Ectopic expression of *VAV1* reveals an unexpected role in pancreatic cancer tumorigenesis

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Summary

Herein, we show that the hematopoietic-specific GEF *VAV1* is ectopically expressed in primary pancreatic adenocarcinomas due to demethylation of the gene promoter. Interestingly, *VAV1*-positive tumors had a worse survival rate compared to *VAV1*-negative tumors. Surprisingly, even in the presence of oncogenic *KRAS*, *VAV1* RNAi abrogates neoplastic cellular proliferation in vitro and in vivo, thus identifying Vav1 as a growth-stimulatory protein in this disease. Vav1 acts synergistically with the EGF receptor to stimulate pancreatic tumor cell proliferation. Mechanistically, the effects of Vav1 require its GEF activity and the activation of Rac1, PAK1, and NF-κB and involve cyclin D1 upregulation. Thus, the discovery of prooncogenic pathways regulated by Vav1 makes it an attractive target for therapeutic intervention.

Introduction

Pancreatic ductular adenocarcinoma is the most common pancreatic tumor, accounting for more than 90% of all pancreatic cancers, and ranks fourth as a cause of death by cancer in the United States (Jaffee et al., 2002). Pancreatic cancer has one of the poorest prognoses among human neoplasms, with an overall 5 year survival rate of 3%. This is due to the highly aggressive and rapidly metastatic nature of the disease and the fact that current diagnostic tools do not detect early stages of the disease, as indicated by the reality that many patients have metastases at the time of diagnosis. Besides surgical resection, which is only possible in a small fraction of patients (less than 20%) and which is rarely curative, current therapies for pancreatic cancer rely on traditional cytotoxic agents with limited effects throughout the course of the disease (Jaffee et al., 2002). Therefore, efforts aimed at understanding the molecular mechanisms underlying the development of pancreatic cancer may lead to prevention and treatment strategies that improve the prognosis of the disease.

Although the underlying etiology and pathophysiology of pancreatic ductal cancer are poorly understood, there is increasing evidence that signaling and transcriptional pathways that control cell proliferation, differentiation, and apoptosis are dysregulated in pancreatic cancer (Bardeesy and DePinho, 2002; Urrutia and DiMagno, 1997). Together, changes in these molecular pathways lead to abnormalities in growth factor-mediated signaling cascades, cell cycle control, cellular migration, and transcriptional regulation. In fact, gain-of-function mutations in the *KRAS* protooncogene are among the most common genetic abnormality associated with pancreatic cancer, approaching a frequency of 95% (Hruban et al., 2001; Jaffee et al., 2002). In addition, overexpression of EGF receptors and their ligands occurs frequently and is associated, like Kras, with the early developmental processes leading to pancreatic cancer development (Hruban et al., 2001; Korc, 1998). Lastly, antiproliferative pathways (e.g., TGF-β and DPC4) and those that regulate programmed cell death (e.g., TP53 and Akt) are frequently altered in pancreatic cancer (Jaffee et al., 2002). Despite this understanding of pancreatic cancer, therapies designed toward some of these pathways (e.g., Kras and EGFR) have remained ineffective (Jaffee et al., 2002). Thus, the identification of additional signaling pathways specifically upregulated in pancreatic

SIGNIFICANCE

Pancreatic cancer is the fourth leading cause of cancer death in the United States and remains virtually incurable. Despite significant advances in our understanding of the molecular mechanisms contributing to the development of pancreatic cancer, current therapies have remained ineffective; thus, new therapies rely on the identification of molecular targets that contribute to pancreatic tumorigenesis. We have identified that *VAV1* is misexpressed in 53% of pancreatic adenocarcinomas, is associated with decreased survival, and contributes to the tumorigenic properties of pancreatic cancer cells. Interestingly, ablation of Vav1 in the presence of oncogenic Kras abrogates neoplastic cell growth, suggesting a dependence on Vav1-regulated pathways for maintenance of the transformed phenotype. Thus, Vav1-regulated pathways may serve as potential therapeutic targets in pancreatic cancer.

cancer may provide novel functional targets for diagnosis, prevention, and treatment of this deadly disease.

The Rho family of GTP binding proteins function as molecular switches going from an inactive GDP bound to an active GTP bound state. When activated, these proteins interact with a variety of effectors to trigger distinct signaling pathways leading to reorganization of the actin cytoskeleton, proliferation, and antiapoptotic pathways. The role of Rho family proteins in human tumorigenesis is becoming appreciated with the identification of the overexpression and hyperactivation of some Rho proteins in human cancers (Sahai and Marshall, 2002). Indeed, numerous cell surface receptors on pancreatic tumor cells, including those aberrantly expressed in the pancreatic tumor microenvironment (e.g., IGF-I, EGF, PDGF) activate Rho family proteins (Matozaki et al., 2000; Sahai and Marshall, 2002). Moreover, Rho family proteins have been shown to be required for *ras*-mediated transformation of 3T3 cells (Qiu et al., 1995a, 1995b); however, gain-of-function mutations in Rho family GTP binding proteins, such as those found for *ras*, have not been observed in human cancers. Thus, it has been speculated that dysregulation of the GDP-GTP cycle by either inhibition of GTPase-activating proteins (GAPs) or expression of guanine nucleotide exchange factors (GEFs), both of which would result in increased bound GTP, may participate in the hyperactivation of Rho family signaling cascades in cancer (Bustelo, 2000; Sahai and Marshall, 2002). Consistent with this idea, many GEFs, including Dbl, Lbc, Lfc, Vav, Net, Ect2, and Tim, were originally isolated as oncogenes using in vitro 3T3 fibroblast transformation assays with DNA from various human tumors, but most were the result of oncogenic activation by truncation during the cloning procedure. Although *bcr* and *larg* are found as translocation partners with *abl* and *mll*, respectively, the role of most Dbl GEFs in regulating the tumorigenic properties of human cancers has remained elusive.

The Vav proteins (Vav1–Vav3) represent a novel family of Dbl GEFs for Rho family GTPases that are regulated by tyrosine phosphorylation downstream of several receptor and nonreceptor tyrosine kinases (Bustelo, 2000; Turner and Billadeau, 2002). While *VAV1* is primarily expressed in bone marrow-derived cell lineages, where it has been extensively studied, *VAV2* and *VAV3* demonstrate a more ubiquitous pattern of gene expression. Interestingly, although truncated versions of Vav proteins lacking the amino terminus have been found to transform 3T3 fibroblasts (Katzav et al., 1989; Movilla and Bustelo, 1999; Schuebel et al., 1996) and synergize with active Ras in transformation (Bustelo et al., 1994; Katzav et al., 1995; Khosravi-Far et al., 1994), their role in human tumorigenesis has thus far not been described. The fact that pancreatic adenocarcinomas frequently overexpress growth factor receptor tyrosine kinases or their ligands (Korc, 1998) and harbor *KRAS*-activating mutations in more than 95% of tumors suggested to us that Vav proteins may participate in the pathogenesis of this disease. We found that *VAV1* is expressed in an ectopic manner in adenocarcinoma of the pancreas and is associated with decreased survival in pancreatic cancer patients. Furthermore, Vav1 contributes to the tumorigenic properties of pancreatic cancer cells by regulating both cellular proliferation and cell survival pathways through the regulation of an EGF-Src-Vav1-Rac1-Pak1-NF-κB-Cyclin D1 signaling axis.

Results and discussion

VAV1 is ectopically expressed in pancreatic cancer and associated with decreased survival

Using isoform-specific oligonucleotides and antibodies, we screened normal human pancreas and eight human pancreatic tumor cell lines for the expression of all three *VAV* isoforms. Although *VAV2* RNA and protein are present in all eight pancreatic tumor cell lines and normal human pancreatic tissue (Supplemental Figures S1A and S1B at http://www.cancercell.org/cgi/content/full/7/1/39/DC1/), we did not detect *VAV3* message in the normal human pancreas but did detect *VAV3* RNA and protein in two of the eight cell lines (MiaPaCa2 and SU86.86; data not shown). Surprisingly, mRNA and protein of the hematopoietic-specific *VAV1* isoform were detected in three cell lines, CAPAN2, CFPAC, and MiaPaCa2, but not in normal human pancreas (Supplemental Figures S1A and S1B). Sequence analysis of *VAV1* cDNA prepared from the *VAV1*-positive tumor cell lines shows that the Vav1 protein expressed in these cell lines is wild-type in nature (data not shown). Significantly, we did not observe the expression of *VAV1* in multiple cell lines from four other epithelial-derived tumor types, including breast, ovarian, lung, and colon. Interestingly, screening of tissue sections prepared from mouse pancreatic cancer models reveals expression of Vav1 in the elastase promoter-driven SV40 T-ag (Ela-Tg) (Ornitz et al., 1987) and the preneoplastic lesions within the $KRAS^{G12D}$/*pdx*-Cre mouse (Hingorani et al., 2003), but not in the normal tissue (Supplemental Figure S1C). In addition, the mouse overexpressing the dominant-negative TGFβ type II receptor (DNR) using the mouse metallothionein I promoter, which displays ductular hyperplasia (Bottinger et al., 1997), shows expression of Vav1 (Supplemental Figure S1C). In order to determine if Vav1 protein expression is observed in primary human tumors, we screened paraffin-embedded tissue sections from 15 normal and 95 pancreatic cancer patients for the expression of *VAV1* using immunohistochemistry. Vav3 was not detected in either the normal pancreas or pancreatic adenocarcinoma specimens (data not shown). In contrast to *VAV2* expression, which is observed in both the acinar and ductal epithelial cells in the normal pancreas and tumor specimens (Supplemental Figure S2), *VAV1* expression was not observed in either the acinar or ductal epithelial cells in the normal pancreas sections but was present in greater than 50% of the tumor specimens analyzed (Figure 1A). Importantly, the expression of *VAV1* in the *VAV1*+ tumor specimens was specific to the epithelial tumor cells and did not stain the underlying stroma.

To determine the clinical significance of *VAV1* ectopic expression, patients with *VAV1*-negative and -positive tumors were compared with respect to demographic (age, gender) and prognostic (tumor size [cm] and stage [I–IV]) variables using chi-square tests for nominal variables and Wilcoxon rank sum tests for continuous and ordinal variables. Of the 95 patients analyzed for Vav1 protein expression, clinical data on 75 patients were available. The mean age of the patients studied was 63.7 ± 11.3, and 57% of the patients were males. Of the patients in which staging was available, the mean size of the resected lesion was 2.7 cm (±1.09). By UICC staging, 11 (16%) were stage I, 15 (22%) were stage II, 37 (54%) were stage III, and 6 (9%) were stage IV. Patients with *VAV1*-positive tumors were less likely to be females (31% versus 69%); otherwise the two groups were comparable. The association between Vav1 protein

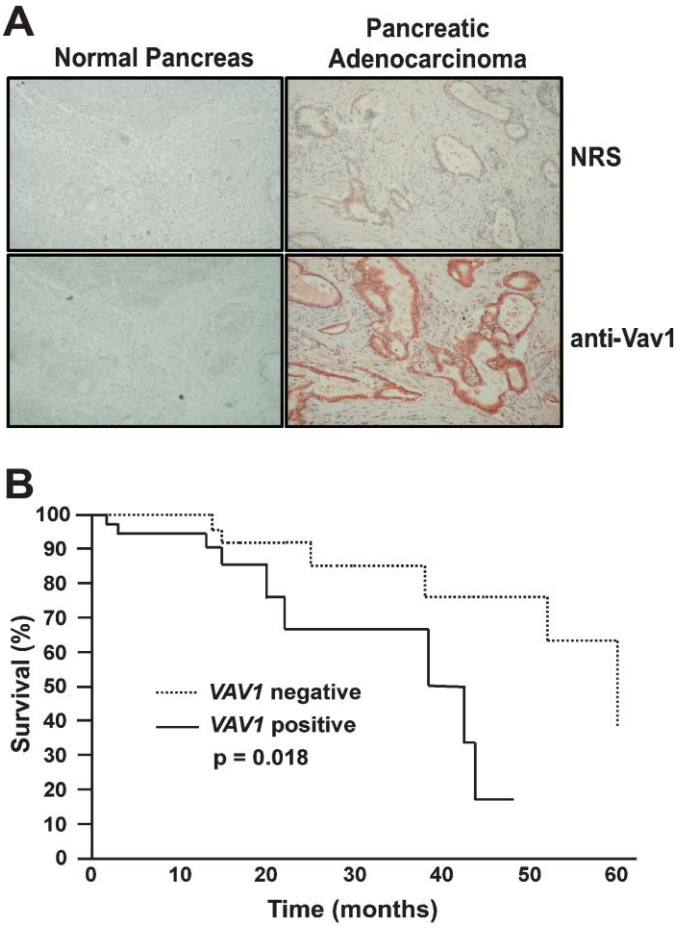

Figure 1. *VAV1* is ectopically expressed in pancreatic adenocarcinoma and associated with decreased survival

A: Immunohistochemical examination of normal human pancreas and primary pancreatic cancers for the expression of *VAV1*. Paraffin-embedded tissue samples were prepared as described in the Experimental Procedures and stained with normal rabbit serum (NRS) as a control or anti-Vav1 polyclonal rabbit antisera.

B: Kaplan-Meier survival curve of *VAV1*+ and *VAV1*− patients.

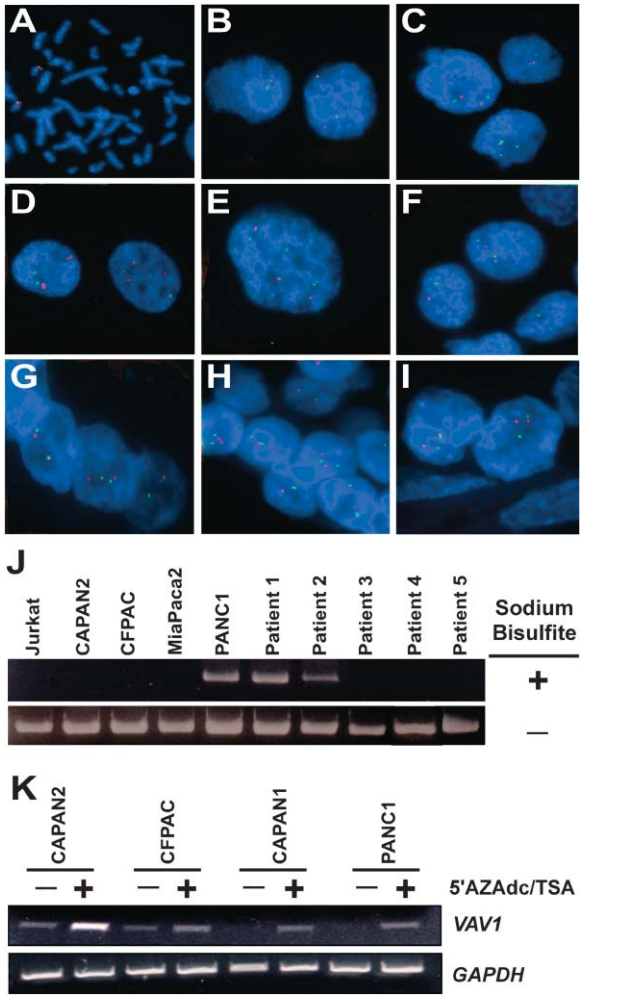

Figure 2. Ectopic expression of *VAV1* results from demethylation of the *VAV1* gene

A–I: Amplification of the *VAV1* gene was assessed in the *VAV1*-expressing cell lines as well as in the primary pancreatic adenocarcinomas analyzed by fluorescent in situ hybridization. The *VAV1* gene maps to chromosome 19p13; a telomere probe to chromosome 19 is shown in green, and the *VAV1* genomic probe is shown in red. A normal metaphase spread demonstrates the localization of the two probes (**A**). The tumor cells lines are CAPAN2 (**B**), CFPAC (**C**), BXPC3 (**D**), PANC1 (**E**), ASPC1 (**F**), and three primary pancreatic cancers (**G–I**).

J: DNA from the indicated pancreatic tumor cell lines and primary pancreatic cancer specimens was left untreated or was treated with sodium bisulfite in order to determine the methylation status of the *VAV1* gene by methylation-specific PCR (MSP). Following the treatment, the DNA was purified and subject to MSP as described in the Experimental Procedures. The methylation-specific products were resolved on 2.5% agarose gels and visualized following staining with ethidium bromide. DNA from the *VAV1*-expressing Jurkat T cell line was used as a control for MSP.

K: The indicated tumor cell lines were left untreated or were treated with 5′AZAdc/TSA for 4 days, and RNA was subsequently extracted. *VAV1* and *GAPDH* gene expression was analyzed by RT-PCR as described in the Experimental Procedures.

status and survival was assessed with Kaplan-Meier curves and a log rank test. On univariate analysis, *VAV1*-negative tumors had a better survival compared to *VAV1*-positive tumors ($p = 0.018$) (Figure 1B). Vav1 protein status was the only significant factor predicting survival ($p = 0.03$) on multivariate analysis, after adjusting for gender, tumor size, and stage. Thus, these data suggest that *VAV1* misexpression may lead to a decreased survival benefit in pancreatic cancer patients.

Ectopic expression of *VAV1* is due to demethylation of the *VAV1* gene promoter

In order to investigate the mechanism for dysregulated expression of *VAV1*, we performed fluorescence in situ hybridization (FISH) analysis on *VAV1*-expressing and nonexpressing pancreatic tumor cell lines. We found that the *VAV1*+ cell lines each contained 2 copies of the *VAV1* gene, as did ASPC1, a cell line that does not express *VAV1* (Figures 2B, 2C, and 2F). However, BXPC3 and PANC1, two *VAV1*− cell lines, contained 3 copies of the *VAV1* gene (Figures 2D and 2E). Moreover, FISH analysis of paraffin-embedded tumor samples has revealed no amplification of the *VAV1* gene (Figures 2G–2I). This suggested to us that gene amplification was not the mechanism by which the *VAV1* gene was being aberrantly expressed in the two *VAV1*+ tumor cell lines and pancreatic cancer tissues.

Since epigenetic abnormalities leading to the activation of oncogenes or inactivation of tumor suppressors play a key role in tumorigenesis, we sought to determine if epigenetic changes in the *VAV1* gene contributed to aberrant gene expression. We first analyzed the methylation status of the *VAV1* gene using methylation-specific PCR (MSP). Following bisulfite treatment of the DNA, MSP amplification of the *VAV1* gene was not detected in the cell lines that express *VAV1* as well as in treated DNA from primary human pancreatic tumors (Figure 2J) but was detected in the *VAV1*− PANC1 cell line (Figure 2J), indicating that the *VAV1* gene is not appropriately methylated in *VAV1*-expressing cell lines and pancreatic tumor specimens. Interestingly, all cell lines, regardless of *VAV1* status, were able to activate a luciferase gene under the control of the *VAV1* promoter (Denkinger et al., 2000) (data not shown), suggesting that transcriptional activators of this gene are present in these cells. We next tested whether treatment with the DNA demethylation agents 5′Aza-dC and TSA would lead to the expression of *VAV1* in nonexpressing pancreatic tumor cell lines. In fact, the *VAV1*− PANC1 and CAPAN1 cell lines express *VAV1* upon 5′Aza-dC and TSA treatment (Figure 2K), indicating that demethylation of the *VAV1* gene can result in expression in these cell lines. Thus, the ectopic expression of *VAV1* in primary pancreatic cancer is the result of an epigenetic modification of the *VAV1* gene.

VAV1 is required for anchorage-independent growth and tumor formation in vivo

The misexpression of *VAV1* prompted us to examine its significance in the tumorigenic properties of pancreatic tumor cells. To this end, we generated short hairpin RNA-targeting vectors toward *VAV1* (shVav1-1 and shVav1-2) in order to acutely silence this gene product and then measured cellular proliferation. Importantly, the shVav1-1 and shVav1-2 targeting vectors deplete Vav1 protein levels in all three *VAV1*+ tumor cell lines and do not affect the ubiquitously expressed *VAV2* (Supplemental Figure S3 at http://www.cancercell.org/cgi/content/full/7/1/39/DC1/). Using the RNA interference vectors toward *VAV1*, we determined the ability of *VAV1*+ CFPAC and MiaPaCa2 pancreatic tumor cells to form colonies in soft agar. Suppression of Vav1 in the oncogenic *KRAS*-containing CFPAC and MiaPaCa2 cell lines results in a substantial reduction in colony formation in soft agar compared to the control-transfected populations (Figure 3A). Importantly, cotransfection of a shRNA-resistant Vav1 cDNA (Vav1r) could completely reverse the anchorage-independent growth characteristics observed in Vav1-depleted MiaPaCa2 cells (Figure 3B). In order to further examine the affect of Vav1 suppression on pancreatic tumorigenesis, we injected Vav1-suppressed or control-transfected cells subcutaneously into the flank of irradiated male athymic nude mice and monitored the increase in tumor volume over a 6 week period. Significantly, suppression of Vav1 in the CAPAN2 cell line results in a dramatic reduction in tumor volume (Figures 3C and 3E), whereas the CFPAC cell line shows a less dramatic, yet reproducible reduction in tumor volume (Figure 3D). Consistent with the effect of Vav1 in tumor formation, we observe a decrease in expression of the angiogenic marker CD34 (Figure 3E), thus supporting the notion that *VAV1* participates in the tumorigenic properties of pancreatic cancer cells. These data indicate that *VAV1* is required for maintenance of the transformed phenotype

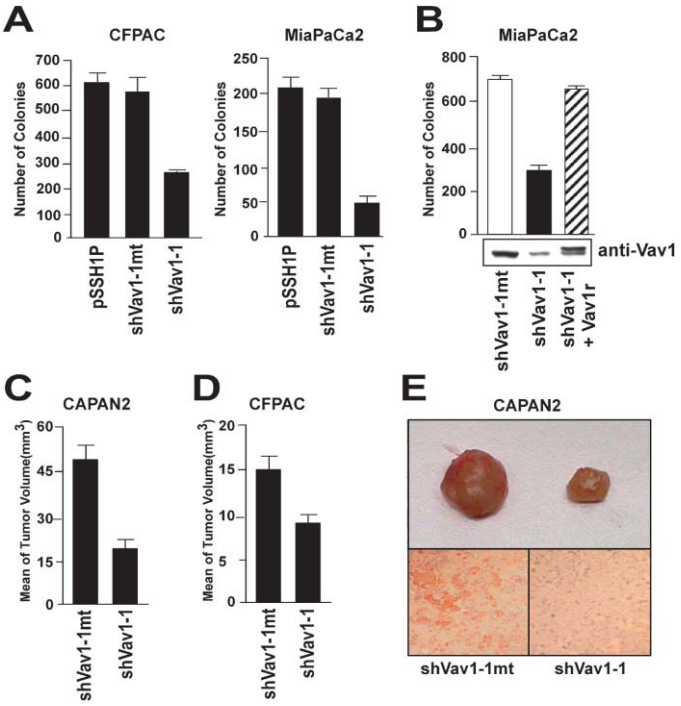

Figure 3. Vav1 ablation leads to reduced tumorigenic properties in vitro and in vivo

A: The *VAV1*-expressing cell lines CFPAC and MiaPaCa2 were transfected with a GFP-expressing plasmid and either a control vector (pSSH1P), a short-hairpin (sh) RNA vector that targets the Vav1 mRNA (shVav1-1), or a mutant Vav1-targeting construct (shVav1mt). One day posttransfection, the cells were sorted for GFP expression, and then 2×10^4 cells were plated in quadruplicate as described in the Experimental Procedures. Two weeks later, the number of visible colonies was scored.

B: MiaPaCa2 cells were transfected with the indicated plasmids, and anchorage-independent growth was analyzed. The inset shows suppression of Vav1 and reexpression of Vav1r in MiaPaCa2 cells.

C–E: CAPAN2 and CFPAC (**D**) cells were transfected with the indicated constructs and injected in the flank of male athymic nude mice (n = 9 for each experimental condition). Weekly measurements were taken from the tumors, and the mean tumor volume was determined after 6 weeks. **E:** A representative example of tumor mass is shown for the CAPAN2 cell line transfected with either the shVav1mt or shVav1-1 targeting construct. Expression of the angiogenic marker CD34 was determined by immunohistochemical analysis of CAPAN2 tumors.

in vivo and in vitro, even in the presence of oncogenic alleles of *KRAS*.

VAV1 regulates cellular proliferation and survival pathways in pancreatic cancer cells

The data above indicate that *VAV1* participates in the tumorigenic properties of pancreatic tumor cells. However, whether depletion of Vav1 was affecting tumor cell proliferation or cell survival was unclear. We therefore examined these two possibilities. Using the Vav1 shRNA vectors, we analyzed cellular proliferation. Depletion of Vav1 in all of the *VAV1*+ tumor cell lines results in a pronounced decrease in cellular proliferation as measured by either BrdU incorporation (Figure 4A) or MTS assay (Supplemental Figures S4A–S4C at http://www.cancercell.org/cgi/content/full/7/1/39/DC1/). A similar effect on cellular proliferation was observed using the shVav1-2 targeting vector (Supplemental Figure S4D). Similarly, a decrease in proliferation as

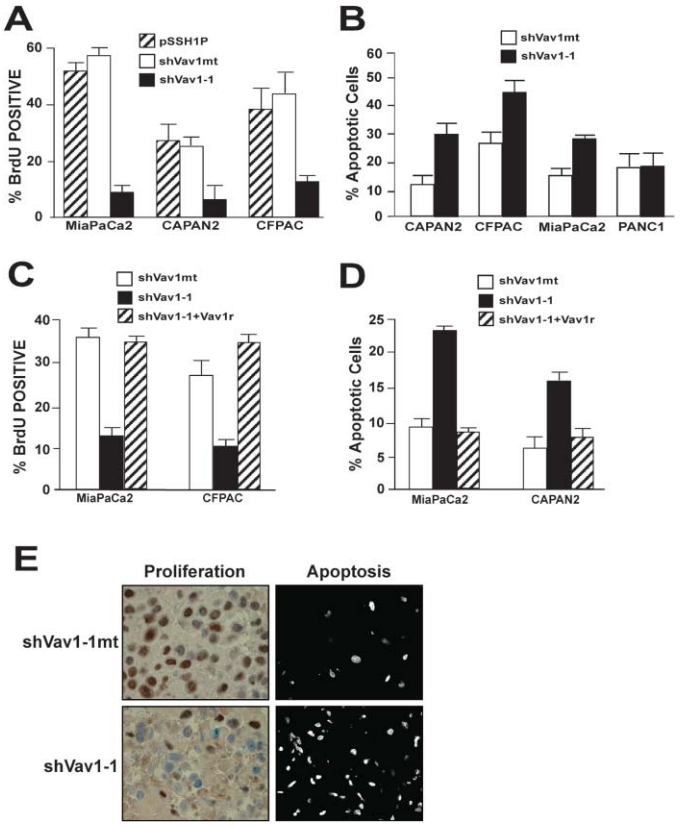

Figure 4. *VAV1* is required for pancreatic tumor cell proliferation and survival

A: The *VAV1*+ CAPAN2, CFPAC, and MiaPaCa2 pancreatic tumor cell lines were transfected with a GFP-expressing vector and the indicated shRNA vectors. BrdU incorporation at 72 hr posttransfection was performed as described in the Experimental Procedures.

B: The indicated cell lines were transfected with GFP and the shVav1mt or shVav1-1 targeting construct. GFP positive cells were scored for apoptotic nuclei using the DNA stain Hoechst 33860.

C: MiaPaCa2 and CFPAC cells were transfected with the indicated plasmids, and BrdU incorporation was measured as described in **A**.

D: MiaPaCa2 and CAPAN2 cells were transfected with the indicated plasmids and apoptosis was measured as described in **B**.

E: Cellular proliferation and apoptosis in CAPAN2 tumors were analyzed by PCNA immunodetection and TUNEL assay, respectively.

measured by the expression of the proliferation marker PCNA was observed in xenograft CAPAN2 tumors in which *VAV1* had been suppressed using RNAi (Figure 4E). Importantly, transfection of neither shVav1-1 nor shVav1-2 affected the proliferation of the *VAV1*− tumor cell lines PANC1, BXPC3, or ASPC1 (Supplemental Figure S5A). Taken together, these data suggest that Vav1 contributes to the proliferative capacity of pancreatic tumor cells. Furthermore, reintroduction of a Vav1-resistant cDNA (Vav1r) into any of the shVav1-1-treated Vav1+ tumor cells rescued the Vav1-suppressed proliferation phenotype (Figure 4C). Similarly, a dominant-negative Vav1 protein (amino acids 1–189; Vav1DN) that is predicted to inhibit Vav1 GEF activity (Abe et al., 1999; Aghazadeh et al., 2000; Movilla and Bustelo, 1999) and T cell activation (data not shown) results in a decrease in proliferation of the *VAV1*+ CAPAN2 and CFPAC tumor cells, but not the proliferation of the *VAV1*− PANC1 and BXPC3 cell lines (Supplemental Figure S5B and data not shown). This is interesting in light of the fact that all three *VAV1*+ tumor lines contain activating alleles of *KRAS*, a gene that is frequently altered in pancreatic cancer and that is associated with the early progression of the disease. Although we do not know at which stage in the development of adenocarcinoma of the pancreas *VAV1* is being misexpressed, it has been previously noted that Ras and Vav can synergize in the transformation of 3T3 cells and that dominant-negative Ras could block the ability of oncogenic Vav to promote the transformation of 3T3 cells (Katzav et al., 1995). Indeed, Vav1 acts synergistically with Kras to stimulate pancreatic tumor cell proliferation (data not shown). Moreover, a dominant-negative version of Vav1 lacking a functional SH2 domain has been observed to not only block transformation of oncogenic Vav, but also inhibit transformation induced by oncogenic Ras (Katzav et al., 1995). Therefore, it is possible that the misexpressed *VAV1* becomes an integral part of the proliferative signaling pathways in the developing cancer and that subsequent removal of Vav1 protein has detrimental effects on the growth characteristics of pancreatic tumor cells.

Vav1 target molecules have been found to be involved in cell survival (Murga et al., 2002). Since the decrease in cellular proliferation could be the result of increased apoptosis, we examined whether depletion of Vav1 altered cell survival in pancreatic tumor cells. In fact, as shown in Figure 4B, removal of Vav1 led to an increase in apoptosis of the *VAV1*+ tumor cell lines but had no effect on the *VAV1*− tumor cell lines. This phenotype could also be reversed by coexpression of the Vav1r cDNA (Figure 4D). Similarly, an increase in apoptosis was observed in xenograft CAPAN2 tumors in which Vav1 had been suppressed using RNAi (Figure 4E). Thus, *VAV1* in pancreatic cancer participates in both cellular proliferation and cell survival pathways, both of which contribute to the tumorigenic properties of pancreatic tumor cells.

VAV2, like *VAV1*, is oncogenic in a 3T3 transformation assay and can couple to multiple nonreceptor and receptor tyrosine kinases (Schuebel et al., 1996, 1998). It was therefore surprising that depletion of Vav1 or inhibition of Vav1 using a dominant-negative construct would alter the proliferative properties of pancreatic tumor cells, since the closely related isoform *VAV2* is present in all of the cell lines. Although the role of *VAV2* in tumor cell proliferation or cell survival has not been determined, the fact that these two family members have the capacity to regulate an overlapping set of Rho family GTPases indicated that these two isoforms may be differentially contributing to the phenotype of pancreatic cancer cells. Since *VAV2* is expressed in all cell lines and primary pancreatic tumors, we wanted to assess whether, like *VAV1*, *VAV2* would be necessary for tumor cell proliferation. To determine if *VAV2* participates in cellular proliferation of pancreatic cancer cells, we generated a *VAV2* shRNA vector and determined if suppression of Vav2 protein levels led to a similar decrease in cellular proliferation in either *VAV1*+ or *VAV1*− tumor cell lines (Supplemental Figures S5C and S5D at http://www.cancercell.org/cgi/content/full/7/1/39/DC1/). In contrast to what was observed for Vav1, depletion of Vav2 protein did not affect cellular proliferation of any pancreatic tumor cell lines examined (Supplemental Figure S5D). Thus, although these two family members share a significant degree of homology at the amino acid and structural level, as well as the ability to regulate an overlapping set of Rho family GTP binding proteins, only *VAV1* is contributing to the growth-promoting pathways in pancreatic cancer cells. Indeed, Vav2 pro-

tein is present in hematopoietic cells but, unlike Vav1, does not participate in the development of the T cell lineage (Turner et al., 1997) but does play a partially redundant role with *VAV1* in B cell development (Doody et al., 2001). Moreover, although the GEF activity of Vav1 and Vav2 are regulated by tyrosine phosphorylation, truncation of the first 65 amino acids of Vav1 results in increased GEF activity and 3T3 transformation, whereas the removal of the entire first 180 amino acids is required to activate the GEF activity of Vav2 and unleash its transforming potential. Thus, whether the mechanism of GEF regulation is involved in the difference observed between these two family members remains to be determined. Lastly, whether *VAV2* is involved in some other aspect of pancreatic tumor cell biology remains to be determined. In fact, *VAV2* has been suggested to participate in cell-cell adhesion through its interaction with E-cadherin binding protein p120Catenin (Fukata and Kaibuchi, 2001; Noren et al., 2001). Thus, it remains possible that *VAV2* may be involved in the migratory and metastatic potential of pancreatic tumor cells.

Vav1 requires its GEF activity to promote pancreatic cancer cell proliferation

To investigate the functional significance of *VAV1* expression in pancreatic cancer, we assessed the effect of Vav1 protein expression on the proliferative potential of *VAV1*− pancreatic tumor cell lines. Compared to control-transfected cells, expression of Vav1 protein in the *KRAS* mutant PANC1, ASPC1, or *KRAS* wild-type BXPC3 cell line resulted in increased proliferation as measured by BrdU incorporation and MTS assay (Figure 5A and data not shown), thus indicating that ectopic expression of *VAV1* is increasing their proliferative capacity. We next sought to determine if the increased proliferation of pancreatic tumor cells by Vav1 requires GEF activity; we expressed a Vav1 protein lacking GEF function in the PANC1 cell line to determine if the activation of Rho GTP binding proteins was required for Vav1-mediated proliferation of pancreatic tumor cell lines. Indeed, the GEF activity was required (Figure 5A and Supplemental Figure S6A at http://www.cancercell.org/cgi/content/full/7/1/39/DC1/). Taken together, these data suggest that GEF activity is required for Vav1-mediated proliferative responses in pancreatic tumor cells.

We next tested whether the expression of known oncogenic versions of *VAV1* can further promote tumor cell proliferation. Interestingly, the expression of either an oncogenic truncation mutant lacking the CH domain (Vav1CH−) or a mutant containing tyrosine to phenylalanine changes at all three residues within the acidic region (Vav1 3YF) did not further increase cellular proliferation compared to that observed by overexpression of wild-type *VAV1* (Figure 5A). Thus, these data indicate that the overexpression of *VAV1* in these epithelial tumor cell lines is sufficient in driving the cell proliferation. This is interesting in light of the fact that wild-type Vav1 is far less transforming in the in vitro 3T3 transformation assay than either the truncation or 3YF mutant (Katzav et al., 1991; Lopez-Lago et al., 2000; Zugaza et al., 2002). However, it is possible that Vav1 can synergize with other molecular pathways that are altered in these pancreatic cancer cell lines in order to promote tumor cell proliferation. Moreover, since neither truncation nor mutational activation of *VAV1* is found in the *VAV1*+ tumor cell lines (Supplemental Figure S1B at http://www.cancercell.org/cgi/content/full/7/1/39/DC1/ and data not shown), such mutations may not

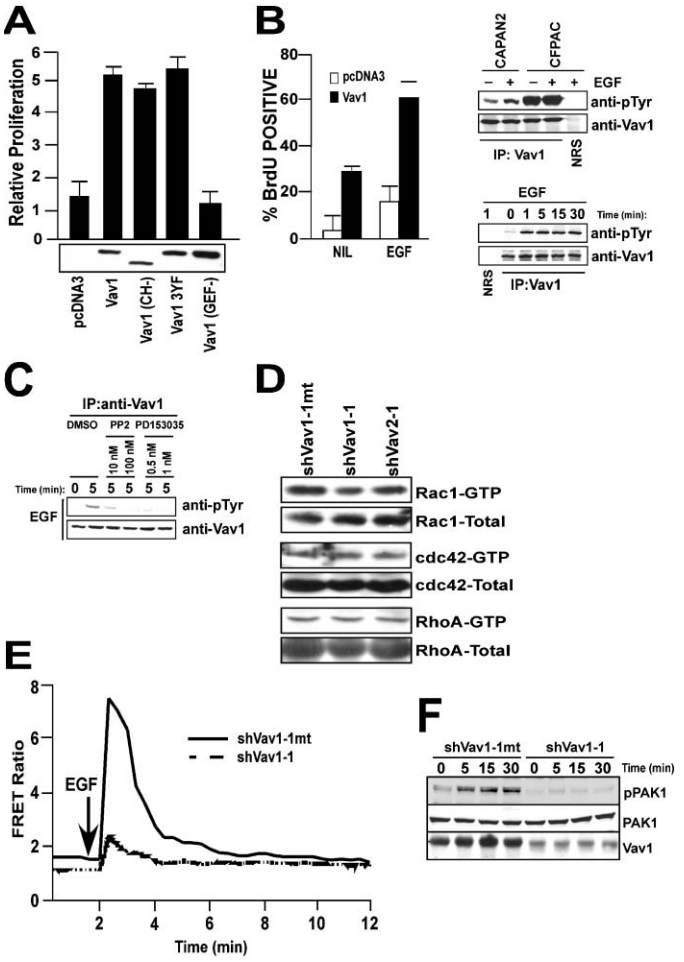

Figure 5. Characterization of the Vav1-mediated cellular proliferation pathway in pancreatic cancer cells

A: The PANC1 cell line was transfected, with either wild-type Vav1 or hyperactive mutants of Vav1 (Vav1 CH−, Vav1 3YF), and cellular proliferation was measured as described above.

B: BXPC3 cells were transfected with Vav1 expression plasmid, and BrdU incorporation was measured in the absence (NIL) or presence of EGF (200 ng/ml) stimulation as described above. Vav1 was immunoprecipitated from serum-starved CAPAN2 and CFPAC cells pre- or post-EGF stimulation for 5 min (top left) or in CAPAN2 over the indicated time course (bottom left). Bound proteins were resolved by SDS-PAGE and immunoblotted using the indicated antibodies.

C: CAPAN2 cells were serum starved and then pretreated with the indicated inhibitors for 1 hr and then stimulated for 5 min with EGF (100 ng/ml). Vav1 proteins were immunoprecipitated, and bound proteins were resolved by SDS-PAGE and immunoblotted using the indicated antibodies.

D: CAPAN2 cells were cotransfected with either a control or Vav1 or Vav2 suppression vector and expression vectors for the indicated FLAG-tagged Rho family GTPases. Forty-eight hours posttransfection, cell lysates were prepared, and GTP-loaded Rac1, Cdc42, and RhoA were pulled down using GST-Pak(PBD), GST-WASp(CRIB), and GST-Rhotekin(RBD), respectively. Bound proteins were detected by anti-FLAG immunoblotting. Total cell lysates (100 μg) were also loaded for expression control of the FLAG epitope-tagged Rho family GTPases.

E: CAPAN2 cells were transfected with either a control or Vav1 suppression vector and the pRaichu-Rac1 FRET construct. Forty-eight hours posttransfection, fluorescence was measured over time (90 s, unstimulated) using a fluorescence plate reader as described in the Experimental Procedures.

F: CAPAN2 cells were transfected with either a control or Vav1 suppression vector, and 48 hr posttransfection cells were stimulated with 100 ng/ml EGF. Cell lysates were prepared, and 100 μg of protein was resolved by SDS-PAGE and probed with the indicated antibodies.

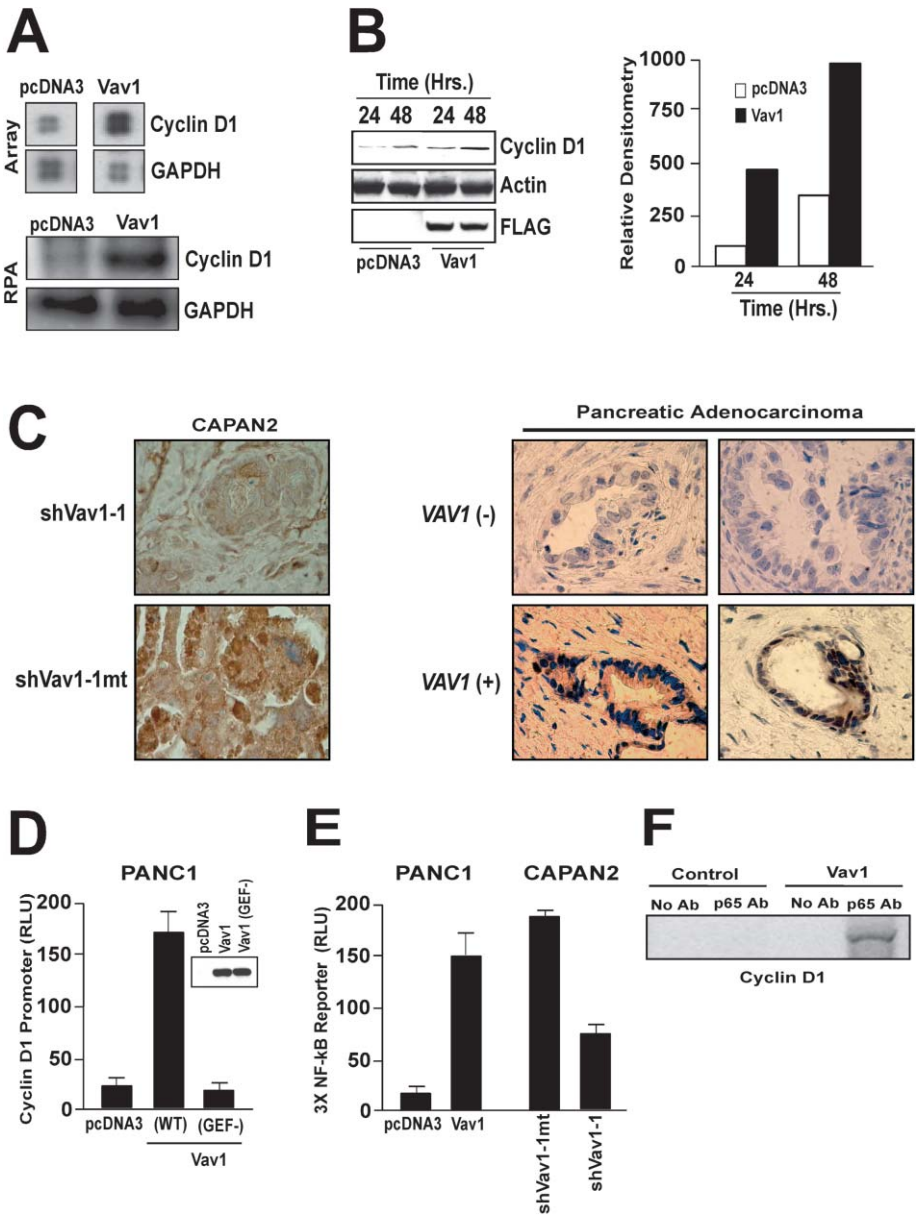

Figure 6. Vav1 increases the expression of cyclin D1 by regulating its promoter activity

A: PANC1 cell lines transfected with Vav1 display an increase in the expression of Cyclin D1 as shown by gene array analysis (top) and RNase protection assay (bottom).

B: Western blot analysis for cyclin D1 levels in whole-cell lysates from PANC1 cells transfected with Vav1 or control vector (left) and densitometry (right).

C: The level of expression of cyclin D1 was determined by immunohistochemistry in CAPAN2 tumors (left) and two Vav1+ and two Vav1− pancreatic cancer tissues (right).

D: PANC1 cells were transfected with a cyclin D1 promoter-luciferase reporter construct and either a control vector, Vav1, or the GEF-deficient Vav1 expression vector. Luciferase activity was measured 2 days posttransfection as described in the Experimental Procedures. Control Western blot shows expression of the Vav1 wild-type and mutant.

E: PANC1 cells were transfected with control vector or Vav1 expression vector and NF-κB luciferase reporter construct (left). CAPAN2 cells were transfected with a control or Vav1 suppression vector and NF-κB luciferase reporter construct (right). Luciferase activity was measured as described above.

F: PANC1 cells were transfected with a control or Vav1 expression vector and chromatin immunoprecipitation assay was performed as described in the Experimental Procedures.

be required to activate Vav1 GEF activity in these tumors. In fact, the GEF activity of Vav1 may be modulated by growth factor-induced tyrosine phosphorylation, since aberrant growth factor receptor expression and autocrine/paracrine production of ligands occur frequently in human tumors and, in particular, in pancreatic cancer (Korc, 1998). Indeed, we have found that Vav1 from serum-starved (48 hr) CAPAN2 and CFPAC cell lines is still tyrosine phosphorylated (Figure 5B). Although stimulation of serum-starved CAPAN2 cells with EGF leads to an increase in the tyrosine phosphorylation of Vav1, we are unable to observe changes in the level of tyrosine phosphorylation of Vav1 from CFPAC cell in response to EGF because of the high basal level of phosphorylation (Figure 5B). Thus, it is possible that the high levels of basal tyrosine phosphorylation observed in these cancer cells led to constitutive activation of Vav1. In fact, the GEF activity of endogenous Vav1 from CAPAN2 and CFPAC cell lines is similar to that of the oncogenic versions (data not shown).

Vav1 regulates the activation of a Rac1-Pak1 pathway in an EGF-stimulated manner

Aberrant expression of both growth factor receptors and growth factors is common during pancreatic cancer cell evolution (Korc, 1998). Indeed, Vav1 becomes rapidly tyrosine phosphorylated following the addition of EGF, insulin, or IGF (Figure 5B and data not shown). Significantly, the addition of EGF to *VAV1*-expressing cells results in increased cellular proliferation (Figure 5B). To determine if Vav1 phosphorylation through the EGF receptor requires Src or binding to tyrosine phosphorylated EGF receptor, we analyzed EGF-stimulated tyrosine phosphorylation of Vav1 from serum-starved CAPAN2 cells. As shown in Figure 5C, Vav1 underwent EGF-stimulated tyrosine phosphorylation

that was blocked by pretreatment of cells with either the Src kinase inhibitor PP2 or the EGFR inhibitor PD153035. These data suggest that tyrosine phosphorylation of Vav1 downstream of the EGFR in pancreatic cancer cells requires both Src and the EGFR.

The results described above suggest that Vav1 GEF activity is required for its ability to promote pancreatic cancer cell proliferation. Therefore, we asked which Rho GTPase family member was most affected by depletion of Vav1. Previous studies have indicated that Vav1 can act as a GEF for Rac1, Cdc42, and RhoA (Schuebel et al., 1998; Movilla and Bustelo, 1999; Abe et al., 2000; Movilla et al., 2001). As shown in Figure 5D, removal of Vav1 in the CAPAN2 cell line led to a decrease in the basal levels of Rac1-GTP but did not affect levels of GTP bound Cdc42 or RhoA. Densitometric analysis reveals a greater than 50% decrease in GTP bound Rac1; shVav1mt (100%) versus shVav1-1 (40%) versus shVav2-1 (82%), whereas GTP bound Cdc42; shVav1mt (100%) versus shVav1-1 (94%) versus shVav2-1 (91%) and GTP bound RhoA; shVav1mt (100%) versus shVav1-1 (104%) versus shVav2-1 (93%) remained largely unchanged. To determine if Vav1 regulates the levels of Rac1-GTP following EGF stimulation, we made use of a previously described Rac1 fluorescence resonance energy transfer (FRET) biosensor that can quantitatively measure Rac1 GTP binding (Itoh et al., 2002). Significantly, whereas *VAV1*+ CAPAN2 cells show an increase in FRET upon addition of EGF, Vav1-suppressed CAPAN2 cells fail to stimulate Rac1-GTP binding following EGF stimulation (Figure 5E). Moreover, consistent with the GST pull-down data shown in Figure 5D, we did not observe significant differences in the ability of Vav1-suppressed cells to regulate Cdc42 and RhoA FRET biosensors in the absence or presence of EGF stimulation (data not shown). We further characterized the downstream signaling pathways regulated by Vav1 using phospho-specific antibodies toward Rac1 target molecules. As shown in Figure 5F, depletion of Vav1 in the CAPAN2 cell line results in a defect in the basal and EGF-stimulated activation of the Rac1 effector molecule Pak1. In addition, we observed a decrease in EGF-stimulated JNK activation in the absence of Vav1, as well as a small but reproducible decrease in ERK activation (Supplemental Figure S6 at http://www.cancercell.org/cgi/content/full/7/1/39/DC1/). Taken together, these data identify Vav1 as an Src target downstream of the EGFR and reveal a role for Vav1 in the regulation of an EGF-stimulated Rac1-Pak1 pathway in pancreatic cancer cells.

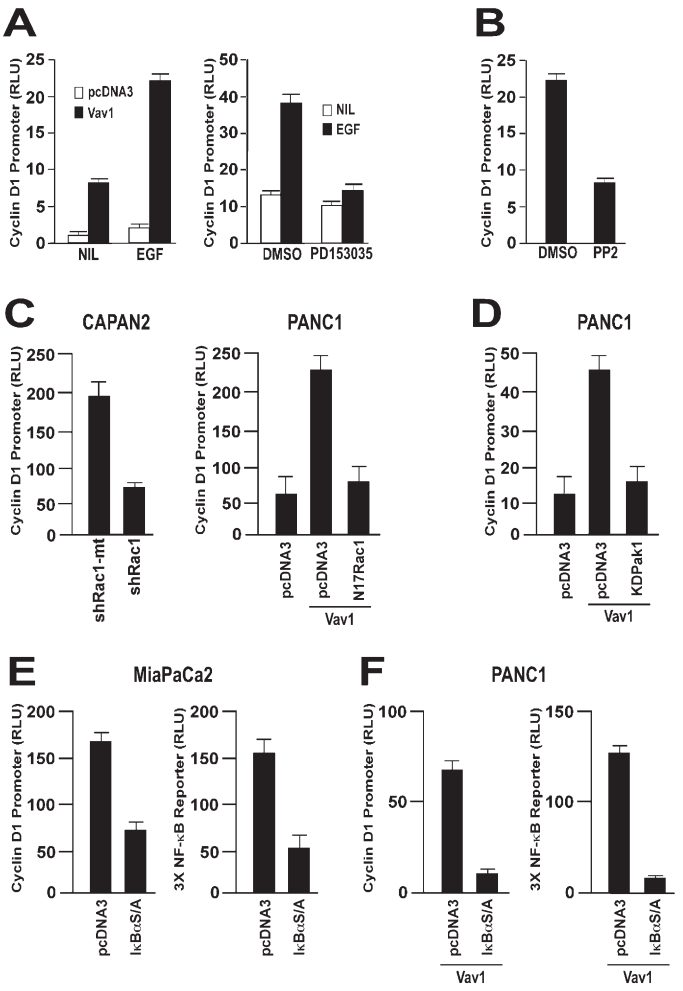

Figure 7. Vav1-mediated activation of the cyclin D1 promoter requires an intact EGF-Src-Vav1-Rac1-Pak1-NF-κB signaling pathway in pancreatic cancer cells

A–C: BXPC3 (**A**) and CAPAN2 (**B and C**) cells were transfected with the indicated vectors, and cyclin D1 promoter activity was measured in the absence (NIL) or presence of EGF (200 ng/ml). In **A** and **B**, cells were treated with 50 nm PD153035 (**A**) or 100 nm PP2 (**B**). CAPAN2 (**C**), MiaPaCa2 (**E**), and PANC1 (**C, D, and F**) cells were transfected with the indicated vectors, and cyclin D1 promoter activity was measured as described above.

Cyclin D1 is a molecular target of *VAV1* in pancreatic tumor cells

To identify downstream molecular targets of Vav1 that are involved in cell proliferation, we performed expression array and RNase protection assays for genes whose protein products are involved in regulating cellular proliferation. As determined by both methods, mRNA levels of cyclin D1 were significantly increased over the control-transfected population (Figure 6A), and this correlated with increased cyclin D1 levels in Vav1-transfected cells (Figure 6B). Similarly, *VAV1*-positive CAPAN2 tumors show increased level of cyclin D1 compared to CAPAN2 tumors where Vav1 was suppressed using RNAi (Figure 6C). Furthermore, pancreatic cancer tissues that were negative for *VAV1* demonstrate lower cyclin D1 levels compared to *VAV1*-positive specimens (Figure 6C). Consistent with a role for Vav1 GEF activity in pancreatic cancer cell proliferation, expression of Vav1 in PANC1 augmented cyclin D1 promoter activity in a GEF-dependent manner (Figure 6D). In addition, expression of Vav1 in both ASPC1 and BXPC3 led to an increase in cyclin D1 promoter activity (data not shown). Taken together, these data indicate that Vav1 can promote tumor cell proliferation in part through the upregulation of cyclin D1.

Vav1 has been shown in several cell systems to modulate gene expression through the regulation of several transcription factors, including AP-1, NFAT, and NF-κB. Indeed, overexpression of Vav1 stimulates increased activity of luciferase reporter constructs containing binding sites for these individual transcription factors (Figure 6E and data not shown). Consistent with the role of Pak1 in the activation of NF-κB, we find that kinase-dead Pak1 can inhibit basal as well as Vav1-mediated activation of NF-κB in pancreatic cancer cells (data not shown). Significantly, depletion of Vav1 by RNAi in CAPAN2 cells results

in decreased activation of the isolated NF-κB reporter (Figure 6E). Furthermore, chromatin immunoprecipitation from cells expressing Vav1 demonstrates NF-κB binding to the cyclin D1 promoter compared to control cells (Figure 6F), suggesting a role for this transcription factor in the Vav1-mediated regulation of cyclin D1 gene expression in pancreatic cancer cells.

Inhibition of Vav1 downstream targets abrogates cyclin D1 promoter activity

Recent data have demonstrated that there is a correlation between EGF, EGFR, and cyclin D1 levels in pancreatic cancer (Poch et al., 2001). Since Vav1 is a target of the EGFR and Vav1 undergoes receptor-mediated tyrosine phosphorylation, we investigated whether EGF could potentiate Vav1-mediated cyclin D1 promoter activity. As shown in Figure 7A, addition of EGF to Vav1-transfected cells potently augments cyclin D1 promoter activity. Consistent with the role of tyrosine phosphorylation regulating Vav1 activity, treatment of CAPAN2 cells with either PP2 or PD153035 abrogated basal and EGF-stimulated cyclin D1 promoter activity (Figures 7B and 7C). We next investigated if the downstream targets of Vav1 were required in the regulation of cyclin D1 promoter activity. Consistent with the notion that Vav1 is regulating Rac1 in pancreatic cancer cells, suppression of Rac1 in CAPAN2 cells leads to decreased cyclin D1 promoter activity similar to what was observed using a dominant-negative mutant of Rac1 (Figure 7C). Similarly, the kinase-dead Pak1 was able to block basal cyclin D1 promoter activity in the CAPAN2 cell line (data not shown). Furthermore, kinase-dead Pak1 was also capable of abrogating Vav1 stimulation of the cyclin D1 promoter in PANC1 cells (Figure 7D). Furthermore, abrogation of NF-κB activation by expression of the dominant-negative IκBαS/A leads to a significant inhibition of cyclin D1 promoter activity in the MiaPaCa2 (Figure 7E) and PANC1 (Figure 7F) cell lines. Taken together, these data identify a receptor-mediated Vav1 signaling pathway in pancreatic cancer cells in which EGF stimulation leads to the tyrosine phosphorylation of Vav1 in a Src-dependent manner, followed by the activation of a Rac1-Pak1-NF-κB signaling cascade driving cyclin D1 promoter activity.

Understanding the etiology of pancreatic cancer is of paramount importance, as it will contribute to our understanding of the molecular pathways that control pancreatic tumor cell proliferation and metastasis. Herein, we have shown ectopic expression of the hematopoietic-specific protein Vav1 in a cancer of epithelial origin. Significantly, we find that pancreatic cancer patients with *VAV1*-expressing tumors have a decrease in survival. Moreover, our data indicate that *VAV1* not only imparts a proliferative advantage to pancreatic tumor cells, but also plays a transforming role, similar to that of *KRAS* (Brummelkamp et al., 2002). In addition, Vav1, Kras, and EGFR signaling pathways can synergize to promote pancreatic tumor cell proliferation. The data herein suggest that *VAV1* participates in pancreatic tumorigenesis and can regulate cellular proliferation through the activation of a Vav1-Rac1-Pak1-NF-κB axis that leads to the increase in cyclin D1 and thus increased pancreatic tumor cell proliferation. The recent observation of *VAV1* expression in neuroblastomas suggests that *VAV1* may participate in human tumorigenesis in sites other than the pancreas (Betz et al., 2003; Hornstein et al., 2003). Whether *VAV1* is involved in the tumorigenic properties of this tumor type will require further investigation.

Our results uncover a novel role for the hematopoietic-specific Rho GEF *VAV1* in the regulation of a prooncogenic pathway in pancreatic tumor cells and demonstrate synergistic effects between this protein and recently established drug targets for this disease such as Kras and the EGFR (Wolff, 2001). In addition, our data would suggest that Pak1 and Src inhibitors could show clinical benefit in the treatment of pancreatic cancer. Therefore, increased knowledge of the mechanisms and signaling pathways controlled by Vav1 in this disease may provide the theoretical framework for the development of novel small molecule inhibitors to combat this deadly disease.

Experimental procedures

Reagents

Unless otherwise specified, all reagents were from Sigma (St. Louis, MO). Rabbit polyclonal antisera toward Vav1, Vav2, and Vav3 have been previously described (Billadeau et al., 2000a, 2000b; Cao et al., 2002). Antisera for phospho-specific and total protein levels of Pak1 (T199/204), JNK, and ERK1/2 were obtained from Cell Signaling Technology Inc. (Beverly, MA). Antibodies for CD34 were obtained from Becton Dickinson, p65 was obtained from Santa Cruz, and PCNA was obtained from BD Bioscience.

Immunohistochemistry

Institutional review board approval was obtained for all studies involving human specimens. Immunohistochemistry was carried out on paraffin-embedded normal and pancreatic cancer specimens using Zymed Histostain Plus as per manufacturer's protocol and is described in the Supplemental Data at http://www.cancercell.org/cgi/content/full/7/1/39/DC1/.

FISH, methylation-specific PCR, and 5-Aza TSA

Details of these techniques are described in the Supplemental Data at http://www.cancercell.org/cgi/content/full/7/1/39/DC1/.

Tumor xenografts

We injected 2.5×10^6 CAPAN2 and CFPAC pancreatic cells subcutaneously into the hind leg of a male athymic nude mice and measured the tumor weekly. We estimated tumor volume (V) from the length (l) and width (w) of the tumor using the formula: $V = (\pi/6) \times [(l + w)/2]3$. Animal experiments were carried out using protocols approved by the Animal Facility at Mayo Clinic College of Medicine.

Proliferation and apoptosis assays

These assays are described in detail in the Supplemental Data at http://www.cancercell.org/cgi/content/full/7/1/39/DC1/.

Immunoprecipitation and Western blotting

Details regarding these methods are described in the Supplemental Data at http://www.cancercell.org/cgi/content/full/7/1/39/DC1/.

Soft agar growth assay

CFPAC or MiaPaca2 cells (1×10^7) were cotransfected with a GFP expression vector (100 ng) and either the control pFRT-H1P, Vav1, or Vav1mt targeting vector as described above. In rescue experiments, a resistant version of the human Vav1 cDNA was also transfected (10 μg). Eighteen hours posttransfection GFP+ cells were sorted by FACS and 2×10^4 GFP+ cells were mixed with 4 ml of RPMI medium containing 10% serum and 0.4% low melting point (LMP) agarose. One milliliter of this mixture was subsequently placed over 1 ml of hardened RPMI medium containing 10% BCS and 1% LMP and allowed to harden at room temperature. The cells were allowed to grow for 2 weeks, after which visible colonies containing greater than 50 cells were counted.

RNase protection assay and expression array

Experimental procedures for the RNase protection assay and Genearray are described in detail in the Supplemental Data at http://www.cancercell.org/cgi/content/full/7/1/39/DC1/.

Luciferase assay

All cells were grown and transfected as indicated above. For luciferase reporter assays, 1×10^6 cells were distributed in triplicate in 24-well plates and allowed to recover for 18 hr. The cells were subsequently washed, and fresh medium containing 1% BCS was added. The cells were then left unstimulated or were stimulated with growth factor as indicated. Samples were harvested and prepared for luciferase assays according to the protocol suggested by the manufacturer (Promega, Madison, WI). All reporter assays were cotransfected with a pRL-TK reporter plasmid (Promega, Madison, WI) to control for intersample variations in transfection efficiency. In the latter case, firefly and pRL-TK-derived *Renilla* luciferase activities were measured in each sample with a Dual Luciferase Assay Kit (Promega).

Rho activation and FRET assay

CAPAN2 cells (10^7) were transfected with 30 μg of the indicated suppression vectors and 2 μg of a FLAG epitope-tagged Rho family protein expression vector, and the GTP bound Rho family proteins were determined as described in the Supplemental Data at http://www.cancercell.org/cgi/content/full/7/1/39/DC1/.

Chromatin immunoprecipitation assay

PANC1 cells were transfected with full-length Vav1 and control vector. At 36 hr posttransfection, cells were crosslinked with formaldehyde for 20 min at 25°C, harvested in SDS-lysis buffer (Upstate Biotechnology, Lake Placid, NY), and sheared to fragment DNA to ~500 bp. Samples were then immunoprecipitated using an agarose-conjugated anti-p65 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or agarose beads alone at 4°C overnight. Following immunoprecipitation, samples were washed and eluted using the Chromatin Immunoprecipitation Kit (Upstate Biotechnology) according to the manufacturer's instructions. Crosslinks were removed at 65°C for 4 hr, and immunoprecipitated DNA was purified using phenol/chloroform extraction (500 μl) and ethanol precipitation. A 200 bp region of the Cylin D1 promoter was detected in immunoprecipitated samples by PCR. PCR products were visualized by 2.5% agarose gel.

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