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High-Throughput Tyrosine Kinase Activity Profiling Identifies FAK as a Candidate Therapeutic Target in Ewing Sarcoma

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Abstract

Limited progress has been made in the treatment of advanced-stage pediatric solid tumors despite the accelerated pace of cancer discovery over the last decade. Tyrosine kinase inhibition is one tractable therapeutic modality for treating human malignancy. However, little is known about the kinases critical to the development or maintenance of many pediatric solid tumors such as Ewing sarcoma. Using a fluorescent, bead-based technology to profile activated tyrosine kinases, we identified focal adhesion kinase (FAK, PTK2) as a candidate target in Ewing sarcoma. FAK is a tyrosine kinase critical for cellular adhesion, growth, and survival. As such, it is a compelling target for cancer-based therapy. In this study, we have shown that FAK is highly phosphorylated in primary Ewing sarcoma tumor samples and that downregulation of FAK by short hairpin RNA and treatment with a FAK-selective kinase inhibitor, PF-562271, impaired growth and colony formation in Ewing sarcoma cell lines. Moreover, treatment of Ewing sarcoma cell lines with PF-562271 induced apoptosis and led to downregulation of AKT/mTOR and CAS activity. Finally, we showed that small-molecule inhibition of FAK attenuated Ewing sarcoma tumor growth *in vivo*. With FAK inhibitors currently in early-phase clinical trials for adult malignancies, these findings may bear immediate relevance to patients with Ewing sarcoma. *Cancer Res; 73(9); 2873–83. ©2013 AACR.*

Introduction

Pediatric solid tumors are frequently associated with aberrant activation of transcription factors or loss of tumor suppressor genes. Neither of these oncogenic mechanisms, however, is readily druggable by traditional pharmacologic approaches. Moreover, the sequencing of pediatric cancer genomes is revealing remarkable stability with surprisingly few additional genetic mechanisms of oncogenesis in these diseases (1, 2). Thus, the search for new pediatric cancer therapeutic targets may need to expand beyond next-generation DNA sequencing.

Targeting aberrantly activated tyrosine kinases is one treatment approach that has had recent success in several types of adult cancers. Selective kinase inhibitor therapy has transformed the care of patients with BCR/ABL-positive chronic myelogenous leukemia (CML; ref. 3), EGF receptor (EGFR)-mutated lung cancer (4), and c-KIT–mutated gastrointestinal stromal tumors (GIST; ref. 5). Over the last year, the B-RAF inhibitor vemurafenib has been approved for patients with B-RAF^{V600E} mutant metastatic or inoperable melanoma, and the ALK inhibitor crizotinib was approved for patients with locally advanced or metastatic ALK–positive non–small cell lung cancer (NSCLC). In pediatric solid tumors, clinical trials testing crizotinib for patients with ALK mutations are ongoing (http://clinicaltrials.gov/; NCT00939770). However, few genetically altered kinase targets in pediatric solid tumors have been identified despite emerging data suggesting that activated kinases may play an important role in the development and maintenance of these diseases. For example, in the pediatric solid tumor Ewing sarcoma, insulin-like growth factor-1 receptor (IGF1R) is activated but not mutated, and clinical trials testing IGF1R-targeting antibodies induced clinical responses in a subset of patients with Ewing sarcoma (6, 7). More recently, high levels of spleen tyrosine kinase (SYK) expression were reported as a result of epigenetic deregulation in the pediatric retinal cancer retinoblastoma. SYK targeting with a small-molecule inhibitor induced retinoblastoma cell death *in vitro* and *in vivo* (2).

In this study, we chose to use a screening modality that could rapidly identify highly activated kinases, agnostic to mechanism, and targetable by available drugs. Therefore, we

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leveraged a recently described, bead-based tyrosine kinase activity assay that profiles the phosphorylation of a large panel of tyrosine kinases, many of which have available inhibitors (8). We focused on Ewing sarcoma, the second most common bone tumor diagnosed in children, where survival remains poor for patients with metastatic and recurrent disease despite intensive regimens (9, 10). New therapeutic approaches are needed to improve the survival of patients with this disease. Using kinase activity profiling, we evaluated the tyrosine kinase landscape in Ewing sarcoma cell lines and identified focal adhesion kinase (FAK/PTK2) as an actionable, highly activated tyrosine kinase in this disease.

Materials and Methods

Cell culture

A673, EW8, SKNEP, EWS834, and RDES cells were grown in Dulbeccos' Modified Eagle's Media (DMEM; Mediatech) with 10% FBS (Sigma-Aldrich). A673 medium was supplemented with 1 mmol/L sodium pyruvate (Invitrogen). TC71 and TC32 were grown in RPMI (Mediatech) with 10% FBS and TTC466, EWS502, and CADO-ES-1 with 15% FBS. Cell lines were provided by Dr. Todd Golub (Broad Institute, Cambridge, MA) except for EWS502 and EWS834, which were a gift from Dr. Jonathan Fletcher (Brigham and Women's Hospital, Boston, MA). All cell lines have unique genotypes and EWS/FLI and EWS/ERG rearrangements were confirmed by RNA sequencing.

Protein extraction and immunoblotting

Whole-cell lysates were extracted in 1× Cell Lysis Buffer (Cell Signaling) supplemented with EDTA-free protease inhibitors and PhosSTOP phosphatase inhibitors (Roche). Western immunoblotting was conducted using standard techniques. Primary antibodies against total FAK, phospho-PYK2 (Y402), total mTOR, phospho-mTOR (S2448), total S6, phospho-S6 (S240/244), total AKT, phospho-AKT (S473), PARP, total ERK, phospho-ERK (T202/Y204), phospho-CAS (Y410), and total SRC were obtained from Cell Signaling. Phospho-FAK (Y397) and total CAS antibodies were from BD Transduction Laboratories, anti-GAPDH from Santa Cruz, anti-vinculin and phospho-SRC (Y416) from Abcam, total PYK2 from Millipore, and anti-actin from Thermo Fisher Scientific.

High-throughput kinase activity profiling

A Luminex immunosandwich assay was conducted on 6 Ewing sarcoma cell lines (EWS502, TC71, TC32, A673, SKNEP, and EW8) and 293FT cells, as previously described (8). Briefly, whole-cell lysates from each cell line were quantified, and equal concentrations of protein were incubated overnight at 4°C with a mixture of 87 validated antibody-coupled Luminex beads (probes) specific for 62 tyrosine kinases. The mixture was then washed and incubated with biotin-labeled 4G10 antibody (Millipore) for 30 minutes at room temperature, washed, and then incubated with 4 μg/mL of SAPE (Molecular Probes) for 10 minutes at room temperature. The conjugates were washed 2 additional times and analyzed on a FlexMAP 3D (Luminex) with xPONENT software (version 4.0; Luminex) to determine the median fluorescent intensity (MFI).

Immunohistochemistry

Fifteen Ewing sarcoma tumor cores were obtained through Institutional Review Board–approved discarded tissue protocols from Massachusetts General Hospital and Children's Hospital Boston. A673 cell lines with and without FAK-directed short hairpin RNA (shRNA) were used for controls. Tumors and controls were examined for phospho-FAK (Y397) protein expression using standard immunohistochemical techniques. A hematoxylin and eosin (H&E)-stained slide was also prepared for each tumor to define the tumor margins. Phospho-FAK expression was scored on a semiquantitative scale based on the percentage of positive staining tumor cells (11).

Lentivirus production and lentiviral transduction

Lentivirus was produced by transfecting HEK-293T cells with the appropriate pLKO.1 lentiviral vector and packaging plasmids (pCMV8.9 and pCMV-VSVG) according to the FuGENE 6 (Roche) protocol. Plasmids were obtained from The RNAi Consortium (Broad Institute). For lentiviral transduction, Ewing sarcoma cells were incubated with 2 mL of virus and 8 μg/mL of polybrene (Sigma-Aldrich) for 2 hours. Cells were selected in puromycin (Sigma-Aldrich) 48 hours posttransduction. shRNA target sequences are listed in Supplementary Table S1.

Small-molecule treatment *in vitro*

Ewing sarcoma cells were plated in 10-cm dishes, allowed to adhere for 24 hours, and then treated with PF-562271 (FAK/PYK2 inhibitor, Haoyuan Chemexpress Co., Ltd.), PD0325901 (MEK inhibitor, Selleck Chemicals), or dasatinib (SRC, BCR/ABL, c-Kit inhibitor, and others, ChemieTek).

Cell viability

ATP content was measured as a surrogate for cell number using the CellTiter-Glo Luminescent Cell Viability Assay (Promega). Luminescence readings were obtained using the FLUOstar Omega microplate reader (BMG Labtech). For experiments with small-molecule treatment, 1.25×10^3 Ewing sarcoma cells were seeded in each well and treated with a range of concentrations. IC_{50} values were calculated from ATP measurements obtained after 3 days of treatment using log-transformed, normalized data in GraphPad Prism 5.0 (GraphPad Software, Inc.). Cell lines were also treated with compound in 6-cm dishes, trypsinized, and counted by light microscopy using trypan blue exclusion. For experiments using shRNA-transduced cells, 1.25×10^3 cells were seeded per well into 384-well plates on day 3 posttransduction. ATP content was measured on days 3, 6, and 8 posttransduction.

Colony formation in methylcellulose matrix

Approximately 3.75×10^3 cells were dissolved into 1.5 mL of methylcellulose matrix (ClonaCell-TCS Medium, Stemcell Technologies), plated into gridded 6-cm plates (Thermo Fisher Scientific), and incubated for at least 10 days. Colonies from 100 squares were counted using a Nikon inverted microscope.

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Flow cytometry

Cells undergoing apoptosis were identified by flow cytometry (FACSCalibur, BD Biosciences) using the ApoAlert Annexin V-FITC Apoptosis Kit (Clontech). For intracellular phosphoprotein staining, cells were fixed and permeabilized using the BD Cytofix/Cytoperm Kit (BD Biosciences) and stained with phycoerythrin (PE) anti-phospho-S6 (S240, BD Biosciences) and analyzed by flow cytometry (BD FACSCanto II).

In vivo studies

Approximately 1×10^6 A673 cells transduced with either a control or 2 different FAK-directed shRNAs were suspended in 30% Matrigel and subcutaneously injected into the flank of 6-week-old female NCr nude mice ($n = 8$ per condition). Tumor volume was measured periodically using calipers to monitor tumor growth (volume $= 0.5 \times$ length $\times$ width^2).

Approximately 4.2×10^6 A673 cells were mixed with 30% Matrigel and injected subcutaneously into 8-week-old female NCr nude mice. When the average tumor volume in all animals reached approximately 100 mm^3, twice-daily treatment was administered by oral gavage with 200 mg/kg PF-562271 (in a volume of 10 ml/kg) or vehicle control (0.5% hydroxypropyl methylcellulose and 0.2% Tween 80 in sterile water; $n = 6$ per condition). Ten-week-old male NSG mice were used for *in vivo* experiments with TC32 cells to improve tumor engraftment with this cell line. In this model, several mice experienced toxicity when treated with PF-562271 at 200 mg/kg twice daily (data not shown). Therefore, after injection of approximately 5×10^6 TC32 cells in 30% Matrigel, animals began treatment with PF-562271 at 100 mg/kg or vehicle control ($n = 8$ per condition) every 12 hours when tumor volumes reached 100 mm^3. This dose was well tolerated, and at day 13, the dose was escalated to 150 mg/kg. Tumor volumes were measured with calipers. Animals were sacrificed when tumor volumes exceeded 2,000 mm^3.

Statistical methods

The Student *t* test or 1- or 2-way ANOVA with Tukey *post hoc* tests were used to compare groups. Statistical analyses were conducted using GraphPad Prism 5.0 or SigmaStat 3.5 (Aspire Software International).

Results

FAK is active in Ewing sarcoma

We profiled the activity of 62 unique tyrosine kinases in 6 Ewing sarcoma cell lines using a Luminex bead-based assay (Fig. 1A; ref. 8). The majority of kinases profiled displayed low phosphorylation levels in Ewing sarcoma cell lines, which was similar to the phosphorylation levels observed in the 293FT cell line control. However, SRC kinase (SRC), extracellular signal–regulated kinases (ERK), and FAK were highly phosphorylated in the panel of Ewing sarcoma cell lines (Fig. 1B; Supplementary Table S2) relative to the control. Treatment of Ewing sarcoma cell lines with the SRC inhibitor dasatinib and the MEK inhibitor PD0325901 had minimal effect on cell viability at concentrations that inhibited phosphorylation of SRC and ERK, respectively (Supplementary Fig. S1). Therefore, we focused our efforts on evaluating the importance of FAK activity in Ewing sarcoma.

First, we show that FAK is highly phosphorylated across a panel of 10 Ewing sarcoma cell lines, including those screened in the profiling assay, by Western immunoblotting (Fig. 1C). Because FAK is known to play a role in adhesion, it was important to confirm that FAK activity was not merely a phenomenon of cell lines growing in culture (12). Therefore, we conducted immunohistochemical (IHC) staining for phosphorylated FAK (Y397) in 15 primary Ewing sarcoma tumor samples using Ewing sarcoma cell lines with or without FAK-directed shRNA as controls (Fig. 1D and Table 1). All but one tumor specimen stained positive for the presence of phosphorylated FAK, showing that FAK is expressed and activated in the majority of Ewing sarcoma tumors.

FAK suppression impairs Ewing sarcoma cell growth and colony formation

To test the dependency of Ewing sarcoma on the presence of activated FAK for cell growth and colony formation, we infected cell lines with lentivirus containing shRNA constructs targeting multiple different regions of the FAK transcript. We identified 5 unique shRNA sequences that robustly downregulated FAK protein levels (Supplementary Fig. S2 and Supplementary Table S1) and chose 2 shRNAs that gave consistent knockdown across our panel of cell lines (Fig. 2A) for further study. The downregulation of FAK through shRNA transduction resulted in significantly impaired cell growth (using ATP content as a surrogate for cell number) across a panel of 4 cell lines (Fig. 2B), including one line with an EWS/ERG rearrangement (TTC466). FAK downregulation also inhibited Ewing sarcoma cell line colony formation in a methylcellulose matrix, showing that FAK activity contributes to anchorage-independent growth in Ewing sarcoma cell lines (Fig. 2C).

A common therapeutic modality for inhibiting kinases is to use small-molecule inhibitors. These compounds, however, do not downregulate total protein as is the case for shRNAs, but rather they inhibit kinase activity. Therefore, it was important to determine whether the inactivation of FAK activity was sufficient to induce the effects observed after shRNA-induced downregulation of FAK. We thus tested the effects of PF-562271, a selective inhibitor of both FAK and proline-rich tyrosine kinase 2 (PYK2), a FAK-related family member, on cell growth and colony formation in Ewing sarcoma cell lines (13). Seven cell lines were treated for 5 days with PF-562271 across a range of concentrations using 2-fold serial dilutions. Treatment with PF-562271 impaired cell viability in all cell lines, with an average IC_{50} of 2.4 μmol/L after 3 days of treatment (Fig. 3A). TC32 and A673 were the 2 most sensitive cell lines, with IC_{50} concentrations of 2.1 and 1.7 μmol/L, respectively. After confirming that this luminescent ATP detection assay is a faithful surrogate for cell number in Ewing sarcoma cell lines (Supplementary Fig. S3), we evaluated the effects of PF-562271 in a time course. FAK inhibition impaired Ewing sarcoma cell growth in all cell lines tested (Supplementary Fig. S4). We then tested four Ewing sarcoma cell lines for their ability to form colonies in a methylcellulose matrix after

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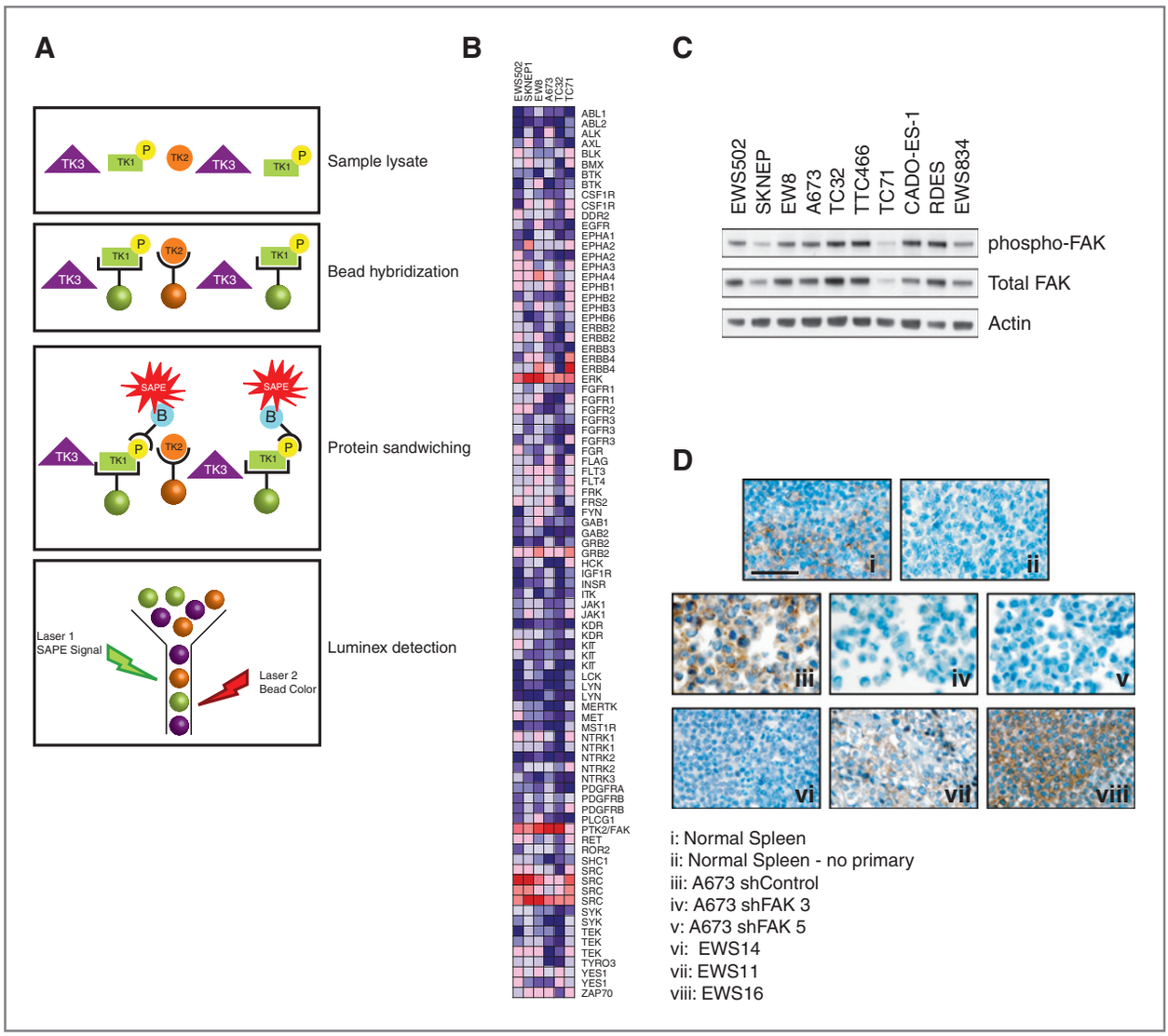

Figure 1. FAK is activated in Ewing sarcoma. A, schematic representation of the Luminex-based kinome profiling assay used to identify FAK (also known as PTK2) as a highly phosphorylated protein tyrosine kinase in Ewing sarcoma. B, heat map illustrating tyrosine kinase phosphorylation across 6 Ewing sarcoma cell lines. Median fluorescent intensity values for each kinase-specific probe in the Ewing sarcoma cell lines were divided by the respective median fluorescent intensity values for the 293FT control. Relative values were then normalized across each cell line on a scale of −3 to 3 and displayed as a heatmap using GenePattern software (version 3.3.3; Broad Institute). SRC, ERK, and FAK are highly phosphorylated in the panel of cell lines tested. C, Western immunoblot showing total FAK and phospho-FAK expression levels across a panel of 10 Ewing sarcoma cell lines using actin as the loading control. D, IHC staining for phospho-FAK in normal spleen with and without phospho-FAK primary antibody (i and ii), A673 cells with and without FAK downregulation by shRNA (iii–v), and Ewing sarcoma primary tumor cores (vi–viii). Scale bar, 1 mm.

24 hours of PF-562271 treatment across a range of concentrations. Cells were continuously exposed to PF-562271 in the methylcellulose matrix. Colony formation was significantly reduced with PF-562271 treatment in a concentration-dependent manner and corresponding to the IC_{50} of each line (Fig. 3B).

Inhibition of FAK induces apoptosis in Ewing sarcoma cell lines

In some cell lines treated with PF-562271, we noted a decrement in cell number over time (Supplementary Fig. S4), indicating that FAK inhibition may also induce apoptosis in Ewing sarcoma. To test this hypothesis, we treated 4 Ewing sarcoma cell lines with PF-562271 and measured apoptosis using Annexin V/propidium iodide staining. Apoptosis was significantly increased in a time- and concentration-dependent manner across all lines tested (Fig. 3C). To confirm this finding, we measured PARP cleavage by Western immunoblotting in Ewing sarcoma cells treated with PF-562271 for 24 hours. There was a concentration-dependent increase in PARP cleavage, which correlated with our flow cytometric findings (Fig. 3D).

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Table 1. Phospho-FAK IHC staining intensity in A673 controls and Ewing sarcoma primary tumor cores

Sample ID	Score 0–2
A673 shControl	1+
A673 shFAK 3	Occasional single cell
A673 shFAK 5	Occasional single cell
EWS1	1+
EWS2	0–1+
EWS3	2+
EWS4	1+
EWS5	1+
EWS6	0–1+
EWS7	1–2+
EWS8	1+
EWS10	1+
EWS11	1+
EWS12	1+
EWS13	1+
EWS14	0
EWS15	0–1+
EWS16	2+

FAK inhibition downregulates mTOR and CAS

The mTOR pathway is reported to be activated in a subset of Ewing sarcoma tumors and is a likely oncogenic driver (14–16). FAK is a known upstream regulator of AKT in normal tissue and models of cancer, suggesting a possible mechanistic link in Ewing sarcoma (17, 18). Therefore, we examined the effects of FAK inhibition on the AKT/mTOR pathway. First, Ewing sarcoma cells were serum-starved overnight to prevent oversaturation of this pathway by serum growth factor stimulation. Then, cells were treated with multiple concentrations of PF-562271 for 6 hours, followed by stimulation with IGF-1 for 2 hours. Treatment with PF-562271 downregulated AKT (S473), mTOR (S2448), and S6 (S240/244) phosphorylation at concentrations that impair cell growth and colony formation (Fig. 4A and Supplementary Table S3). We next confirmed that S6 phosphorylation was downregulated in cell lines treated with PF-562271 in a dose response by intracellular phospho-specific flow cytometry (Fig. 4B).

FAK is also reported to regulate the activity of Crkl-associated substrate (CAS) and the MEK/ERK pathway in some cellular contexts (12, 19, 20). Therefore, we examined the effects of FAK inhibition on CAS and ERK activity in A673 and TC32 cell lines after treatment with PF-562271. We found that FAK inhibition resulted in downregulation of CAS phosphorylation but not ERK (Fig. 4C). We then identified 3 unique CAS-directed shRNAs targeting different regions of the CAS transcript that robustly downregulate CAS expression (Fig. 4D and Supplementary Table S1). CAS downregulation in A673 cells had only a modest effect on cell growth and a variable effect on colony formation (Fig. 4E), suggesting that CAS inhibition alone cannot account for the effects observed with FAK inhibition in Ewing sarcoma.

FAK contributes to tumor establishment and growth in xenograft models of Ewing sarcoma

To confirm that loss of FAK impairs tumor initiation *in vivo*, FAK levels were downregulated via shRNA in A673 cells and injected subcutaneously in NCr nude mice. Because FAK suppression is selected against over time, we limited the study to 3 weeks. FAK downregulation significantly impaired tumor establishment and growth in this xenograft model of Ewing sarcoma (Fig. 5A).

We also tested whether the inhibition of FAK activity by treatment with PF-562271 could inhibit progression in established tumors using 2 xenograft models of Ewing sarcoma. NCr nude and NSG mice were subcutaneously injected with A673 and TC32 cells, respectively, and allowed to develop measurable tumors. The animals were then treated with either vehicle or PF-562271 until the animals were sacrificed. Treatment with PF-562271 significantly inhibited tumor growth compared with the controls showing that FAK activity contributes to tumor growth in Ewing sarcoma (Fig. 5B and C).

Discussion

Ewing sarcoma is a rare pediatric cancer with an incidence of approximately one case per million people in the United States (21). Although progress has been made in treating this disease with conventional chemotherapy combined with surgery or radiation for local control, outcomes for relapsed and metastatic disease are notably poor (9, 10, 22). Furthermore, an emerging literature on the long-term side effects of these conventional treatment modalities in pediatric cancer survivors leaves us unsatisfied with the current standard of care (23). However, for many rare pediatric cancers, the tumor driver events are too often the expression of an aberrant transcription factor, such as the EWS/FLI rearrangement in Ewing sarcoma (24), or loss of a tumor suppressor gene, both challenging to target using conventional drug screening approaches. Therefore, it is critical to prioritize efforts to identify more tractable targets in these rare, but devastating, childhood diseases.

Here, we took an alternative approach to identify new, potentially more tractable therapeutic targets in Ewing sarcoma. In light of the recent successes targeting kinases in the treatment of patients with cancer, we focused on this druggable target space. We profiled a panel of tyrosine kinases, inclusive of the majority of kinases with available inhibitors, in a collection of Ewing sarcoma cell lines. Because virtually all tyrosine kinases are phosphorylated when activated, tyrosine kinase phosphorylation was used as a proxy for kinase activity in this assay. One strength of this approach is that kinase activation will be identified independent of the mechanism of activation. While many cancer-promoting kinase activation events involve copy number gain or DNA mutation/rearrangement, there are recent examples of kinase activation via nongenetic events. For example, SYK was reported to be overexpressed in retinoblastoma via epigenetic regulation (2) and the tyrosine kinase Met (MET) is activated in many cancers by overexpression of the ligand, hepatocyte growth factor (HGF; ref. 25).

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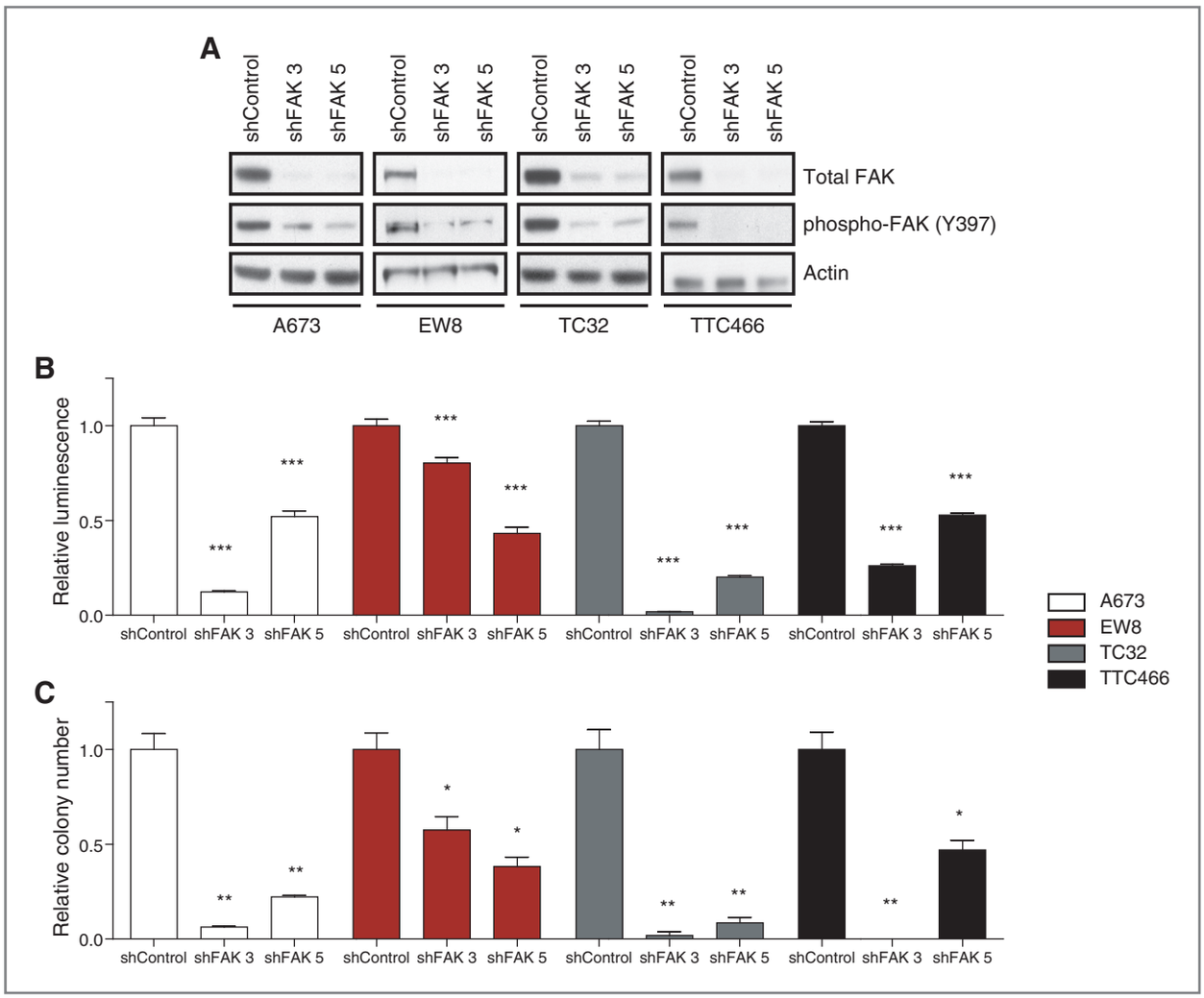

Figure 2. Suppression of FAK protein levels impairs cell growth and colony formation. A, Western immunoblots showing downregulation of FAK levels by transduction with FAK-directed shRNAs. B, effects of genetic downregulation of FAK on cell growth measured by a luminescent ATP detection assay. Relative luminescence was calculated by dividing each day 8 luminescence value by the average day 3 value. Each value was then divided by the average value of the shControl-treated cells. C, colony formation in methylcellulose relative to a control shRNA. Shown are the mean (14–16 replicates for viability and 2–4 for colony formation) ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by 1-way ANOVA with Tukey *post hoc* test.

SRC kinase, ERK, and FAK were highly phosphorylated in this screen. While SRC kinase and ERK pose attractive targets, chemical inhibition at concentrations eliminating phosphorylation had minimal effect on cell viability in the Ewing sarcoma cell lines evaluated. Therefore, our study focused on the validation of FAK as a potential therapeutic target in Ewing sarcoma. FAK is a non–receptor tyrosine kinase and central regulator of integrin signaling that mediates many normal cellular functions including adhesion, migration, survival, growth, and differentiation. FAK activation is initiated through a variety of extracellular signals that allow the cell to adapt to changes in the surrounding environment (26, 27). Activation of FAK leads to autophosphorylation at Y397, triggering an association with the SH2 domain of many signaling proteins including SRC, phosphoinositide 3-kinase (PI3K), phospholipase C, gamma 1 (PLCG1), and growth factor receptor–bound protein 7 (GRB7; refs. 28–31). Aberrant upregulation of FAK activity is a frequent event in cancer, promoting cell growth, survival, and invasion (32–34), and FAK activation is also associated with poor outcomes and increased metastatic potential (35). Downregulation of FAK activity in numerous cancer models impairs survival *in vitro* and inhibits tumor growth in xenograft models (13, 33).

Previous studies in Ewing sarcoma have alluded to a potential role for adhesion molecules in the pathogenesis of this disease. A gene expression–based study identified panels of highly expressed genes associated with poor prognosis in Ewing sarcoma. Among the gene sets identified was a collection of genes regulating cell adhesion, supporting a role for adhesion molecules in Ewing sarcoma (36). In a second study, FAK transcript levels were found to be elevated in a panel of Ewing sarcoma family cell lines compared with normal

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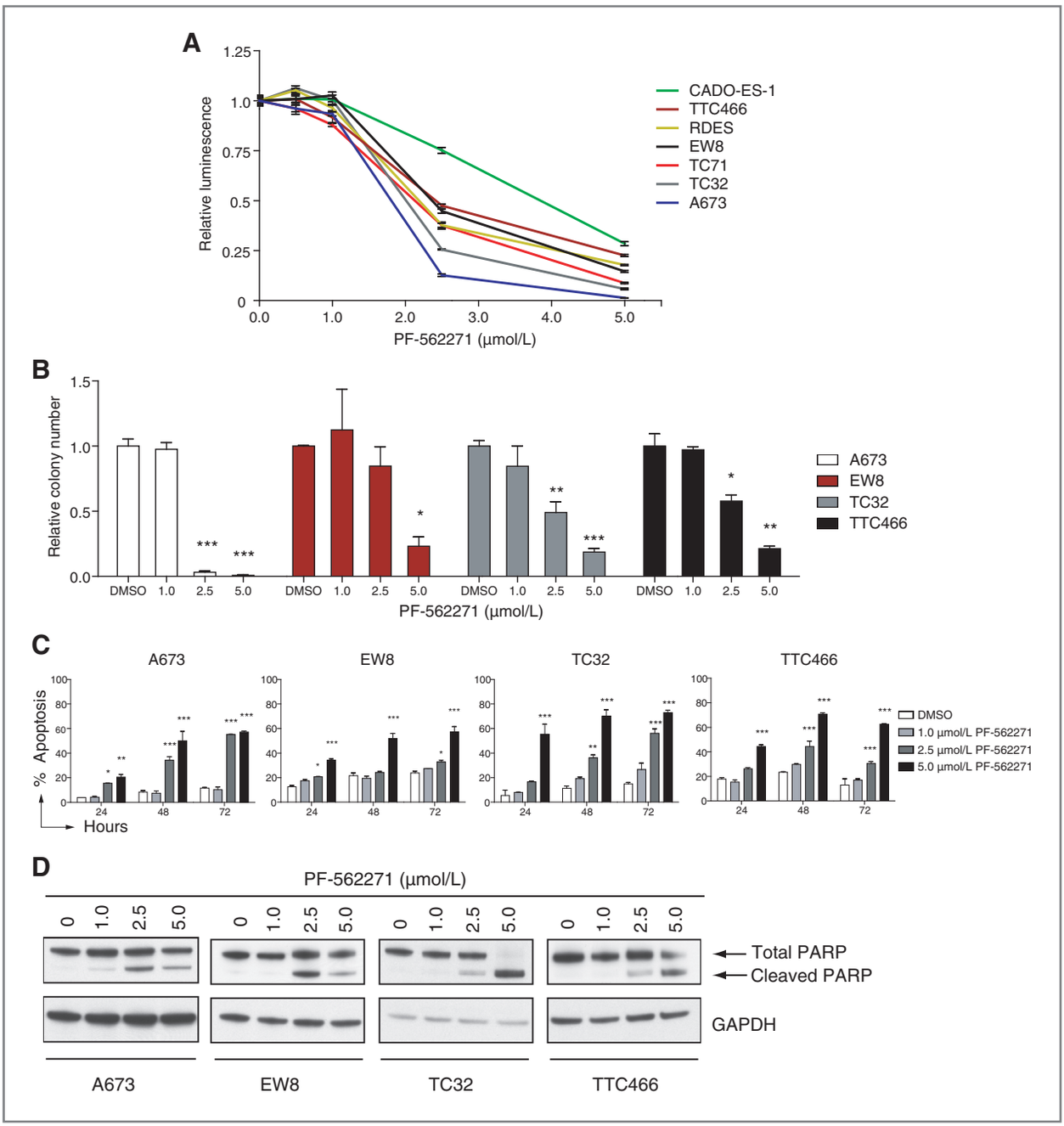

Figure 3. Small-molecule inhibition of FAK impairs cell viability and colony formation and induces apoptosis. A, effects of three days of treatment with PF-562271 on Ewing sarcoma cell lines (average IC_{50} = 2.4 μmol/L). Relative luminescence was calculated by dividing each luminescence value by the average dimethyl sulfoxide (DMSO) value. Shown are the mean of 8 replicates ± the SEM. B, effects of PF-562271 treatment on colony formation in Ewing sarcoma cell lines relative to DMSO (1-way ANOVA, Tukey *post hoc* test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Mean relative colony number (±SEM) of 4 replicates is shown. C, effects of PF-562271 treatment on apoptosis in a time- and concentration-dependent manner in Ewing sarcoma cell lines (2-way ANOVA, Tukey *post hoc* test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Shown are the mean percent of cells undergoing apoptosis (±SEM) of 2 replicates. D, Western immunoblots depict the protein levels of total PARP and cleaved PARP following 24-hour treatment with PF-562271. Glyceraldehde-3-phosphate dehydrogenase (GAPDH) was included as the loading control.

fibroblasts (37). Another study found that endoglin expression in Ewing sarcoma tumors is associated with increased expression of several proteins, including FAK, and is associated with poor outcome (38). Recently, a phosphoproteomics screen identified FAK phosphorylation in sarcoma cell lines including an Ewing sarcoma cell line (39).

The *FAK* (*PTK2*) gene is located on chromosome 8. *FAK* gene amplification has been reported in several malignancies and

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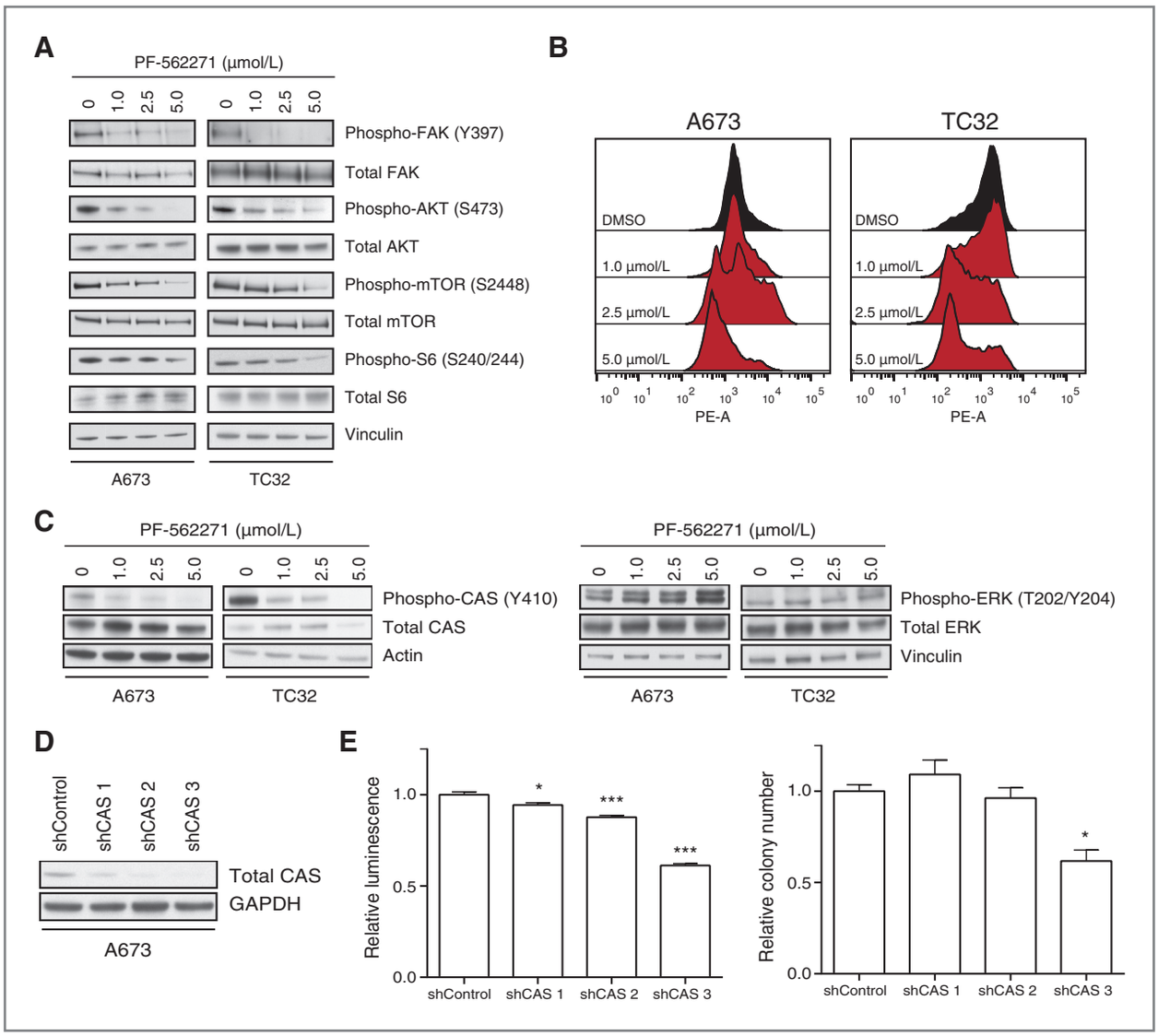

Figure 4. FAK inhibition downregulates the AKT/mTOR pathway and CAS activity. A, protein levels measured by Western immunoblotting for AKT/mTOR pathway proteins in A673 and TC32 cells serum-starved overnight, treated with PF-562271 for 6 hours, and then stimulated with IGF-1 for 2 hours. Vinculin was used as the loading control. B, histograms of intracellular phospho-S6 (S240) levels in live cells stained with anti-phospho-S6 and measured by flow cytometry in A673 and TC32 cells treated overnight with PF-562271. C, Western immunoblots showing downregulation of phospho-CAS but not phospho-ERK in A673 and TC32 cells after treatment with PF-562271. D, downregulation of CAS by 3 unique shRNA constructs has a modest effect on (E) cell growth and colony formation in methylcellulose relative to a control shRNA (1-way ANOVA, Tukey *post hoc* test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Relative luminescence was calculated by dividing each day 6 luminescence value by the average day 3 value. Each value was then divided by the average value of the shControl-treated cells. Relative luminescence and relative colony number are shown as the mean of 14 replicates and 2 replicates, respectively (±SEM).

has variable association with increased expression of FAK protein (40, 41). Interestingly, in Ewing sarcoma, chromosome 8 copy number gain is a frequent genetic event, potentially implicating FAK in the pathogenesis of this disease (42). However, despite reports of FAK expression in Ewing sarcoma cell lines, to our knowledge, this is the first study to show that FAK is highly phosphorylated in both Ewing sarcoma cell lines and Ewing sarcoma primary tumor samples, and it is the first to describe the dependency of Ewing sarcoma on FAK activity. Our *in vitro* and *in vivo* studies using both FAK-directed shRNA and chemical inhibition of FAK, show that Ewing sarcoma is dependent on FAK activity for growth, colony formation, tumor establishment, and tumor progression.

Efforts to develop targeted therapy for Ewing sarcoma are still in their infancy. However, studies have shown that downregulation of the AKT/mTOR pathway has antitumor activity, and inhibiting this pathway may be a reasonable approach to targeting this disease (14, 15). In fact, there has been modest success in treating relapsed and refractory Ewing sarcoma

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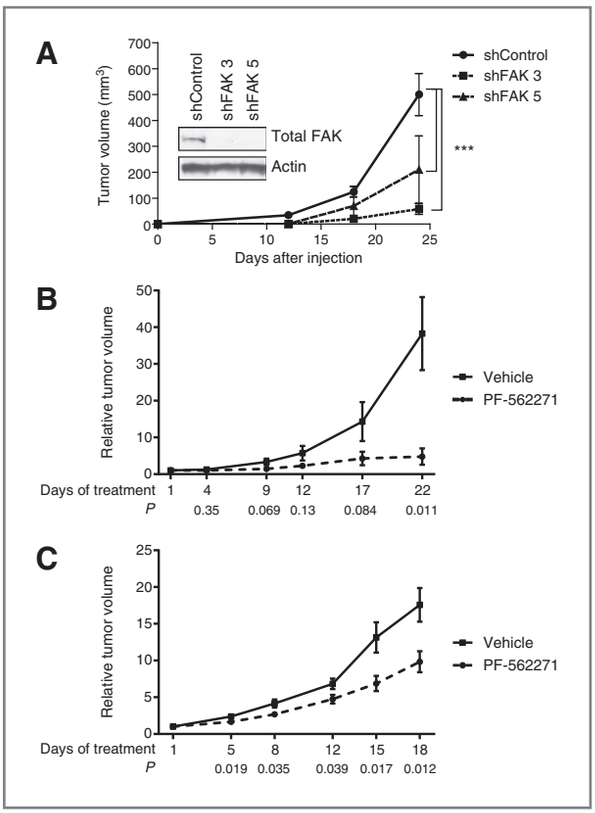

Figure 5. FAK inhibition impairs tumor engraftment and tumor progression *in vivo*. A, average size (mean ± SEM) of subcutaneous tumors in NCr mice. Tumors were grown from subcutaneous injection of A673 cells transduced with control or FAK-directed shRNA. Tumor volume was measured periodically until sacrifice 24 days postinjection. Western immunoblotting displays the total FAK levels from the control and knockdown lines in cells before injection. Tumor volume was significantly reduced 24 days postinjection (two-way repeated measures ANOVA; ***, $P < 0.001$). B and C, PF-562271 treatment significantly reduced tumor growth after subcutaneous injection of A673 and TC32 cells, respectively, in human tumor xenograft models of Ewing sarcoma. Relative tumor volumes were calculated by dividing the tumor volume measurement for each animal by the corresponding day 0 measurement for that animal. *P* values were calculated by the Student *t* test for each time point. Shown are the mean relative tumor volumes ± SEM.

tumors with IGF1R-targeted antibody therapy (7). Studies have shown that the inhibition of IGF1R leads to downregulation of AKT and mTOR in Ewing sarcoma (43). Furthermore, there is an emerging literature that champions the targeting of multiple nodes in a critical cancer pathway by using a combination of inhibitors to improve response and prevent the development of resistance (44). An RNAi screen in Ewing sarcoma determined that downregulation of the mTOR pathway sensitized Ewing sarcoma cells to IGF1R inhibition, and a recent phase I study showed tumor responses in a subset of patients with refractory Ewing sarcoma using IGF1R- and mTOR-targeted combination therapy (45, 46). While FAK activation may have multiple cancer-promoting effects in Ewing sarcoma, treatment of Ewing sarcoma cells with PF-562271 downregulated the activity of the AKT/mTOR pathway, suggesting that the inhibition of FAK is not only an attractive approach to treating Ewing sarcoma but also may offer a logical candidate for combination therapy with IGF1R- or AKT/mTOR-targeted treatment. Because PF-562271 is also reported to inhibit PYK2 (13), which is variably expressed in Ewing sarcoma cell lines (Supplementary Fig. S5), another possibility is that, in a subset of Ewing sarcoma cell lines, PYK2 inhibition contributes to the downregulation of the AKT/mTOR pathway.

In exploring other reported downstream effectors of FAK, we also found that treatment with PF-562271 in Ewing sarcoma cells inhibited CAS. Genetic downregulation of CAS, however, had only a modest effect on cell growth and colony formation in Ewing sarcoma cells, suggesting that CAS inhibition alone is not sufficient to explain the effects of FAK inhibition *in vitro*. CAS has been shown to play a role in cancer cell invasion and migration (47). One study reported that in a glioblastoma cell line ectopically expressing PTEN, overexpression of CAS restored invasion and migration but did not fully rescue cell growth (48). Because of the reported role of FAK/CAS in cancer metastasis, it will be important to determine in future studies whether this axis is relevant to the metastasis of Ewing sarcoma tumors. Moreover, additional pathways downstream of FAK, not explored in the current study, may be contributing to the anti–Ewing sarcoma phenotype seen with FAK inhibition.

Finally, it is important to note that there are several recently completed or ongoing early-phase clinical trials using FAK inhibitors to treat adult patients with solid tumors (http://clinicaltrials.gov/; NCT00666926, NCT00996671, NCT00787033, NCT01138033). Early reports for one of these inhibitors suggest that these drugs are well tolerated. Interestingly, several patients had disease response by positron emission tomography (PET) and prolonged stabilization of disease while on treatment (49). While it is still too early to draw conclusions from these clinical trials, the availability of clinically relevant FAK inhibitors, combined with the results described in our study, suggest the potential for rapid translation to the clinic for patients with Ewing sarcoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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High-Throughput Tyrosine Kinase Activity Profiling Identifies FAK as a Candidate Therapeutic Target in Ewing Sarcoma

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