregulation following ErbB2 depletion may also occur via an Src-dependent mechanism. Pharmacologic or RNAi inhibition of Src in FG cells with and without ErbB2 suppressed PEAK1 expression at both the transcript and protein levels (Fig. 5G and H). Taken together, our findings show that KRas activation induces the upregulation of Src, PEAK1, and ErbB2 to form a feed-forward, self-sustaining tyrosine kinase amplification loop that promotes PDAC growth and metastasis.

**Src/PEAK1/ErbB2 signaling drives trastuzumab and gemcitabine resistance in pancreatic cancer cells**

Because Src and ErbB2 inhibitors are currently in clinical trials for pancreatic cancer and we have shown that inhibition of Src activity and ErbB2 expression modulate PEAK1 protein levels, we sought to determine whether Src (dasatinib) or ErbB2 (trastuzumab) inhibition might elicit compensatory expression of PEAK1, Src, and/or ErbB2 genes. Importantly, inhibition of PEAK1 expression did not induce compensatory transcription of Src or ErbB2 (Fig. 6A). In contrast, whereas dasatinib inhibited PEAK1 expression, it significantly increased transcription of both ErbB2 and Src (Fig. 6A, middle and right). This compensatory upregulation of ErbB2 in response to dasatinib may have important clinical implications. However, we show here that increased ErbB2 activity above endogenous levels in PDAC cells does not promote increased tumor growth (Supplementary Fig. S4I–S4L). Therefore, as dasatinib potently blocks both Src activity and PEAK1 expression, even in the context of upregulated ErbB2, it is likely that this therapeutic approach will provide some benefit to patients (4, 9). On the other hand, directly inhibiting ErbB2 expression and function with trastuzumab alone may not be a viable therapeutic option because it induced PEAK1 expression (Fig. 6A, left, and 6B), which in turn, can drive Src activity and PDAC growth (Figs. 5C–E and 6B, and Supplementary Fig. S4G and S4H). Taken together, these data suggest that targeting PEAK1 may be a preferred method for treating PDAC as it minimizes compensatory signaling through Src and ErbB2 kinases.
overexpression of PEAK1 in HPNE-KRas cells did not significantly change their response to trastuzumab, PEAK1 knockdown enabled trastuzumab to elicit growth-inhibitory effects under anchorage-dependent conditions (Fig. 6D). Rescuing PEAK1 expression in the HPNE-KRas cells reversed this effect, causing these cells to regain their trastuzumab-resistant phenotype (Fig. 6D). Furthermore, PEAK1 knockdown reduced the IC50 values of trastuzumab (Fig. 6E) and a commercially available ErbB2-targeting antibody (Supplementary Fig. S5C) by approximately 100- and 10-fold, respectively, whereas PEAK1 overexpression in HPNE cells made them nonresponsive to trastuzumab in vitro (Supplementary Fig. S5D). These findings support a mechanism by which elevated PEAK1/Src signaling compensates for loss of ErbB2 function in PDAC and, therefore, contributes to resistance of ErbB2-targeted therapies.

As mentioned earlier, the standard therapy regimen for patients with metastatic pancreatic cancer is gemcitabine. However, most patients with advanced metastatic disease show little response to this treatment (1, 30). Because Src activity has recently been identified as a contributing factor to gemcitabine resistance in pancreatic cancer (9) and we show that Src inhibition with dasatinib sensitized FG cells to gemcitabine resistance in pancreatic cancer (9), we explored whether targeting Src might also sensitize PDAC cells to gemcitabine treatment. In this regard, shPEAK1 FG cells were 10 times more sensitive to gemcitabine treatment (Fig. 6F) than in control cells (Fig. 6F). In agreement with these data, previous studies have shown a significant increase in PEAK1 expression in patients with lung cancer who are resistant to gemcitabine treatment (Supplementary Fig. S5F and Table S1). Taken together with our earlier results showing that PEAK1 mediates the formation and function of a triple-kinase complex between Src, PEAK1, and ErbB2 to promote PDAC progression, these findings suggest that the development of combination therapies that include PEAK1 inhibition or suppression may provide maximum benefit for patients.

Discussion

We originally identified PEAK1 as a tyrosine phosphorylated protein enriched in the pseudopodium of migrating cells (12, 31). PEAK1 localizes to integrin-mediated focal adhesions and actin stress fibers, where it regulates the cytoskeleton and shape changes necessary for cell migration. Here, we report that PEAK1 also regulates cancer cell growth and progression. Although it is not yet known how PEAK1 regulates cancer growth and cancer cell motility, PEAK1 can receive input signals from integrin adhesions and growth factor receptors, which send vital proliferation, survival, and motility information from the surrounding extracellular environment to the cell’s interior (32–34). In this regard, we have previously shown that growth factors promote PEAK1 tyrosine phosphorylation in a Src-dependent manner (12, 31). Also, PEAK1 overexpression in cancer cells is sufficient to promote extracellular signal–regulated kinase (ERK) activation and phosphorylation of the focal adhesion proteins p130CAS and paxillin (12, 31). Together, these findings suggest that PEAK1 is an important cytoskeletal regulator that controls cancer cell growth and migration.

PEAK1 is upregulated in many solid tumors and blood-borne cancers (Fig. 1A and Supplementary Table S1). This suggests that PEAK1 plays fundamental roles in tumor progression downstream of the diverse oncogenic insults found in human malignancies. While the precise role of PEAK1 in these malignancies needs to be defined further, we show here that in PDAC, activating mutations in the KRas oncogene drive the aberrant upregulation of PEAK1 via Src-dependent transcriptional regulation. This can affect cancer progression in 2 ways. First, PEAK1 upregulation increases Src kinase activity above that already induced by KRas activation (Fig. 5E and Supplementary Fig. S4H). PEAK1 has a highly conserved Src consensus phosphorylation site and Src SH2–binding domain at Y665. Thus, it is possible that KRas drives the initial Src activity that promotes both increased PEAK1 protein expression and the phosphorylation of Y665. We suspect that this increases Src binding to PEAK1–Y665 via its SH2 domain, which in turn stabilizes Src in the open kinase-active conformation, and further promotes tumorigenesis (ref. 35; Supplementary Fig. S6).

Second, PEAK1 mediates Src/ErbB2 binding and ErbB2 phosphorylation at Y877 (Fig. 5E and F and Supplementary Fig. S4H and S4M). Phosphorylation of ErbB2–Y877 is Src-dependent and can enhance the oncogenic potential of ErbB2 (26). Therefore, it is possible that enhanced ErbB2 activation, by PEAK1/Src signaling, can also contribute to cancer growth and progression (Supplementary Fig. S6). If this is the case, depletion of ErbB2 should reduce tumorigenesis. Surprisingly though, depletion of ErbB2 protein from PDAC cells enhanced tumorigenesis in a PEAK1/Src-dependent fashion (Fig. 5C–E and Supplementary Fig. S4G and S4H). Furthermore, overexpression of the constitutively active NeuT receptor did not modulate PEAK1 expression or increase tumor growth (Supplementary Fig. S4I–S4L). This suggests that elevating ErbB2 activation above endogenous levels in pancreatic cancer cells does not further contribute to tumor growth and that ErbB2 overexpression is permissive during later stages of PDACs (36). Interestingly, it has been previously reported that oncogenes known to contribute to tumorigenesis in some tumor types have no phenotype when overexpressed in other cancers (37, 38). Furthermore, it will be important that future studies investigate the role of ErbB2 during early pancreatic neoplasia. One explanation of these results is that the loss of ErbB2 protein from the PEAK1/Src kinase complex makes this active complex available to target new substrates (such as other growth factor receptors or intracellular effector molecules) that can also drive cancer. Alternatively, ErbB2 inactivation could inhibit an unidentified negative feedback pathway that normally permits or holds PEAK1/Src activity in check within tumor cells. In this regard, we show that ErbB2 suppression via RNAi or trastuzumab increases PEAK1 expression and Src signaling, leading to sustained phosphorylation of the residual ErbB2 protein (Figs. 5E–H and 6A and Supplementary Fig. S4H). Yet, it is still possible that other feedback mechanisms are in action, and the specific protumorigenic mechanism(s) of PEAK1/Src hyperactivation downstream of ErbB2 inactivation as well as the role of PEAK1 in other cancers need to be further elucidated. In any case, our
findings clearly show that PEAK1 is necessary for PDAC progression whether or not ErbB2 is present.

Because our data suggest that ErbB2 plays a permissive, oncogene-sensing role in PDAC, our findings that trastuzumab resistance results from compensatory PEAK1/Src signaling have important implications for this disease. The therapeutic effectiveness of trastuzumab is currently being tested in human PDAC clinical trials in combination with gemcitabine (2, 6, 7, 29). However, the majority of patients show no benefit from trastuzumab treatment. Thus, our finding that PEAK1/Src signaling can compensate for the loss of ErbB2 activity may provide a plausible explanation for the poor clinical response to trastuzumab and are in agreement with previous studies. In our studies, trastuzumab initiated compensatory PEAK1-dependent signaling (Fig. 6A–E and Supplementary Fig. S5A–S5D). Thus, PEAK1 may be a preferred target against which novel therapies can be developed for patients with PDACs, either alone or in combination with existing therapies. Finally, PEAK1 inhibition sensitized PDAC cells to gemcitabine treatment (Fig. 6F). Because gemcitabine represents the standard of care for patients with PDAC, therapeutic interventions that target PEAK1 may improve patient outcome in combination with gemcitabine therapy.

In summary, KRas-induced PEAK1 mediates pancreatic cancer growth, metastasis, and therapy resistance via activation of Src kinase. While many factors contribute to the pathogenesis of PDACs, a detailed understanding of the molecular components, such as PEAK1, that can drive cancer progression, metastasis, and therapy resistance may lead to new avenues for disease diagnosis and treatment.

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

Authors' Contributions
Conception and design: J.A. Kelber, S. Kaushal, Y. Wang, R.L. Klemke
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.A. Kelber, T. Reno, S. Kaushal, C. Mettidi, T. Wright, K. Stoletov, J.M. Weems, F.D. Park, E. Mose, Y. Wang, A.M. Lowy, M. Bouvet, R.L. Klemke
Analysis and interpretation of data (e.g., statistical analysis, bios-statistics, computational analysis): J.A. Kelber, S. Kaushal, T. Wright, A.M. Lowy, R.L. Klemke
Writing, review, and/or revision of the manuscript: J.A. Kelber, T. Reno, C. Mettidi, K. Stoletov, J.M. Weems, R.M. Hoffman, R.L. Klemke
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.A. Kelber, T. Wright, R.L. Klemke
Study supervision: J.A. Kelber, R.L. Klemke

Acknowledgments
The authors thank members of the Klemke, Bouvet, and Lowy laboratories and Dr. Peter Gray for instructive feedback and discussions about the manuscript. The authors thank Dr. Cynthia Snyder who assisted with the histopathology analysis of tumor sections. The authors also thank Elizabeth Hampton, Ryan Matson, and Tiffany Taylor for assistance with molecular biology and cell culture protocols.

Grant Support
This work was supported by the NIH-RRACDA (NIH - Institutional Research and Academic Career Development Award) Postdoctoral Fellowship GM08652 (J.A. Kelber) and NIH grants CA097022 and CA129231 (R.L. Klemke), CA132971 (M. Bouvet) and CA157692 (A.M. Lowy).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received October 31, 2011; revised February 24, 2012; accepted February 24, 2012; published OnlineFirst May 16, 2012.

References


