

Intravital FLIM-FRET Imaging Reveals Dasatinib-Induced Spatial Control of Src in Pancreatic Cancer

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Abstract

Cancer invasion and metastasis occur in a complex three-dimensional (3D) environment, with reciprocal feedback from the surrounding host tissue and vasculature-governing behavior. In this study, we used a novel intravital method that revealed spatiotemporal regulation of Src activity in response to the anti-invasive Src inhibitor dasatinib. A fluorescence lifetime imaging microscopy–fluorescence resonance energy transfer (FLIM-FRET) Src biosensor was used to monitor drug-targeting efficacy in a transgenic p53-mutant mouse model of pancreatic cancer. In contrast to conventional techniques, FLIM-FRET analysis allowed for accurate, time-dependent, live monitoring of drug efficacy and clearance in live tumors. In 3D organotypic cultures, we showed that a spatially distinct gradient of Src activity exists within invading tumor cells, governed by the depth of penetration into complex matrices. In parallel, this gradient was also found to exist within live tumors, where Src activity is enhanced at the invasive border relative to the tumor cortex. Upon treatment with dasatinib, we observed a switch in activity at the invasive borders, correlating with impaired metastatic capacity *in vivo*. Src regulation was governed by the proximity of cells to the host vasculature, as cells distal to the vasculature were regulated differentially in response to drug treatment compared with cells proximal to the vasculature. Overall, our results in live tumors revealed that a threshold of drug penetrance exists *in vivo* and that this can be used to map areas of poor drug-targeting efficiency within specific tumor microenvironments. We propose that using FLIM-FRET in this capacity could provide a useful preclinical tool in animal models before clinical translation. *Cancer Res*; 73(15); 4674–86. ©2013 AACR.

Introduction

Maximizing the use of preclinical disease models in drug discovery requires the development of new innovative approaches for the study of drug delivery at the molecular level in live tissue. Examining cancer behavior in an intact host setting allows us to understand, in a more physiologic context, the

aberrant regulation of critical events that drive tumor progression. Intravital imaging is providing new insights on how cells behave in a more native microenvironment, thereby improving our understanding of disease progression (1, 2). In combination with adaptations of *in vitro* molecular techniques to three-dimensional (3D) model systems, intravital imaging is helping to bridge the gap in our understanding of key biologic events that govern cancer progression and therapeutic response *in vivo* (2–4).

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal forms of human cancer, with 90% of patient deaths occurring within 1 year of diagnosis due to surgically unresectable, locally advanced, or metastatic disease being present at the time of clinical diagnosis (5). Systemic therapies are largely ineffective in inoperable disease, leading to an overall 5-year survival rate of less than 5% (6, 7). In addition, the tumor microenvironment is characterized by poor vascularity and extensive deposition of extracellular matrix (ECM), limiting the accumulation and perfusion of drug delivery within the tumor tissue (8–10). Consequently, the development of more effective strategies to efficiently target and treat pancreatic cancer is required.

Recently, we have used a genetically engineered [*Kras*^{G12D/+}; *Trp53*^{R172H/+}; *Pdx1-Cre* (KPC)] mouse model of pancreatic cancer in which Cre-lox technology is used to target *Kras*^{G12D} and mutant *p53*^{R172H} to the pancreas via the *Pdx1* promoter (11). This results in the formation of invasive and metastatic PDAC

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that recapitulates the pathology of human disease (12, 13). Using this model, we have previously shown that introduction of a mutant p53^{R172H} allele drives a metastatic program over and above the tumor-promoting effects of p53 loss (14). We and others have also shown that mutations in p53 provide a gain-of-function role that drives metastasis, via disruption of cell-cell and cell-matrix adhesion, leading to invasion and spread of disease (14–20).

A number of new therapeutics have been developed to exploit the deregulation of nonreceptor tyrosine kinases such as Src in cancer and are currently under clinical investigation (21). Elevated expression and/or activity of Src has been shown to contribute to various types of invasive tumor cell behavior including evasion of apoptosis, enhancement of proliferation, and the deregulation of cell-cell and cell-matrix adhesions, associated with numerous forms of cancer including pancreatic cancer (22–24). In line with this, we have recently shown a correlation between Src upregulation and activation with reduced survival in human pancreatic cancer, and showed that the level of Src and phospho-Src are important indicators of vascular invasion, lymph node positivity, and prognosis in human PDAC (25). Importantly, we also established that the small-molecule Src kinase inhibitor, dasatinib, which is currently being clinically evaluated in combination with chemotherapy in locally advanced PDAC (26), inhibited invasion of primary PDAC cells generated from this model, and significantly reduced the development of metastases by approximately 50% (25).

As the current therapeutic failure of agents in the treatment of pancreatic cancer may arise from a potentially reversible impairment in drug delivery to the tumor (27), it remains to be determined whether improvements in drug targeting and effective delivery could enhance the encouraging antimetastatic profile of dasatinib and improve survival in this aggressive disease (25).

Here, we have adopted a fluorescence resonance energy transfer (FRET) biosensor, previously used to examine the dynamic regulation and activation state of Src kinase *in vitro* (28), as a preclinical tool to assess drug delivery and efficacy in live tumors. Using fluorescence lifetime imaging microscopy (FLIM) to measure FRET, we have rapidly analyzed and quantitatively measured Src activity at a single/subcellular level *in vivo* and detected effects intractable to standard techniques. We have investigated aspects of the 3D tumor environment that may contribute to poor drug targeting, such as the proximity of cells to the host vasculature or their location with regards to the tumor cell core or invasive border. Critically, we exploit this detailed spatial and temporal mapping of drug response to monitor the heterogeneity of tumor cells in response to new combination therapy that targets the stromal ECM architecture to favor effective drug delivery within solid tumor tissue.

Materials and Methods

Cell culture

Primary mouse PDACs were derived from tumors harvested from *Pdx1-Cre-GFP*, *LSL-KRas*^{G12D/+}, *LSL-Trp53*^{R172H/+} mice (14). PDACs were cultured in Dulbecco's Modified Eagle Medi-

um (DMEM) supplemented with 10% FBS and 2 mmol/L L-glutamine (Invitrogen) and transfected with the ECFP-YPet Src-FRET biosensor using Polyfect as described in the manufacturer's protocol (Qiagen; ref. 28). Cells were selected using 0.6 mg/mL G418 and stable pools generated using standard procedures.

Multiphoton time-correlated single photon counting FLIM

Imaging was conducted on a Nikon Eclipse TE2000-U inverted microscope with an Olympus long working distance 20× 0.95 NA water immersion lens. The excitation source used for all image acquisition was a Ti:Sapphire femtosecond pulsed laser (Cameleon), operating at 80 MHz and tuned to a wavelength of 830 nm. For more details, see Supplementary Data.

Fluorescence lifetime imaging of Src-FRET biosensor *in vivo*

Following trypsinization, 1×10^6 cells were resuspended in 100 μ L Hank's Balanced Salt Solution (HBSS; Invitrogen) and subcutaneously injected into the rear flank of a nude mouse. Tumors were allowed to develop for 7 days. Animals were kept in conventional animal facilities and all experiments were carried out in compliance with U.K. Home Office guidelines. To permit imaging, mice were terminally anesthetized using an anesthetic combination of 1:1 hypnorm-H₂O + 1:1 hypnovel-H₂O. Following induction of anesthesia, the subcutaneous tumor was surgically exposed and the mouse restrained on a 37°C heated stage. Multiphoton excited FLIM measurements were conducted *in vivo* using the system described earlier. Typical scan parameters were 150 μ m × 150 μ m field of view over 512 pixels × 512 pixels scanned at 400 Hz with the acquisition of 100 frames. The pixel dwell time was 5 microseconds with a total acquisition time of approximately 120 seconds. Typical laser power as measured at the sample plane was approximately 30 mW. It was found that PDAC cells expressing the Src biosensor could be imaged at depths of up to 150 μ m in live tumor tissue. To visualize vasculature, quantum dots (Qtracker-655; Life Technologies) were tail vein injected into mice 10 minutes before imaging (10 μ L in 190 μ L HBSS). For control- or drug-treated tumor, at least 300 cells were measured. Columns, mean; error bars, $\pm$ SE; *P* value by unpaired Student *t* test.

Frequency domain FLIM

In vitro fluorescence lifetime measurements were conducted using a Lambert Instruments fluorescence attachment on a Nikon Eclipse TE 2000-U microscope equipped with a ×60 objective and a filter block consisting of a 436/20 excitation filter, a T455LP dichroic mirror, and a 480/40 emission filter. For more details, see Supplementary Data.

Organotypic invasion assay

Organotypic assays were conducted as described previously (29). Briefly, approximately 7.5×10^4 /mL primary human fibroblasts were embedded in a 3D matrix of rat tail collagen I. Rat tail tendon collagen was prepared by extraction with 0.5 mol/L acetic acid to a concentration of approximately 2 mg/mL.

Detached, polymerized matrix (2.5 mL) in 35-mm Petri dishes was allowed to contract for 6 to 8 days in complete media (DMEM, supplemented with 10% FCS; Invitrogen) until the fibroblasts had contracted the matrix to 1.5 cm diameter. Subsequently, 4×10^4 Src biosensor-expressing PDAC cells were plated on top of the matrix in complete media and allowed to grow to confluence for 4 to 5 days. The matrix was then mounted on a metal grid and raised to the air-liquid interface resulting in the matrix being fed from below with complete media that was changed every 2 days. After 6 to 8 days invasion, the cultures were imaged as described or fixed using 4% paraformaldehyde and processed for hematoxylin and eosin (H&E) staining. For drug treatment, 100 nmol/L of dasatinib (Bristol-Myers Squibb) was added to the media below the matrix during the 6- to 8-day period as described. For cyclopamine treatment, 2.5 to 10 μ mol/L was added directly after matrix detachment before contraction (30, 31). Representative images of at least 3 independent experiments are shown, total of 985 cells assessed by FLIM-FRET. Columns, mean; error bars, $\pm$ SE; *P* value by unpaired Student *t* test.

Drug treatment *in vitro* and *in vivo*

Dasatinib (a kind gift from Bristol-Myers Squibb) was administered daily by oral gavage in 80 mmol/L citrate buffer (10 mg/kg) or 100 nmol/L *in vitro* (19, 25, 32). Cyclopamine (LC Laboratories) was administered by oral gavage (25 mg/kg; 33) or 2.5 to 10 μ mol/L *in vitro*. PP1 (Sigma-Aldrich; 10 μ mol/L) and EGF (R&D Systems; 50 ng/mL) was used *in vitro*.

Collagen organization measurements using gray-level co-occurrence matrix analysis

Using ImageJ, summed intensity projections were formed from raw 16-bit TIFF z-stacks of the collagen second harmonic generation (SHG) data. Three 100 μ m $\times$ 100 μ m regions of interest were selected to assess the collagen present. These subregions were saved for analysis using an in-house modified version of the UMB gray-level co-occurrence matrix (GLCM) features plug-in (http://arken.umb.no/~kkvaal/eamtexplorer/imagej_plugins.html). The modifications made allow for looped operation of the plug-in for a user-defined number of neighbor index points and for 0°, 90°, 180°, and 270° directions of comparison. This allows for the calculation of the average texture parameters for each image. The plug-in was sequentially applied to each 100 μ m $\times$ 100 μ m region of interest. Once all the images were analyzed, the normalized texture parameters (contrast, uniformity, correlation, homogeneity, and entropy) were calculated for each images and imported into GraphPad Prism (version 5.00 for Mac OS X, GraphPad Software; www.graphpad.com). The mean parameters were plotted (averaging over all four directions) along with the SEM against the neighbor index. Data are shown as points and dashed lines represent the nonlinear biexponential fit to each dataset (34).

Results

Generation of a FLIM-FRET-based genetic model of pancreatic cancer

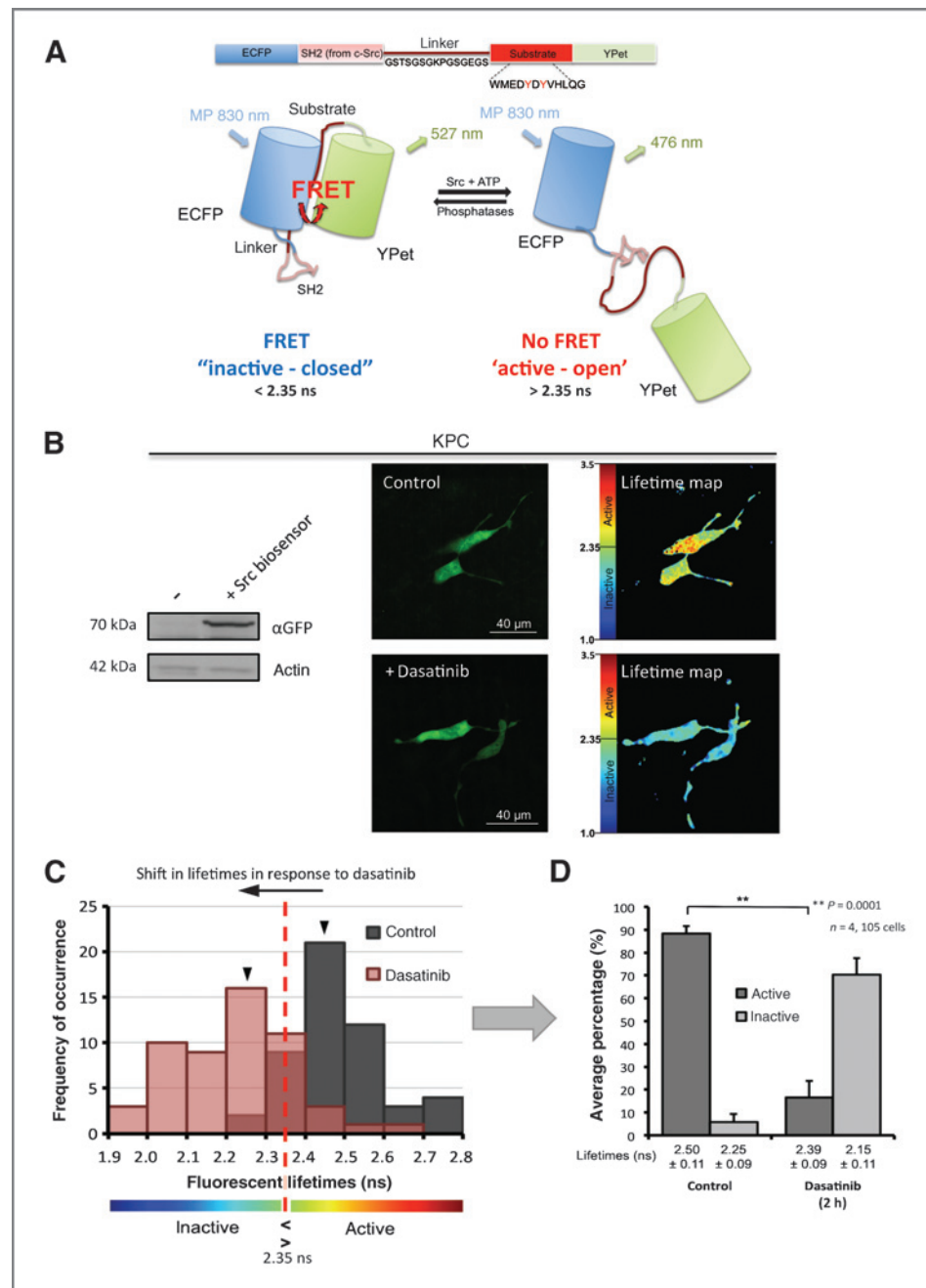
To study the dynamic regulation of Src activity in pancreatic cancer *in vivo*, we first established primary PDAC cells from the

KPC mouse model (14). Using these primary PDAC cells, we generated stable cells expressing a ECFP-YPet variant of the Src-FRET biosensor (28). The biosensor is composed of ECFP, an SH2 domain, a flexible linker and a Src substrate peptide derived from the c-Src substrate p130cas, linked to YPet (Fig. 1A). When the substrate is unphosphorylated the biosensor adopts a compact conformation in which the fluorophores are in close proximity, resulting in high FRET efficiency. Upon Src-induced phosphorylation, the substrate peptide binds to the SH2 domain and separates YPet from ECFP, thereby decreasing FRET (Fig. 1A; ref. 28). The loss of FRET upon Src activation is consistent with the intramolecular interaction of the phosphorylated substrate with the SH2 domain, and the subsequent change in FRET efficiency can be measured using fluorescent lifetime imaging of the donor fluorophore, ECFP (Fig. 1A and B). In the lifetime color maps, low basal Src activity is represented as blue, whereas high Src activity is represented as warm red/yellow colors, and areas with low signal-to-noise ratio in which lifetime measurements cannot be achieved are black (Fig. 1B). A critical advantage of this approach is that the biosensor allows us to visualize the dynamic and reversible equilibrium of Src activity within single cells in real-time. The resulting PDACs expressing the Src biosensor therefore serve as an excellent genetic and fluorescent model system to quantify the spatial and temporal regulation of Src activity in pancreatic cancer (Fig. 1A and B; refs. 11–13).

Application of the Src biosensor to investigate drug targeting

To establish whether FLIM-FRET analysis of the Src biosensor could be used to rapidly and accurately distinguish subtle changes in Src activity in response to therapeutic intervention, PDAC cells were subjected to dasatinib treatment in culture. Cells were treated with 100 nmol/L of dasatinib, a dose previously shown to be biologically effective in the KPC model (25) and the activity of Src within individual cells was determined using FLIM-FRET. Following 120 seconds of FLIM acquisition, a distribution of fluorescent lifetimes was obtained, where a peak in lifetime within control cells was evident between 2.4 and 2.5 nanoseconds (Fig. 1C, gray). Dasatinib treatment induced a shift in this lifetime distribution, yielding a lower peak of 2.2 to 2.3 nanoseconds (Fig. 1C, pink and overlay, red). Using this shift in lifetime distribution peaks, we stratified these readouts and used a cutoff of > 2.35 nanoseconds to classify cells as "active," whereas lifetimes of < 2.35 nanoseconds were classed as "inactive" (Fig. 1C and D and Supplementary Fig. S1). This enabled us to analyze the status of individual cells with respect to features of their environment, and capture behavior that would be masked by average measurements of fluorescence within a field of cells. We next conducted rapid pharmacodynamic measurements of dasatinib efficacy in PDAC cells by generating a single cell, FLIM-FRET-based, dose-response curve (Fig. 2A) supported by standard Western blotting (Fig. 2B). The ability to monitor Src activity on a single cell basis and the fast acquisition provided by multiphoton-based FLIM (19) shows the advantage and potential application of this technique as a dynamic, single cell biomarker for drug-targeting studies.

Figure 1. Use and characterization of Src biosensor for monitoring drug targeting. **A**, schematic of multiphoton-based FLIM-FRET acquisition of the ECFP-YPet reversible Src biosensor upon the activity of Src kinase or phosphatases, adapted from ref. 28. **B**, representative fluorescence image of KPC (11)-derived primary PDAC cells expressing the Src biosensor (green) with corresponding lifetime maps of Src activity $\pm$ 100 nmol/L dasatinib. **C** and **D**, quantification and distribution of fluorescent lifetime in response to 100 nmol/L dasatinib treatment for 2 hours. Stratified readouts from the Src biosensor were used as a threshold for cells being classified as "active" lifetimes $>$ 2.35 nanoseconds or "inactive" lifetimes $<$ 2.35 nanoseconds, based on the distribution between pre/post treatment peaks (arrowheads). Gray, control; pink, dasatinib; red, overlay. Columns, mean; bars, SE. **, $P = 0.0001$ by unpaired Student t test.



Temporal monitoring of drug targeting using a FRET biosensor

To determine whether the Src biosensor could be used to monitor fluctuations in target activity and drug clearance in a time-dependent manner, we carried out an *in vitro* washout experiment in which PDAC cells were subjected to 100 nmol/L dasatinib for 30 minutes, and subsequently monitored Src activity in response to dasatinib withdrawal using FLIM-FRET over 6 hours. The efficacy of dasatinib treatment was shown by a significant decrease in the fraction of Src-active cells upon dasatinib treatment (Fig. 2C; decreasing from $54.8\% \pm 3.7\%$ to $35.7\% \pm 6.9\%$). Following washout, a rapid recovery in the

fraction of Src-active cells was observed after 1 hour, which peaked at 3 hours ($P = 0.014$) and subsequently returned toward control levels within 6 hours (Fig. 2C). The capacity to track Src activity overtime *in vitro* prompted us to gauge whether we could use this approach to investigate temporal drug targeting and clearance under more functional and physiologic conditions in live animal tumors.

Monitoring drug targeting and clearance in a live microenvironment using FLIM-FRET

We have previously used multiphoton-based FLIM to quantify protein activity at depths of approximately 150 μ m within

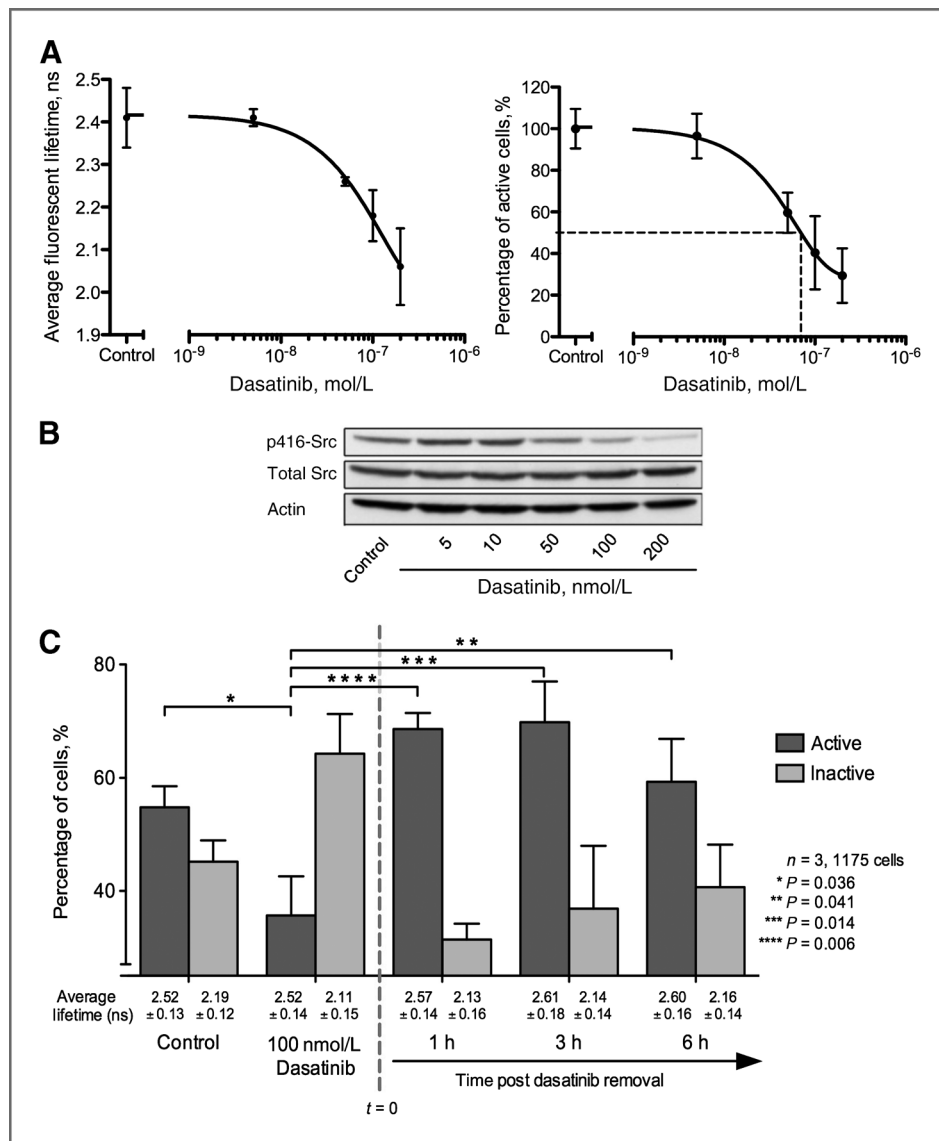


Figure 2. Application of FLIM-FRET for rapid and dynamic drug targeting studies. A, quantification and distribution of Src biosensor lifetime measurements in response to increasing concentrations of dasatinib ranging from 5 to 200 nmol/L (left). FLIM-FRET generated dose-response curve to dasatinib treatment *in vitro* (right). B, corresponding Western blot analysis of Src autophosphorylation using anti-phospho-Src Y416, -Src, and -actin antibodies, respectively, in response to dasatinib treatment for 2 hours. C, single cell FLIM-FRET quantification permitting the detailed tracking of fluctuations in active/inactive fraction of Src following pretreatment and subsequent washout of 100 nmol/L dasatinib over a 6-hour time period. Columns, mean; bars, SE. *, $P = 0.036$; **, $P = 0.041$; ***, $P = 0.014$; ****, $P = 0.006$ by unpaired Student *t* test.

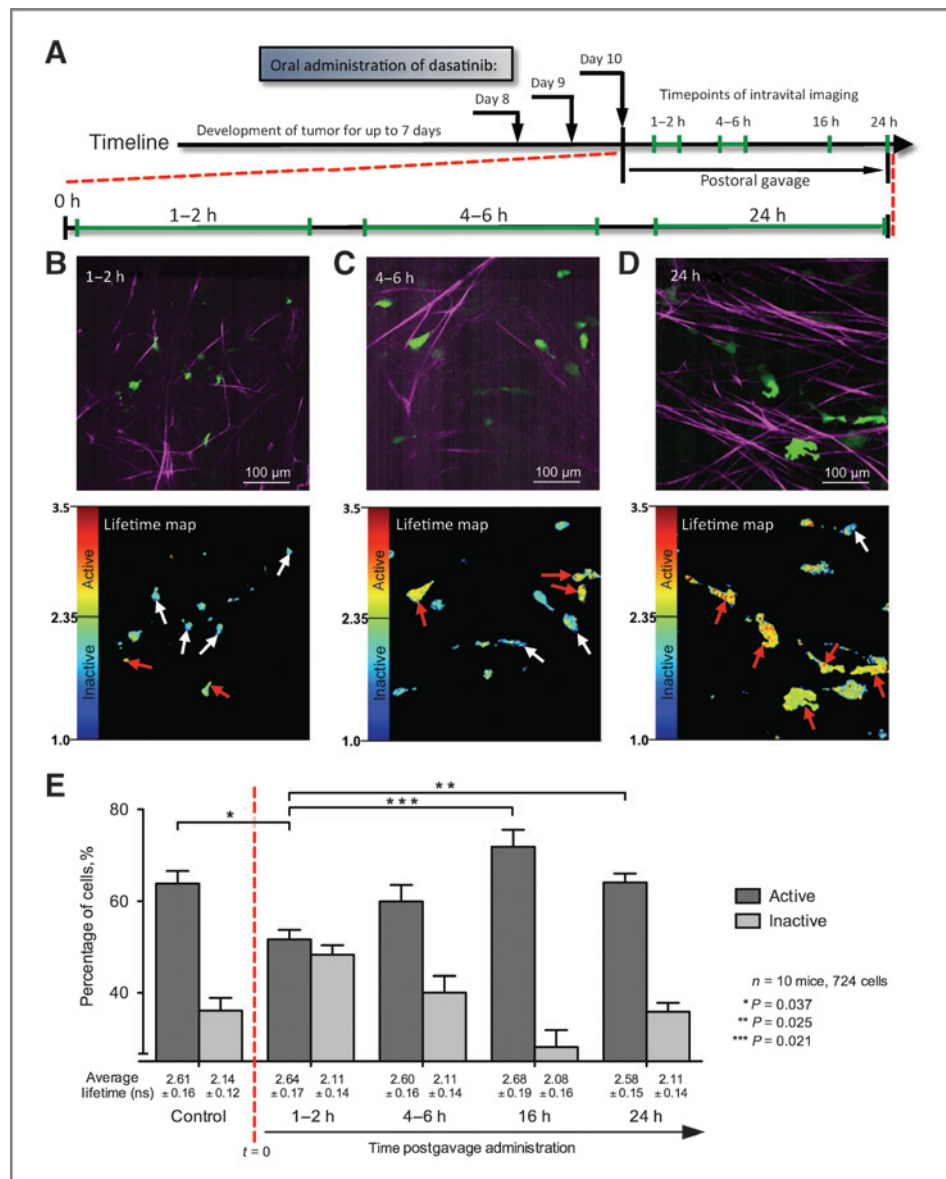
solid tumor tissue, providing a powerful tool to directly examine cancer progression *in situ* (19). This allowed us to readily investigate cells surrounded by ECM, stroma, and vasculature in a live setting upon drug treatment. Here, we subcutaneously injected mice with primary PDAC cells, and once tumors were established, subjected these mice to daily oral gavage of 10 mg/kg of dasatinib for 3 days (Fig. 3A). Inactivation of Src activity was evident under these conditions, as previously described (19, 25, 32). After the final administration of dasatinib, Src activity was subsequently monitored using FLIM-FRET at time points of 1 to 2, 4 to 6, 16, and 24 hours posttreatment (see imaging regimen Fig. 3A). After the final dose of dasatinib, a rapid recovery of Src activity was observed, reaching a maximum at 16 hours postdrug treatment before returning to control levels within 24 hours (Fig. 3B–D; quantified in (Fig. 3E; $P = 0.021$). The rapid recovery of Src activity to control levels as early as 4 to 6 hours post-dasatinib (Fig. 3E) could potentially

explain the partially ineffective targeting of pancreatic tumors we previously observed after a single daily oral dose of 10 mg/kg in dasatinib-treated KPC mice, which resulted in a reduction of metastasis of approximately 50% (25). Our FLIM-FRET analysis suggests that treating mice more frequently to counteract this early recovery may warrant future investigation in this genetically engineered mouse model (25).

Spatial regulation of Src activity in tumor subpopulations in 3D

The *in vivo* fluorescent lifetime assessment of the Src biosensor (Fig. 3) represents single cell measurements taken from cells located throughout the tumor mass. The activity of proteins involved in cancer progression can, however, also be locally regulated by different environmental cues within tumor cell subpopulations such as areas of invasive borders or within hotspots in the tumor microenvironment. By

Figure 3. Monitoring drug-targeting efficacy and clearance in a live tumor microenvironment using Src biosensor. **A**, schematic depicting drug treatment and *in vivo* FLIM-FRET imaging regimen. After 7 days of tumor development, dasatinib was administered at 10 mg/kg by oral gavage once daily for 3 days. Upon the last administration of dasatinib, four post-administration lifetime measurements of Src activity were obtained using *in vivo* FLIM-FRET analysis ranging from 1 to 2, 4 to 6, 16, and 24 hours posttreatment. **B–D**, representative *in vivo* fluorescence images of PDACs expressing the Src-reporter (green) with SHG signal from host ECM components (purple) at 1 to 2 hours (left), 4 to 6 hours (middle), and 24 hours (right), post-oral administration of dasatinib. Corresponding *in vivo* lifetime maps (bottom) showing the capacity to monitor single cell fluctuations in the fraction of active/inactive cells over the time course of dasatinib treatment in live tumor tissue. Red arrows, active tumor subpopulation; white arrows, inactive cells. **E**, quantitative FLIM-FRET imaging post-dasatinib treatment (1–2, 4–6, 16, and 24 hours) shows the capacity to quantify fluctuations and the distribution in drug target activity over time in a live tumor environment. Columns, mean; bars, SE. *, $P = 0.037$; **, $P = 0.025$; ***, $P = 0.021$ by unpaired Student *t* test.



adapting *in vitro* optical techniques, such as fluorescence recovery after photobleaching (FRAP) or photoactivation (PA) to *in vivo* applications, we have previously shown context-dependent differences in protein behavior governed by local signals during cancer progression *in vivo* (32, 35). We therefore sought to determine whether FLIM-FRET could be used to monitor response to drug treatment in spatially distinct tumor subpopulations.

Complex 3D matrices such as organotypic assays have been used to mimic cell–ECM interactions *in vivo* (30, 36, 37). They offer a source of growth factors and appropriate integrin engagement, resulting in bidirectional signaling between tumor cells and the surrounding stromal fibroblasts during invasion (Fig. 4A and Supplementary Movies S1 and S2). We therefore used organotypic matrices as previously described (29, 38, 39) to provide a controlled platform for assessment of

Src activity in invading tumor cells before *in vivo* analysis. Organotypic matrices consisting of human fibroblasts and rat tail fibrillar collagen I were established, which when exposed to an air–liquid interface induce a chemotactic gradient that allows overlaid PDAC cells to invade over time (Fig. 4A). Consistent with our recent *in vitro* and *in vivo* data (14, 19, 20, 25), KPC-derived PDACs expressing the Src reporter invaded into the matrix within 8 days (Fig. 4B). To investigate Src activity during different stages of invasion, we measured activity at 20 μm intervals up to 120 μm within the matrix (Fig. 4C and D). Interestingly, the fraction of active Src cells increased in proportion to the depth invaded (Fig. 4D). This spatially distinct gradient of Src activity within invading tumor cell subpopulations, suggests that in a 3D context, a switch in Src activity may be regulated by cues from within the local microenvironment during invasion.

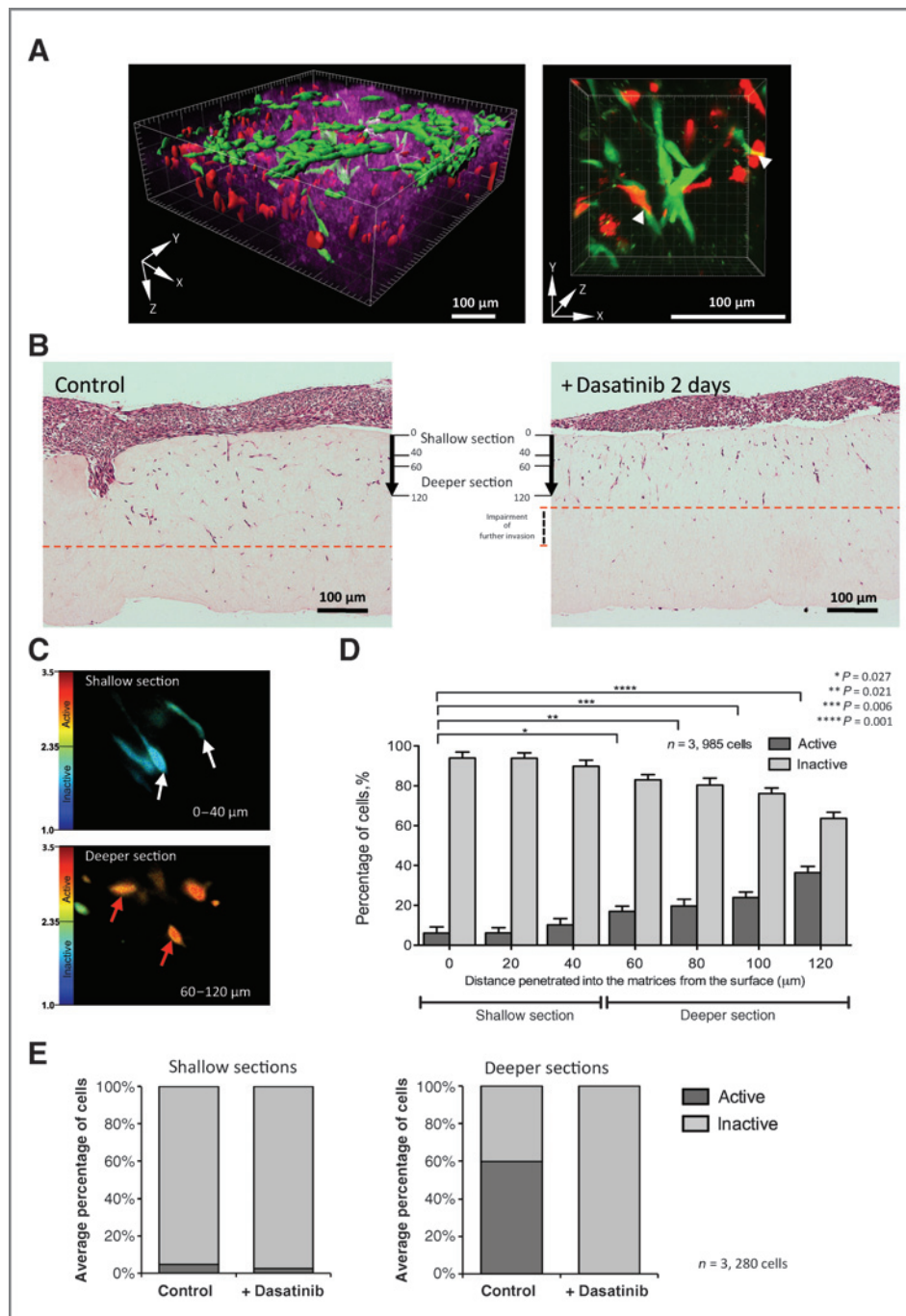


Figure 4. Spatial regulation of Src activity within 3D tumor subpopulations. **A**, primary PDAC cells (green) interacting with stromal fibroblasts (red) and SHG signal from collagen I (purple) during 3D invasion on organotypic matrix over 8 days. White arrowhead (right hand z-projection panel) indicates interactions between cocultured tumor cells and fibroblasts. **B**, H&E-stained sections of PDAC cell invasion after 6 ± 2 days postinvasion dasatinib treatment. **C**, corresponding lifetime map of Src activity in PDAC cells in upper section of matrix (0–40 μm) or at depth within the matrix (60–120 μm). **D**, quantification of lifetime measurements of Src activity at distinct depths within the matrix ranging from the surface at 0 to 120 μm in intervals of 20 μm . **E**, quantification of fluorescence lifetime measurements of Src activity in tumor subpopulations at the surface or within the matrix $\pm$ 100 nmol/L dasatinib. The distribution of active and inactive cells within the top or bottom of the matrix are expressed (%) of total tumor tissue population. Columns, mean; bars, SE. *, $P = 0.027$; **, $P = 0.021$; ***, $P = 0.006$; ****, $P = 0.001$ by unpaired Student *t* test.

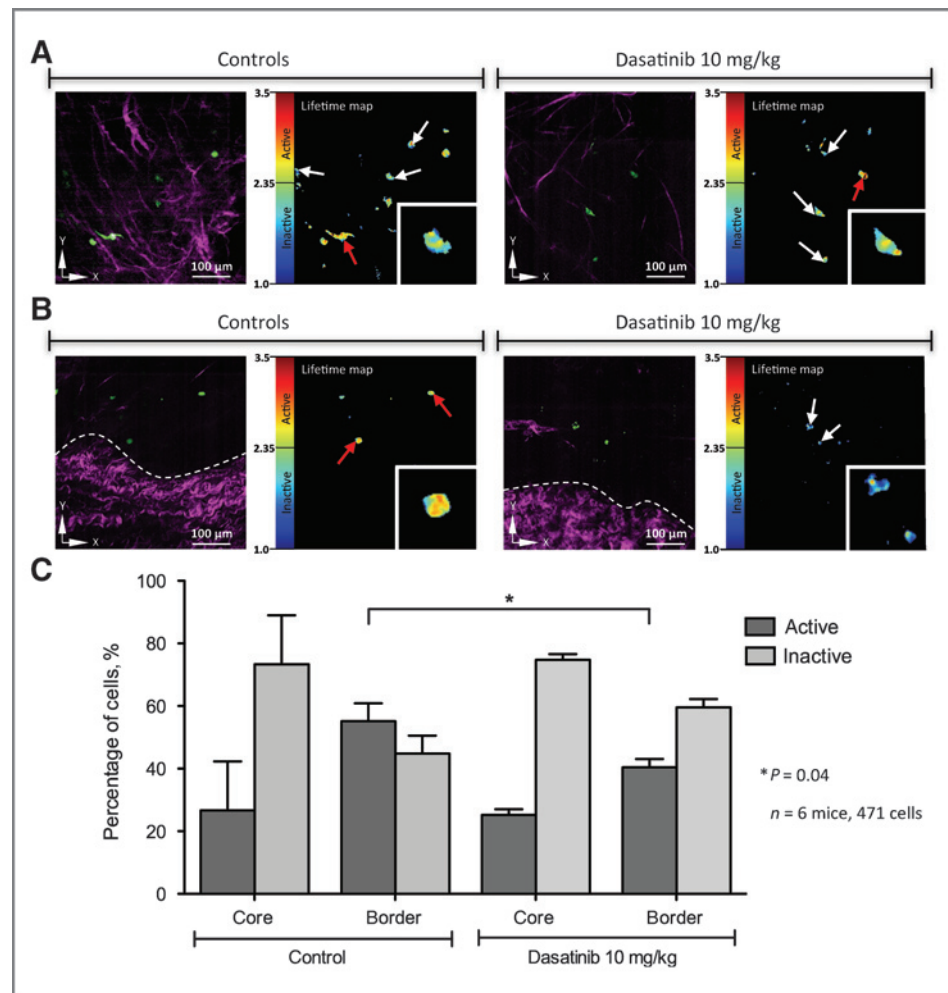
Next, we assessed the response to dasatinib treatment of tumor subpopulations in the upper section of the organotypic matrix (0–40 μm) compared with those at depth (60–120 μm). Cells were allowed to invade for 6 days followed by dasatinib treatment for the last 2 days. The fraction of active Src cells in the upper section did not significantly change upon dasatinib treatment, whereas the fraction of active Src cells at depth was significantly reduced (Fig. 4E). This also correlated with a reduction in invasion after treatment (Fig. 4B; Supplementary Fig. S2; refs. 19, 25). It is therefore possible to use FLIM-FRET to

visualize activity within spatially distinct subpopulations of tumor cells and gauge context- and site-specific drug-targeting efficiencies during processes such as invasion. We therefore investigated Src activity in distinct regions within living solid tumors.

Spatial regulation of Src within distinct tumor microenvironments *in vivo*

We have previously shown that primary PDAC cells expressing mutant p53^{R172H} form subcutaneous tumors with highly

Figure 5. Src activity is subject to spatial regulation within tumor subpopulations in live tumor microenvironments. A and B, representative *in vivo* fluorescence images of PDAC cells (left) expressing the Src-reporter (green) with SHG signal from host ECM components (purple). Corresponding *in vivo* lifetime maps (right) showing the distinct activation state of Src within the tumor center or invasive border $\pm$ 10 mg/kg oral administration of dasatinib (white dashed lines indicate position of invasive border). C, *in vivo* quantification of the activation state of cells (%) population within distinct regions of the tumor environment $\pm$ 10 mg/kg dasatinib. Columns, mean; bars, SE. *, $P = 0.04$ by unpaired Student *t* test.



invasive borders, whereas cells in which p53 is deleted, form noninvasive, encapsulated tumors (19). To identify distinct tumor regions, we used features of the tissue stroma visualized using SHG imaging. Central regions of the tumor were characterized by anisotropic distribution of collagen fibers, whereas border regions were readily identified by isotropic organization as previously described (34). GLCM analysis was used to distinguish between the tumor center and border based on features of the SHG collagen image (Supplementary Fig. S3; ref. 34). In line with our results using organotypic matrices (Fig. 4), we observed that Src activity correlated with regions of invasion in mutant p53 PDACs *in vivo* (Fig. 5). In control-treated PDAC tumors, cells within the tumor core were found to be predominantly inactive (Fig. 5A and C), whereas cells within invasive borders were predominantly active (Fig. 5B and C). Upon dasatinib treatment, the fraction of active Src cells within the tumor center did not significantly change ($26.6\% \pm 15.7\%$ vs. $25.2\% \pm 1.8\%$ of active cells). However, at the invasive border, a switch from a predominantly active to inactive state was observed ($55.2\% \pm 5.7\%$ vs. $40.4\% \pm 2.7\%$). These results show that cells within different microenvironments have differential sensitivity to therapeutic treatment across the tumor pop-

ulation and that FLIM-FRET can potentially be used to map areas of drug-targeting efficiency.

Drug delivery and targeting efficacy in live tumors is governed by proximity to vasculature

Poor vasculature or limited drug perfusion to tumor tissue is thought to play a critical role in drug delivery and efficacy (27). Because tumor cells that are located in areas of poor drug exposure may recolonize or subsequently repopulate to form micro-metastases following treatment, it would be beneficial to identify regions of poor drug delivery within various solid tumor environments using FLIM-FRET. Next, we determined whether single cell analysis of Src activity could be used to assess the targeting of dasatinib with respect to the host vasculature.

Mice bearing primary PDAC tumors were generated as before and treated with 10 mg/kg of dasatinib for 3 days (Fig. 3A). Before FLIM-FRET imaging, quantum dots (Qtracker-655) were intravenously injected to act as a contrast agent to identify tumor vasculature (Fig. 6A and Supplementary Movie S3). This enabled us to analyze Src activity within four key zones: 0–25, 25–50, 50–100, and >100 μ m from vasculature. Using this approach, we found that a spatial distribution of Src

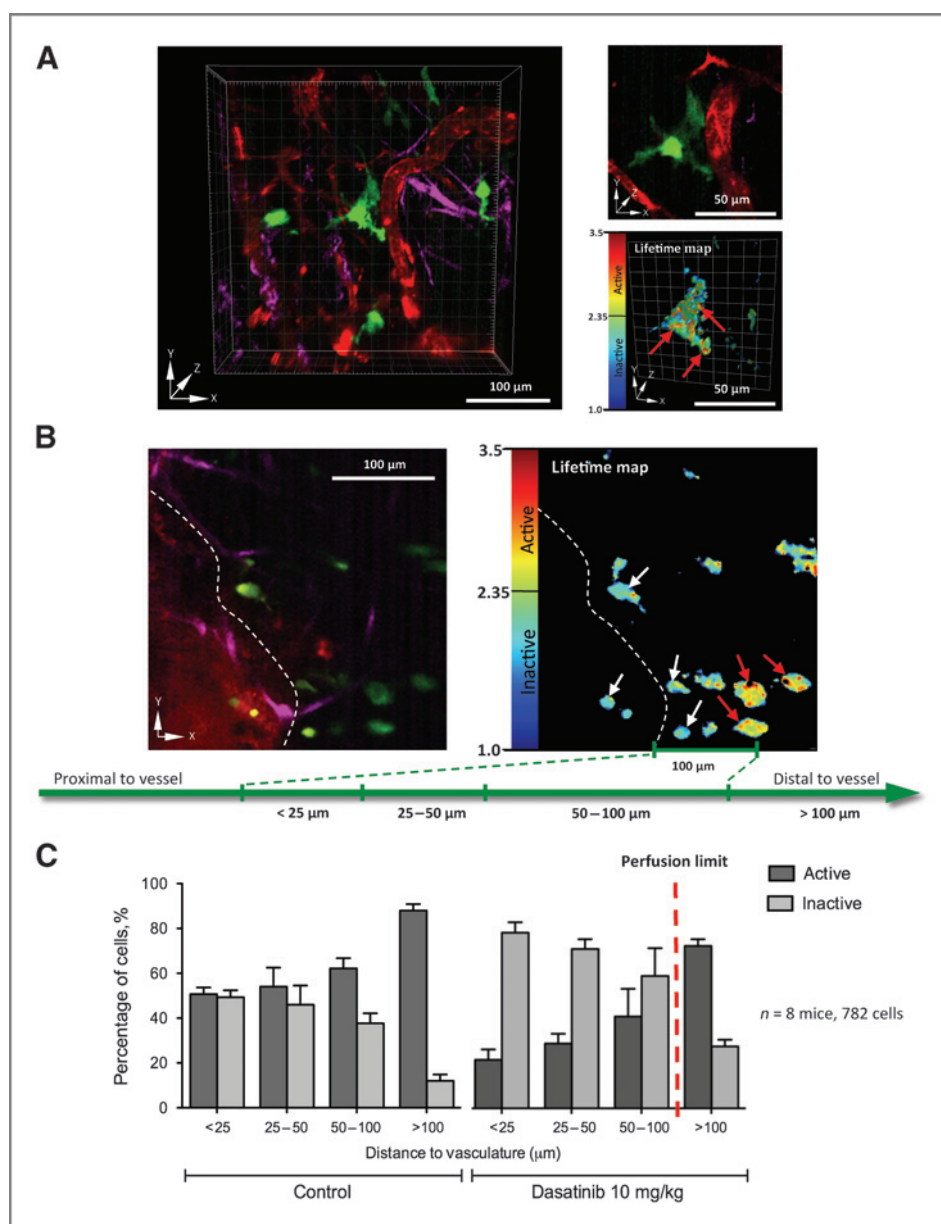


Figure 6. Quantification of drug delivery *in vivo* with respect to tumor vasculature using FLIM-FRET. **A**, representative *in vivo* fluorescence image of PDAC cells expressing the Src biosensor (green) in the context of host tumor vasculature, labeled with Qtracker 655 quantum dots (red) and SHG signal from host ECM components (purple). Corresponding *in vivo* lifetime map shows the capacity to monitor distinct activation states of Src in the context of the drug perfusion from tumor blood supply. **B**, representative *in vivo* fluorescence image of primary PDAC cells expressing the Src biosensor (green) in context of host vasculature (red) with corresponding *in vivo* lifetime map in the presence of 10 mg/kg dasatinib treatment. Vascular imaging regimen determining the proximity of cells to the local vasculature within four key zones including 0–25, 25–50, 50–100, and >100 μm from blood supply. **C**, *in vivo* quantification of Src-targeting activity in the context of tumor blood supply showing a spatially restricted propagation of Src inactivation upon 10 mg/kg oral administration of dasatinib. Columns, mean; bars, $\pm$ SE.

activity exists in this context, in line with previous results (Figs. 4 and 5). Src activity increased in proportion to the distance from blood supply (Fig. 6C, control) and using FLIM-FRET analysis we could accurately measure the response to dasatinib treatment in this spatial setting. Src activity in cells proximal to blood supply (0–25 μm) was readily reduced in response to dasatinib, resulting in the predominant form of Src in cells being converted from an active to inactive state (Fig. 6C; < 25 μm $\pm$ dasatinib). A similar effect was observed within 25–50 μm from vasculature (Fig. 6C; 25–50 μm $\pm$ dasatinib), reaching a limiting threshold at 50–100 μm from the vasculature (Fig. 6C; 50–100 μm $\pm$ dasatinib). Critically, PDAC cells beyond 100 μm from vasculature were inefficiently targeted, such that dasatinib treatment increased the inactive pool by 15.6% compared with control-treated mice, but the predominant

pool of cells remained in an active state (Fig. 6C; >100 μm $\pm$ dasatinib). Using this approach, we can therefore measure the limitations of drug targeting *in vivo* and pinpoint areas (Fig. 5) or thresholds (Fig. 6) of nontargeting and poor drug delivery in live tumor tissue. Applying this target validation technology with combination therapy aimed at improving drug delivery, we could potentially document site-specific and time-dependent improvements in response to new therapeutic regimens.

Measuring the efficacy of combination therapy using FLIM-FRET *in vivo*

Targeting the tumor architecture to favor drug penetration has recently been used to improve drug delivery in pancreatic cancer (27). In particular, the role that tumor-associated matrix and stromal tissue plays in the perfusion deficit found

in pancreatic cancer has been an active area of research (8–10, 40, 41). In the KPC mouse described here, therapy to deplete the tumor-associated ECM using the hedgehog signaling inhibitor IP-926 increased tumor perfusion and the therapeutic index of chemotherapeutic agents within the pancreas, and increased the overall survival in these mice when used in combination with chemotherapy (10). Moreover, the enzymatic depletion of the abundant ECM component, glycosaminoglycan hyaluronan (31), by PEGPH20 has recently been shown to improve the intratumor delivery of chemotherapeutic agents *in vivo* (8, 9). These studies, in part, suggest that the current therapeutic failure of agents in the treatment of pancreatic cancer may arise from a potentially reversible impairment in tumor drug delivery. Combination therapy to disrupt the deposition of ECM and sensitize tumors before treatment may improve the current efficacy of dasatinib in this disease (8–10, 42). As proof-of-principle, we investigated whether targeting the tumor ECM using the hedgehog signaling inhibitor cyclopamine (33) before dasatinib treatment could improve drug delivery within the tumor tissue and examined whether FLIM-FRET could be used as a tool to accurately quantify and monitor the limitations of this process.

To assess whether cyclopamine treatment reduces ECM content (10), we initially subjected organotypic matrices to cyclopamine treatment during matrix formation. Treatment with cyclopamine led to a dose-dependent reduction in stromal fibroblast-driven collagen I contraction (Fig. 7A and B). Moreover, the fibrillar and cross-linked collagen I content in organotypic matrices was significantly reduced as quantified by SHG imaging [as previously described (43)] and GLCM (34) analysis, respectively (Fig. 7C and D; Supplementary Fig. S4 and Supplementary Movies S4 and S5). Next, we assessed whether cyclopamine alone had any effect on Src activity within PDAC cells *in vitro*. Following cyclopamine treatment ranging from 2.5 to 20 $\mu\text{mol/L}$, Src activity was unaffected as determined by FLIM-FRET and Western blotting (Fig. 7E and F).

To assess the effects of cyclopamine *in vivo*, mice were injected subcutaneously with primary PDAC cells, and the tumors were allowed to form before treatment with cyclopamine alone for 3 days by oral gavage. Cyclopamine targeting of the stroma and ECM normalized the tumor microenvironment *in vivo* resulting in a loss of the spatial distribution of Src activity observed in relation to the vasculature (compare Figs. 7G with 6C, control). As cyclopamine treatment alone had no effect on Src activity in tumor cell *in vitro* (Fig. 7E and F), this suggests that targeting the tumor stroma *in vivo* can affect the activity of tumor cells indirectly via targeting environmental cues from the host surroundings such as ECM components.

Combination therapy using cyclopamine pretreatment and dasatinib was then examined and target inhibition within three key zones from tumor vasculature (25–50, 50–100, and >100 μm) was determined. At 25–50 μm from vasculature, there was a significant increase in the proportion of cells that switched to a predominantly inactive state upon cyclopamine + dasatinib treatment compared with dasatinib alone (compare Fig. 6C dasatinib alone with Fig. 7H + cyclopamine; 25–50 μm). A small, though not significant improvement in drug targeting in cells at 50–100 μm was also observed upon cyclopamine pre-

treatment [compare Fig. 6C dasatinib alone with Fig. 7H + cyclopamine; 50–100 μm (inactive $59.05\% \pm 12.35\%$ (SEM) vs. $64.97\% \pm 16.51\%$ (SEM), respectively, $P = 0.407$)]. Importantly, the effect of cyclopamine + dasatinib at 100 μm from the vasculature was equivalent to cyclopamine alone at this distance (compare Fig. 7G to Fig. 7H). Thus, indicating that we have reached the limitation of this combination therapy as no further improvement in drug efficacy at this distance can be achieved using this combination. As cyclopamine was shown to have no direct effect on Src activity in tumor cells alone (Fig. 7E and F), this suggests that indirectly targeting the tumor architecture can affect Src activity within tumor cells. The capacity to monitor spatial regulation of protein species using FLIM-FRET technology can therefore be used as a tool to determine the boundaries of combination therapy and allow for improved targeting and therapeutic outcome in preclinical models within the limitations of the tissue microenvironment of interest.

Discussion

To improve upon the current high attrition rates and late-stage failure in drug development, more innovative and informative approaches to candidate drug selection and clinical design are required (44). Failure to translate drug candidates into clinical benefit suggest that conventional early drug discovery strategies and preclinical models are suboptimal and may poorly predict the heterogeneous drug response to effectively guide clinical dosing or combination strategies (45).

Here, we show the use of FLIM-FRET in the preclinical analysis of a dynamic biomarker *in vivo* during the assessment of therapeutic drug treatment. Detailed topologic analysis at cellular and subcellular resolution in live tumor tissue has enabled precise detection and single cell quantification of subtle changes in protein activity over time that cannot faithfully be achieved using standard fixed endpoint approaches applied to *in vitro* cultured models or *ex vivo* immunohistochemical analysis (2, 44). Using a genetically engineered model of pancreatic cancer that recapitulates the human disease and a novel therapeutic with shown activity in pancreatic cancer, we have quantified the improvement in drug response associated with combination therapy and illustrate the application and advantages of FLIM-FRET in the drug target validation process. The distinct spatial- and environment-based regulation of Src observed here increases the possibility that other cancer types may harbor inefficiently targeted regions governed by unique microenvironments found within the local tumor tissue. Combination of preclinical mouse models with the rapidly expanding portfolio of new FRET-based biosensors (46–49) could prove useful for assessing the response to therapy of a wide variety of potential drug targets. Furthermore, intravital FLIM-FRET imaging could provide a pixel-by-pixel map of protein activity in response to drug targeting in other forms of cancer or disease states.

Advances in systems biology approaches to drug discovery consider the complexity of disease and offer alternative strategies for target selection, validation, and drug profiling (44, 45). In the fluorescence lifetime imaging field, advances have led to

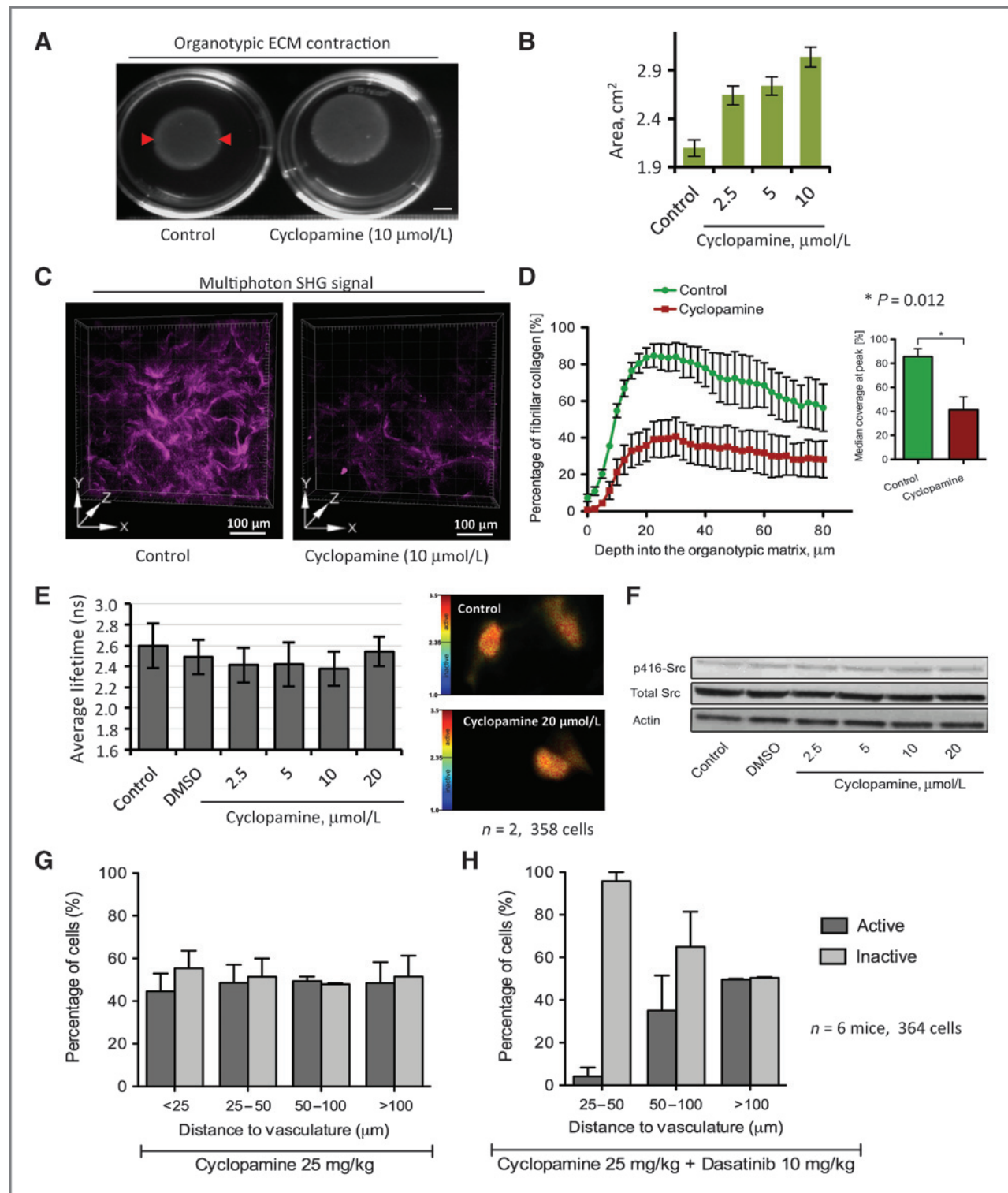


Figure 7. Combination therapy targeting stroma compartment improves drug delivery to solid tumors and targeting efficiency can be monitored using intravital FLIM-FRET imaging. **A** and **B**, representative image and quantification of fibroblast-induced organotypic ECM remodeling and contraction $\pm$ increasing dose of cyclopamine (0–10 $\mu\text{mol/L}$). **C**, representative maximum SHG-projection image of organotypic fibrillar collagen I ECM content (purple) $\pm$ 10 $\mu\text{mol/L}$ cyclopamine. **D**, z-stack quantification (0–80 μm) of SHG-based fibrillar collagen I content $\pm$ 10 $\mu\text{mol/L}$ cyclopamine as described previously (43). **E**, *in vitro* quantification of Src activity $\pm$ cyclopamine treatment (0–10 $\mu\text{mol/L}$) using FLIM-FRET. **F**, *in vitro* Western blot analysis of Src activity $\pm$ cyclopamine treatment (0–10 $\mu\text{mol/L}$) using anti-phospho-Src Y416, Src and actin antibodies, respectively. **G** and **H**, FLIM-FRET quantification documenting site-specific changes in Src response to new combination treatment regimen (50 μm resolution). Columns, mean; bars, $\pm$ SE.

high-speed FLIM being developed to monitor protein activity or protein–protein interactions *in vitro* in a format suitable for high-content chemical or molecular screening (44, 50). Both time domain- or frequency domain-based FLIM have been adapted to allow for fast FLIM in a 96-well high-throughput platform and as such have permitted a multiplexed FRET-readout of distinct and independent protein species that can be temporally and spatially resolved *in vitro* (51, 52). This, along with recent work using FLIM to identify tyrosine phosphorylation networks in response to EGFR signaling in a high-throughput setting (53), highlights the potential application of FLIM for resolving transient compensatory and adaptive signaling events in response to a variety of drug treatment regimens. Early use of FLIM-FRET imaging to intermediate 3D and *in vivo* setting, as described here, may reveal further layers and context-dependent detail about drug response not feasible *in vitro* (54).

Finally, the dynamic tumor analysis described here can be applied to other strategies aimed at altering drug delivery, such as approaches to enhance the vascular patency of blood vessels before treatment to increase perfusion within the tumor tissue (8, 9). The transient normalization of tumor vessels in response to short-term antiangiogenic agents has also been shown to temporarily enhance drug delivery to tumors (55). There is therefore a clear limit to how much we can alter the ECM or blood supply, such that we do not compromise the integrity and structure of the organ/tissue of interest. Use of this imaging technology with combination therapy may help determine the appropriate therapeutic window for altering the equilibrium between the tumor tissue and microenvironment, while maintaining maximum drug delivery (Supplementary Fig. S5). Finally, the intravital imaging approach applied in this study to assess protein behavior can also be applied in other

biologic frameworks where heterogeneity may determine therapeutic response, thereby assisting in the design, scheduling, and streamlining of efficient drug delivery in a wide variety of disease conditions.

Disclosure of Potential Conflicts of Interest

T.R.J. Evans has commercial research grant, honoraria from Speakers Bureau, and is consultant/advisory board member of Bristol-Myers Squibb. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Nobis, J.P. Morton, T.R.J. Evans, P. Timpson

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Nobis, M.C. Frame, P. Timpson

Writing, review, and/or revision of the manuscript: M. Nobis, E.J. McGhee, J.P. Morton, T.R.J. Evans, V.G. Brunton, M.C. Frame, N.O. Carragher, O.J. Sansom, P. Timpson, K.I. Anderson

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Intravital FLIM-FRET Imaging Reveals Dasatinib-Induced Spatial Control of Src in Pancreatic Cancer

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