An integrin $\alpha_v\beta_3$–c-Src oncogenic unit promotes anchorage-independence and tumor progression

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Integrins regulate adhesion-dependent growth, survival and invasion of tumor cells. In particular, expression of integrin $\alpha_v\beta_3$ is associated with progression of a variety of human tumors. Here we reveal a previously undescribed adhesion-independent role for integrin $\alpha_v\beta_3$ in pancreatic cancer and other carcinomas. Specifically, $\alpha_v\beta_3$ expressed in carcinoma cells enhanced anchorage-independent tumor growth in vitro and increased lymph node metastases in vivo. These effects required recruitment of c-Src to the $\beta_3$ integrin cytoplasmic tail, leading to c-Src activation, Crk-associated substrate (CAS) phosphorylation and tumor cell survival that, unexpectedly, was independent of cell adhesion or focal adhesion kinase (FAK) activation. Pharmacological blockade of c-Src kinase activity or decreased expression of endogenous $\alpha_v\beta_3$ integrin or c-Src not only inhibited anchorage-independent growth but also suppressed metastasis in vivo, yet these manipulations did not affect tumor cell migration or invasion. These data define an unexpected role for an integrin as a mediator of anchorage independence, suggesting that an $\alpha_v\beta_3$-c-Src signaling module may account for the aggressive behavior of integrin $\alpha_v\beta_3$-expressing tumors in humans.

Although anchorage-independent growth is a hallmark of transformed cells, tumor growth and metastasis depend on tumor cell interactions with the extracellular matrix, mediated by the integrin family of adhesion receptors. Integrins promote a wide range of adhesion-dependent effects in tumor cells including proliferation, survival, migration or invasion and chemotherapeutic resistance attributed to activation of FAK$^2$–$^5$, which recruits other signaling molecules including c-Src$^6$, a kinase whose activity is associated with enhanced malignancy$^7$. After adhesion, c-Src phosphorylates CAS, a large adaptor protein implicated in cell invasion and survival$^8$–$^{10}$.

Integrin $\alpha_v\beta_3$ is expressed on the most aggressive tumor cells in a variety of cancers, including melanoma and carcinomas of the prostate, breast, cervix and pancreas. In melanoma, integrin $\alpha_v\beta_3$ expression initiates the transition from the benign radial growth phase to the malignant vertical growth phase$^{11,12}$. In both breast and prostate carcinomas, integrin $\alpha_v\beta_3$ mediates bone metastasis through enhanced tumor cell adhesion$^{13–16}$. Expression of integrin $\alpha_v\beta_3$ correlates with disease progression and shorter survival in individuals with cervical carcinoma$^{17}$. In pancreatic ductal adenocarcinoma, integrin $\alpha_v\beta_3$ expression occurs in approximately 58% of human tumors and is associated with increased lymph node metastasis$^{18}$.

Integrins provide context-dependent cues to both normal and transformed cells that paradoxically promote both cell survival and initiate apoptosis. Although expression of some integrins enhances malignancy, expression of others inhibits malignant progression$^{19,20}$. We recently demonstrated that, in some tumors, the expression of an unligated integrin induces apoptosis through recruitment and activation of caspase-8, a process termed integrin-mediated death (IMD)$^{21}$.

Tumors lacking caspase-8 were resistant to IMD and showed increased metastatic potential$^{22}$. Here we describe a new role for an integrin as a mediator of anchorage independence and suggest this may account for the enhanced malignancy associated with $\alpha_v\beta_3$ expression in pancreatic carcinoma and a wide array of other tumors.

**RESULTS**

Expression of $\alpha_v\beta_3$ correlates with metastatic potential

We compared integrin $\alpha_v\beta_3$ expression in multiple matched pairs of primary tumor and lymph node metastases from individuals with pancreatic or breast cancer. In pancreatic cancer specimens, cells in the primary tumor showed heterogeneous staining for integrin $\alpha_v\beta_3$; however, most of the tumor cells in the lymph nodes were integrin $\alpha_v\beta_3$ positive in six of seven individuals (Fig. 1a and Supplementary Fig. 1a,b). In breast cancer specimens, integrin $\alpha_v\beta_3$ was expressed in 28 of 50 primary tumors (56%); however, we observed expression of this integrin in 36 of 50 matched lymph node metastases (72%) (Fig. 1b). In several examples, integrin $\alpha_v\beta_3$ expression was enriched in the lymph node metastases relative to the primary tumor (Fig. 1b). These data suggest that integrin $\alpha_v\beta_3$ expression may identify the more metastatic cells within these tumors.

To directly address the role of integrin $\alpha_v\beta_3$ in tumor malignancy, we injected integrin $\alpha_v\beta_3$–positive or integrin $\alpha_v\beta_3$–negative GFP-labeled human pancreatic carcinoma cells into the pancreas of nude mice and evaluated primary tumor growth and spontaneous metastasis. Compared to FG (fast-growing) human pancreatic tumor cells, which lack $\alpha_v\beta_3$, FG-$\beta_3$ cells ectopically expressing integrin $\alpha_v\beta_3$ (Supplementary Fig. 2) showed higher primary tumor growth.

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mass at both 6 and 8 weeks after injection (Fig. 2a) and significantly enhanced spontaneous metastasis to the hepatic hilar and mesenteric lymph nodes (Fig. 2b). We confirmed lymph node metastases by anatomical location (Fig. 2c,d), GFP fluorescence (Supplementary Fig. 3a) and histological evaluation (Supplementary Fig. 3b). Of note, 25% of the mice with FG-β3 tumors developed severe ascites and wasting, emulating the morbidity associated with late stage human pancreatic carcinoma, which we did not observe in mice with FG tumors lacking αvβ3 integrin (data not shown). In support of these findings, knockdown of endogenous β3 in Panc-1 pancreatic cancer cells (Supplementary Fig. 4a) significantly inhibited metastasis to the liver hilar lymph nodes (Fig. 2e) and caused a modest decrease in primary tumor mass (Supplementary Fig. 4b). In summary, these results indicate that integrin αvβ3 expression enhances the primary tumor growth and metastasis of these carcinoma cell lines.

To discern a potential mechanism to account for these findings, we analyzed the relative level of cell proliferation, apoptosis and vascular density in FG versus FG-β3 primary tumors. In FG-β3 tumors, the number of apoptotic cells was reduced by 62% compared to control FG tumors lacking integrin αvβ3 (Fig. 2f,g), yet we could not detect any difference in proliferation (Fig. 2h) or vascular density (Supplementary Fig. 5a–c). These data indicate that αvβ3 expression is associated with increased tumor cell survival.

**αvβ3 enhances adhesion-independent activation of c-Src**

Typically, integrins initiate signaling via cell adhesion to the extracellular matrix, where they interact with immobilized matrix proteins and cluster in the plane of the membrane. This facilitates the assembly of a focal contact containing the integrin together with tyrosine kinases such as FAK or c-Src and adaptor proteins such as CAS that mediate downstream signaling, leading to a wide array of cellular activities. FG cell adhesion to fibronectin depended entirely on integrin αvβ3, whereas FG-β3 cell adhesion was mediated by either integrin αvβ1, or integrin αvβ3 (Supplementary Fig. 6a). After adhesion to fibronectin, we identified two prominent phosphoproteins of approximately 60 and 130 kDa in the FG-β3 Triton-insoluble lysate relative to the FG control (Fig. 3a). We analyzed the 60-kDa phosphoprotein by immunoblotting with an antibody directed against activated Src family kinases (SKF). We detected a considerable increase in SKF pTyr416 immunoreactivity in FG-β3 lysates (Fig. 3b) that we verified in focal contacts by immunostaining of adherent, permeabilized cells (Supplementary Fig. 6b). The 130-kDa phosphoprotein most probably represents the SKF substrates FAK (125 kDa) and CAS (130 kDa), as both showed enhanced phosphorylation in adherent FG-β3 cells (Supplementary Fig. 7a,b). Thus, whereas both αvβ1 and αvβ3 integrins mediate fibronectin adhesion of FG-β3 cells, only αvβ3 colocalizes with an activated SKF in these cells.

To determine which SKF was associated with integrin αvβ3, we examined Triton-insoluble lysates for the SKF isoforms c-Src, Yes and Fyn. This analysis identified c-Src as the only isoform associated with integrin αvβ3 expression in a subpopulation of human carcinoma cells, and its expression correlates with lymph node (LN) invasion. (a,b) Representative images of immunohistochemical staining for the integrin β3 subunit in matched pairs of primary tumors (left) and lymph node metastases (right) from individuals with pancreatic (n = 7; a) and breast (n = 50; b) cancer. Scale bars, 50 μm.
activates c-Src. To evaluate this further, we immunoprecipitated integrin αβ3 from FG-β3 cells, followed by immunoblotting for c-Src. Integrin αβ3 and c-Src formed a complex (Fig. 3c) that was abolished when we deleted the C-terminal four amino acids from the β3 cytoplasmic tail (759x) (Supplementary Fig. 8a), as previously reported for the platelet integrin αIIbβ3 (ref. 24). These results suggest that integrin αβ3 recruits c-Src in a manner that depends on the terminal four amino acids of the β3 subunit.

We further investigated the integrin αβ3-mediated activation of c-Src by analyzing the kinetics of SFK activation in response to cell adhesion. Consistent with our previous findings (Fig. 3b), expression of integrin αβ3 in either FG or Panc-1 cells increased SFK activity after adhesion (Fig. 3d). However, to our surprise, integrin αβ3 also increased SFK activation in cells maintained in suspension (Fig. 3d and Supplementary Fig. 8b). Unlike in adherent cells, SFK activation in cells maintained in suspension occurred independently of FAK activity (Supplementary Fig. 8c). These findings indicate that integrin αβ3 recruitment of c-Src may promote anchorage-independent signaling distinct from the response induced by this integrin in adherent cells, as measured by FAK activation.

αβ3 promotes anchorage independence through c-Src

Growth in anchorage-independent environments is a hallmark of tumor cell transformation and is suggested to have a role in metastasis25,26. Given our findings that αβ3 activates c-Src in nonadherent FG-β3 cells, we considered whether this might provide an anchorage-independent growth advantage in soft agar. Notably, we found that FG-β3 cells formed approximately twice as many colonies as FG cells (Fig. 4a,b), yet these cells showed no change in their growth rate when maintained in adherent culture conditions (Supplementary Fig. 9a). In fact, ligation of integrin αβ3 did not contribute to the anchorage-independent growth advantage of FG-β3 cells, as neither blockade of integrin αβ3 with the function-blocking monoclonal antibody LM609 (refs. 27,28) nor expression of a β3 D119A mutant incapable of binding ligand29 inhibited colony formation (Supplementary Figs. 9b and 10a–c). We obtained similar results after integrin αβ3 expression in the αβ3-negative MiaPaca-2 human pancreatic cell line (Supplementary Fig. 11a), whereas knockdown of endogenous β3 in Panc-1 cells significantly reduced their anchorage-independent growth (Fig. 4c). These effects were also extended to tumor cells of distinct histological origin, as integrin αβ3 expression mediated similar effects on soft agar colony formation in both breast and cervical cancer cell lines (Supplementary Fig. 12a–f). Enhanced colony formation was associated with increased survival of integrin αβ3-expressing cells (Fig. 4d), as observed in vivo (Fig. 2f–h), and not from increased proliferation (Supplementary Fig. 11c). In contrast, FG and FG-β3 cells attached to fibronectin showed identical levels of apoptosis in response to either gemcitabine (Fig. 4e) or a Fas-specific antibody (Fig. 4f), suggesting that integrin αβ3 provides a specific survival benefit under anchorage-independent growth conditions.

To investigate whether integrin αβ3-mediated, anchorage-independent survival was c-Src dependent, we placed cells in suspension culture in the presence or absence of dasatinib, a clinically approved SFK inhibitor. Treatment of FG-β3 cells with dasatinib reduced colony formation of FG-β3 cells to the level observed for FG cells (Fig. 5a), suggesting that c-Src activity has a role in the αβ3 anchorage-independent growth advantage of FG-β3 cells. Notably, dasatinib had no effect on FG cell anchorage-independent growth, despite markedly inhibiting SFK activity in these cells (Supplementary Fig. 13). We also observed a similar result in MP-2 cells (Supplementary

Figure 3 Integrin αβ3 promotes anchorage-independent activation of c-Src. (a) Western blot detecting phosphophorysotyrosine (PY) in Triton-insoluble lysates from FG and FG-β3 cells adherent to fibronectin. IB, immunoblot. (b) Immunoblot detecting pY416 SFK, c-Src, Yes and Fyn showing enrichment of pY416 SFK and c-Src in the FG-β3 Triton-insoluble lysate. (c) Co-immunoprecipitation of c-Src with αβ3 from the Triton-insoluble fraction of FG-β3 cells plated on the αβ3 substrate vitronectin. IB, immunoprecipitation. (d) Western blots of whole-cell lysates from suspended and fibronectin-adherent FG and FG-β3 cells, showing increased levels of pY416 SFK in suspended FG-β3 cells (asterisk). A similar comparison in Panc-1 cells expressing nonsilencing and β3 shRNA showed decreased pY416 SFK in both suspended and adherent β3 knockdown cells. n = 3 independent experiments.
Fig. 5 Integrin α3β3 induces anchorage-independence through c-Src phosphorylation of CAS. (a) Measurement of FG and FG-β3 cell colony formation in the presence of vehicle (DMSO) or various doses of the SFK inhibitor dasatinib. n = 3 independent experiments. **P = 0.0044. (b) Examination of the effect of c-Src knockdown (c-src sh) on FG and FG-β3 growth in soft agar relative to control cells expressing the nonsilencing shRNA (n-s). n = 3 independent experiments. *P < 0.05. (c) Western blot detecting pY165 CAS, pS473 Akt and pThr202/pTyr204 ERK in suspended FG and FG-β3 cells with or without c-Src knockdown. n = 3 independent experiments. (d) Immunoblot for CAS in FG and FG-β3 cells transiently transfected with control or CAS siRNA oligonucleotides, showing efficient CAS knockdown by the siRNA. Hsp60 is shown as a loading control. (e) Soft agar colony formation with control and CAS-knockdown FG and FG-β3 cells from the same transfection as shown for (d). A representative experiment is shown. n = 3 independent experiments. *P < 0.05. (f) Assessment of colony formation in soft agar with FG and FG-β3 cells stably expressing either vector alone or a CAS mutant with all fifteen substrate domain tyrosines mutated to phenylalanines (CAS Y1–15F). A representative experiment is shown. n = 3 independent experiments. **P < 0.05. Data are means ± s.e.m.

Fig. 11b). Consistent with the lack of FAK activation in suspended FG and FG-β3 cells, treatment with either of two different FAK inhibitors failed to lower colony number in either cell type (Supplementary Fig. 14a,b). In support of this pharmacological data, knockdown of c-Src in FG-β3 cells (Supplementary Fig. 15) specifically inhibited integrin α3β3–mediated colony formation to the level observed in FG cells (Fig. 5b). Next, we considered whether the c-Src–α3β3 complex in FG-β3 cells might have a role in integrin α3β3–mediated, anchorage-independent colony formation. To test this, we expressed a truncation mutant of integrin β3 (759x) that fails to interact with c-Src (Supplementary Fig. 8a). Cells expressing this mutant failed to enhance soft agar colony formation compared to cells expressing the wild-type receptor (Supplementary Fig. 16).

In adherent cells, FAK localizes to integrin focal contacts where it recruits and activates c-Src, resulting in phosphorylation of c-Src substrates, including CAS, and promotion of cell proliferation and migration. Although these effects occur in FG-β3 cells attached to fibronectin (Supplementary Fig. 7a,b) or vitronectin (data not shown), FG-β3 cells maintained in suspension show increased CAS phosphorylation (Fig. 5c) in the absence of FAK activation (Supplementary Fig. 8c). However, CAS phosphorylation under these conditions was c-Src dependent, as it was abolished by knockdown of c-Src (Fig. 5c). FG-β3 cells in suspension also exhibited increased phosphorylation of Akt and ERK in a manner independent of c-Src (Fig. 5c). These findings indicate that α3β3 expression activates both c-Src–dependent and c-Src–independent signaling pathways, yet only the c-Src–dependent pathway leads to increased anchorage-independent growth and CAS phosphorylation.

We next considered whether CAS was required for integrin α3β3–mediated colony formation in soft agar. Knockdown of CAS with small interfering RNA (siRNA) oligonucleotides specifically lowered colony number in FG-β3 cells compared to FG cells (Fig. 5d,e). We further considered whether c-Src–dependent phosphorylation of CAS was required for integrin α3β3–mediated colony formation. To test this hypothesis, we expressed a dominant-negative mutant version of CAS in both FG and FG-β3 cells in which all fifteen c-Src tyrosine phosphorylation sites within the CAS substrate domain were mutated to phenylalanines (CAS Y1–15F) (Supplementary Fig. 18a). Cells expressing this mutant showed no such increase in colony formation (Fig. 5f). These findings indicate that integrin α3β3–mediated activation of c-Src promotes increased anchorage-independent growth on the basis of its capacity to phosphorylate the CAS substrate domain.

c-Src mediates α3β3 tumor cell survival and metastasis

to determine whether integrin α3β3–mediated c-Src activation can lead to increased tumor malignancy, we injected FG and FG-β3 cells expressing nonsilencing or c-Src short hairpin RNAs (shRNAs) into the pancreases of nude mice and analyzed tumor growth and metastasis after 8 weeks. Although c-Src knockdown reduced primary tumor mass in both FG and FG-β3 cells (Fig. 6a), it specifically blocked the metastatic advantage of the FG-β3 cells (Fig. 6b). Mechanistically, this seems to be due to effects on c-Src–dependent cell survival (Fig. 6c) but not proliferation (Fig. 6d). The c-Src binding site on the β3 tail is crucial for the in vivo effects of integrin α3β3 expression, as FG-759x cells formed tumors that were only 3% the mass of FG-β3 cells (Supplementary Fig. 18a,b). These data indicate that the in vivo effects of integrin α3β3 expression require c-Src and its interaction with the β3 cytoplasmic tail.

To validate the therapeutic relevance of our findings, we compared the SFK and Abl inhibitor dasatinib with the Abl inhibitor imatinib for their ability to decrease tumor burden and metastasis of α3β3–expressing tumors. We established orthotopically injected FG-β3 tumor cells for 2 weeks before dosing with vehicle (twice daily), 30 mg per kg body weight dasatinib (twice daily) or 30 mg per kg body weight imatinib (once a day) by oral gavage for 4 weeks. Dasatinib treatment inhibited primary tumor mass relative to the vehicle control, whereas imatinib had no effect (Fig. 6e). Although the incidence of metastasis to the hepatic hilar lymph node was relatively unchanged (incidence of metastasis: dasatinib, 7 of 12 mice; vehicle, 9 of 12 mice; imatinib, 10 of 12 mice), the mass of the metastatic lesions was significantly reduced (Fig. 6f,g).

Previous studies have linked integrin α3β3 expression or c-Src activation with increased tumor cell migration and invasion. 27,31 Although
integron \( \alpha_3 \beta_3 \)-bearing cells were more migratory on both vitronectin and fibronectin (Supplementary Fig. 19a–c), integrin \( \alpha_3 \beta_3 \) failed to potentiate invasion of FG cells into Matrigel (data not shown). Knockdown or pharmacological inhibition of c-Src did not suppress the migration of either FG or FG-\( \beta_3 \) cells (Supplementary Fig. 19a–c), despite inhibiting both anchorage-independent growth and metastasis (Figs. 5b and 6b). These findings indicate that integrin \( \alpha_3 \beta_3 \) recruitment and activation of c-Src increases the malignant properties of pancreatic tumor cells without influencing their ability to migrate.

**DISCUSSION**

Anchorage independence is a hallmark of transformed cells and is suggested to have a role in the growth of solid tumors and survival of circulating tumor cells. However, tumor cell adhesion and migration on extracellular matrix proteins, mediated by members of the integrin family, are linked to tumor cell growth and malignancy. Once ligated, integrins activate FAK and other downstream signaling molecules, leading to anchorage-dependent survival and proliferation. However, unligated integrins can negatively influence the malignant properties of tumor cells through activation of apoptotic pathways inducing a form of death known as IMD. The tumor cells studied here have developed mechanisms to escape IMD that contribute to their metastatic behavior.

Integrin \( \alpha_3 \beta_3 \) expression is linked to metastasis in several cancers, including melanoma, as well as breast, prostate, cervical and pancreatic carcinomas, and enhances tumor cell migration, survival and increased growth factor release. We present the unexpected result that integrin \( \alpha_3 \beta_3 \) contributes to tumor progression and metastatic potential by enhancing anchorage-independent growth. This effect requires integrin \( \alpha_3 \beta_3 \) recruitment and activation of c-Src in a manner that is independent of tumor cell adhesion or the activation of FAK. Of note, integrin \( \alpha_3 \beta_3 \) expression increased colony formation and cell survival in soft agar, even in the presence of a function-blocking antibody that prevents ligation to either soluble ligands. In addition, expression of a mutant integrin incapable of binding ligand also showed increased anchorage independence. We observed a similar increase in cell survival in integrin \( \alpha_3 \beta_3 \)-bearing pancreatic tumors grown in mice, suggesting that integrin \( \alpha_3 \beta_3 \)-mediated survival contributes to both anchorage independence in vitro and tumor malignancy in vivo. Accordingly, knockdown of endogenous integrin \( \beta_3 \) decreased the anchorage independence and metastasis of pancreatic cancer cells.

To our surprise, we found that integrin \( \alpha_3 \beta_3 \) promotes c-Src-dependent, but FAK-independent, phosphorylation of the CAS substrate domain in nonadherent cells. CAS phosphorylation promotes adhesion-mediated cell survival through FAK and c-Src recruitment to integrin containing focal contacts. In fibroblasts transformed with the v-Src or v-Crk oncogenes, CAS forms complexes with these molecules in a phosphorylation-dependent manner, and deletion of CAS prevents v-Src–mediated transformation, implicating CAS in oncosogenesis. We show that knockdown of CAS or expression of a nonphosphorylatable form of CAS abolished the \( \alpha_3 \beta_3 \)-c-Src–mediated colony formation in soft agar.

Anchorage independence and tumor progression commonly result from oncogene expression. For example, the v-Src oncoprotein potently stimulates anchorage-independent growth in fibroblasts, and v-Src is associated with enhanced cell invasion. Expression of an activated mutant of c-Src together with integrin \( \alpha_3 \beta_3 \) promotes the transformation of a mouse pseudoepithelial cell line, suggesting cooperativity between mutually activated Src and \( \alpha_3 \beta_3 \) integrin. However, in some circumstances, normal cellular derivatives of oncogenes, such as c-Src, also contribute to tumor progression by stimulating cell migration and invasion. Here we define a previously undescribed integrin-mediated pathway leading to activation of c-Src, promoting increased anchorage independence and tumor cell malignancy that does not affect cell migration.

Previous studies have shown that the platelet integrin \( \alpha_{\text{IIb}} \beta_3 \) can recruit and activate c-Src in a manner that depends on the C-terminal portion of \( \beta_3 \) cytoplasmic tail. We show that \( \alpha_3 \beta_3 \)-expressing tumor cells also recruit and activate c-Src, and, similar to the platelet studies,
a β3 truncation mutant (759x) prevented c-Src recruitment to the integrin. Cells expressing this truncation mutant failed to increase anchorage-independent growth in vitro or metastasis in vivo. Although c-Src associates with integrin αβ3, we could not detect c-Src recruitment to other integrins in these cells, suggesting that the β3 integrin is unique in this regard. Thus, we conclude that unligated integrin αβ3 and its ability to recruit c-Src contribute to the malignant properties of pancreatic cancer, suggesting that αβ3-c-Src can function as an oncogenic unit, thereby contributing to tumor malignancy.

Expression of integrin αβ3 is associated with the metastatic potential of several cancers. Although antagonists of integrin αβ3 have proven efficacious as angiogenesis inhibitors in mouse tumor models and are now in phase 3 clinical trials in patients with glioblastoma, our studies suggest that direct inhibition of αvβ3 ligation on tumor cells may provide limited clinical benefit, given that integrin αβ3 activates c-Src in a ligand-independent manner. As such, we define a previously undescribed oncogenic signaling module comprised of unligated integrin αβ3 and c-Src that occurs in a subset of tumors resistant to IMD. Furthermore, our study shows that dasatinib, a clinically approved SFK inhibitor, or c-Src knockdown not only blocked integrin αβ3-mediated, anchorage-independent growth of pancreatic cancer cells in vitro but also suppressed their metastatic properties in vivo. This suggests that c-Src kinase inhibition may represent a therapeutic approach for those highly malignant tumors known to express integrin αβ3.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/

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AUTHOR CONTRIBUTIONS

J.S.D. designed the project, performed most of the experiments, analyzed the data and wrote the manuscript. L.A.B. helped design and conduct the orthotopic tumor experiments. D.J.S. designed and conducted the dasatinib treatment study. M.H. initiated and performed the CAS experiments, whereas S.K.L. planned, conducted and analyzed the experiments involving the Src-β3 interaction. D.T. analyzed and interpreted the immunohistochemistry and histology experiments. S.I.S. helped conceive of the study and analyzed the data. D.A.C. initiated the study, analyzed the data, supervised the overall project and wrote the manuscript.

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ONLINE METHODS

Immunohistochemistry. We cut 8-µm sections from formalin-fixed, paraffin-embedded primary tumor specimens from 18 human subjects diagnosed with pancreatic ductal adenocarcinoma (seven with matching lymph node metastases). This study was approved by the UCSD Human Research Protections Program, and informed consent was obtained from all subjects. We also stained a breast cancer tissue microarray containing 50 matched pairs of primary tumors and lymph node metastases (BR1004; BioMax). We deparaffinized and digested the sections with protease K (Invitrogen) for 15 min at 25 °C, before quenching with 0.3% H2O2 and 0.3% normal goat serum. After washing, we blocked the sections in normal goat serum and probed with 1-in-100–diluted primary antibody overnight at 4 °C. We then incubated the sections for 45 min with a biotinylated secondary antibody (1 in 2,000) followed by 30 min in Vectastain Elite ABC Reagent (Vector Labs). Staining was performed with diaminobenzidine substrate (Vector Labs) for 1–2 min before counterstaining with hematoxylin and mounting.

Orthotopic pancreatic tumors. We generated tumors by injection of GFP-labeled human pancreatic carcinoma cells (1 × 106 tumor cells in 50 µl of sterile PBS) into the tails of the pancreases of 6- to 8-week-old male nude mice (purchased from the UCSD Animal Care Program nude mouse colony). See Supplementary Methods for details regarding the generation of cell lines. After 6 or 8 weeks, we resected both the primary tumors and the hepatic hilar lymph nodes. We determined primary tumor mass by measuring the wet weight of the resected tumors. We reported metastasis as the incidence of GFP-expressing cells present in the resected lymph nodes. For the dasatinib treatment experiment, we injected 36 mice with GFP-labeled FG-β3 cells and randomized them into three groups of twelve. We allowed tumors to establish for 2 weeks before beginning dosing. We dosed the mice by oral gavage with the citric acid vehicle (twice daily), 30 mg per kg body weight dasatinib (twice daily) (Chemietek) or 50 mg per kg body weight imatinib (once a day) (Chemietek) for 4 weeks before killing the mice and measuring the mass of the primary tumors and hepatic hilar lymph nodes. All research was conducted under protocols approved by the University of California–San Diego Animal Subjects Committee and is in accordance with the guidelines set forth in the US National Institutes of Health Guide for the Care and Use of Laboratory Animals.

In vivo apoptosis and proliferation. We analyzed both apoptosis and proliferation on optimal cutting temperature medium (Tissue Tek)–embedded frozen primary tumor sections. We assessed apoptosis in vivo by TUNEL staining with the ApopTag Red kit (Millipore). We examined proliferation by immunofluorescent staining for Ki-67 using the manufacturer’s instructions (Abcam). We measured both TUNEL and Ki-67–positive cells by TUNEL staining with the ApopTag Red kit (Millipore) and with hematoxylin and mounting. For Ki-67 we considered a positive interaction between the drug and the cell line. For all analyses, we evaluated statistics by Chi-square analysis. We evaluated colony formation in three groups of twelve. For dasatinib treatment experiments, we grew colonies in vehicle (DMSO) or 50 nM, 250 nM or 1 µM dasatinib diluted in DMSO. We replaced the medium with fresh inhibitor every other day. To knockdown CAS, we transfected 5 × 106 FG or FG-β3 cells with 250 nM of control or CAS-specific siRNA oligonucleotides (Qiagen) in 100 µl of Nucleofector V (Amaxa). We embedded the transfected cells in soft agar 48 h after transfection.

Suspension viability. To directly assay for anchorage-independent survival and proliferation, we cultured 1 × 106 FG or FG-β3 cells in suspension on 1% agar-coated wells in DMEM and 10% FBS for 24 and 48 h before trypsinizing, staining with trypan blue and counting viable and nonviable cells on a hemocytometer.

Statistical analyses. All data, except the metastasis experiments, are presented as the means ± s.e.m., and we evaluated statistical differences by Student’s t test. For metastasis, bars represent the incidence as a percentage of total mice, and we evaluated statistics by Chi-square analysis. We evaluated colony formation in the presence of dasatinib by two-way repeated measure analysis of variance, to identify a positive interaction between the drug and the cell line. For all analyses, we considered P < 0.05 to be statistically significant.

Additional methods. Additional methodology is described in the Supplementary Methods.

Triton-soluble and Triton-insoluble lysates. To isolate focal adhesions, we allowed serum-starved FG and FG-β3 cells to specifically adhere and spread for 2 h on dishes coated with 5 µg ml−1 fibronectin (Sigma). We gently washed away nonadherent cells with PBS and lysed the remaining adherent cells in Triton lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 50 mM NaF, 1× protease inhibitor cocktail (Roche), 2 mM phenylmethylsulphonyl fluoride and 2 mM sodium orthovanadate) to generate the Triton-soluble lysate. We prepared the Triton-insoluble lysate by washing the lysed cells twice with ice-cold PBS before adding RIPA lysis buffer (100 mM Tris pH 7.5, 150 mM NaCl, 0.1% deoxycholate, 0.1% SDS, 50 mM NaF, 1× protease inhibitor cocktail (Roche), 2 mM phenylmethylsulphonyl fluoride and 2 mM sodium orthovanadate) and concentrating the lysate to a minimal volume.

Soft agar assays. We suspended cells in 0.3% agar diluted in complete medium (DMEM with 4.5 g l−1 glucose (Mediatech) supplemented with 2 mM L-glutamine, 1% penicillin-streptomycin-fungizone, and 10% fetal bovine serum and cultured them on a bottom layer of 1% agar in complete medium in 48- or 24-well dishes. We then added additional medium and cultured cells for 7–10 d before counting colonies consisting of at least five cells from 10× fields or whole wells. For dasatinib treatment experiments, we grew colonies in vehicle (DMSO) or 50 nM, 250 nM or 1 µM dasatinib diluted in DMSO. We replaced the medium with fresh inhibitor every other day. To knockdown CAS, we transfected 5 × 106 FG or FG-β3 cells with 250 nM of control or CAS-specific siRNA oligonucleotides (Qiagen) in 100 µl of Nucleofector V (Amaxa). We embedded the transfected cells in soft agar 48 h after transfection.

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Statistical analyses. All data, except the metastasis experiments, are presented as the means ± s.e.m., and we evaluated statistical differences by Student’s t test. For metastasis, bars represent the incidence as a percentage of total mice, and we evaluated statistics by Chi-square analysis. We evaluated colony formation in the presence of dasatinib by two-way repeated measure analysis of variance, to identify a positive interaction between the drug and the cell line. For all analyses, we considered P < 0.05 to be statistically significant.

Additional methods. Additional methodology is described in the Supplementary Methods.

Soft agar assays. We suspended cells in 0.3% agar diluted in complete medium (DMEM with 4.5 g l−1 glucose (Mediatech) supplemented with 2 mM L-glutamine, 1% penicillin-streptomycin-fungizone, and 10% fetal bovine serum and cultured them on a bottom layer of 1% agar in complete medium in 48- or 24-well dishes. We then added additional medium and cultured cells for 7–10 d before counting colonies consisting of at least five cells from 10× fields or whole wells. For dasatinib treatment experiments, we grew colonies in vehicle (DMSO) or 50 nM, 250 nM or 1 µM dasatinib diluted in DMSO. We replaced the medium with fresh inhibitor every other day. To knockdown CAS, we transfected 5 × 106 FG or FG-β3 cells with 250 nM of control or CAS-specific siRNA oligonucleotides (Qiagen) in 100 µl of Nucleofector V (Amaxa). We embedded the transfected cells in soft agar 48 h after transfection.