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Spatial regulation of RhoA activity during pancreatic cancer cell invasion driven by mutant p53

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Abstract

The ability to observe changes in molecular behavior during cancer cell invasion *in vivo* remains a major challenge to our understanding of the metastatic process. Here, we demonstrate for the first time an analysis of RhoA activity at a subcellular level using FLIM-FRET imaging in a live animal model of pancreatic cancer. In invasive mouse pancreatic ductal adenocarcinoma (PDAC) cells driven by mutant p53 (p53R172H), we observed a discrete fraction of high RhoA activity at both the leading edge and rear of cells in vivo which was absent in two-dimensional in vitro cultures. Notably, this pool of active RhoA was absent in non-invasive p53(fl) knockout PDAC cells, correlating with their poor invasive potential in vivo. We used dasatanib, a clinically approved anti-invasive agent that is active in this model, to illustrate the functional importance of spatially regulated RhoA. Dasatanib inhibited the activity of RhoA at the poles of p53R172H cells *in vivo* and this effect was independent of basal RhoA activity within the cell body. Taken together, quantitative in vivo fluorescence life-time imaging illustrated that RhoA is not only necessary for invasion, but that subcellular spatial regulation of RhoA activity, as opposed to its global activity, is likely to govern invasion efficiency in vivo. Our findings reveal the utility of FLIM-FRET in analyzing dynamic biomarkers during drug treatment in living animals, and they also show how discrete intracellular molecular pools might be differentially manipulated by future anti-invasive therapies.

Keywords

Mutant p53; RhoGTPases; intravital-imaging; FLIM; FRET

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Introduction

Coordinated activity of the RhoGTPases RhoA, Rac1 and Cdc42 has been extensively studied during cell motility and migration *in vitro* (1). RhoA is known to control actomyosin-based contractility and retraction at the rear of the cell while Rac and Cdc42 regulate lamellipodia and filopodia formation at the front of the cell, respectively (2, 3). The ability to visualize the precise spatial and temporal activity of RhoGTPase family members using fluorescent reporters *in vitro* has rapidly enhanced our understanding of their distinct roles in cell motility (4-7). This approach has also provided an insight into their role in other key biological processes and diseases such as cancer (4, 8, 9). Recently, several elegant studies have focused on RhoA activity during the initial events of protrusion at the leading edge and poles of cells *in vitro* (5, 10-12). In particular, using fluorescence resonance energy transfer (FRET)-reporters of individual RhoGTPases simultaneously within the same cell, RhoA activity was shown to synchronize with leading edge protrusion both spatially and temporally (11). This, in combination with the recent manipulation of Rac1 and Cdc42 activity using light-switchable probes, has confirmed that RhoA activity plays a key role in protrusion in addition to its previously reported role in cell retraction during cell motility (12). Altered expression or activity of RhoA has been positively correlated with numerous forms of human metastatic disease (8, 13-15), and taken with these novel findings regarding RhoA activity at the cell poles during motility, prompted us to assess for the first time the spatial regulation of RhoA activity during cancer cell invasion in live animals.

Pancreatic cancer is one of the most lethal forms of human cancer, with an overall 5-year survival rate of less than 5% (16, 17). Initiating *KRAS* mutations occur in approximately 90% of human PDACs (18-20) while *TP53* mutations arise in 50-75% of human pancreatic cancers (21). Previously, we have utilized a recently developed mouse model of invasive and metastatic pancreatic cancer (22) in which Cre-Lox technology is used to target $Kras^{G12D}$ and mutant $p53^{R172H}$ to the mouse pancreas via the *Pdx1* promotor. This results in the formation of preinvasive pancreatic intraepithelial neoplasms (PanINs) which develop into invasive and metastatic PDACs (22). Using this model, we have recently demonstrated that mutations of *Trp53*, rather than loss of p53, drives invasion and metastasis in pancreatic cancer, thereby indicating a gain-of-function role for the accumulation of mutant p53 (23). Moreover, we and others have shown that mutant p53 drives invasion and metastasis in cancers other than the pancreas, including colon, lung and breast cancer (24, 25). Recently, cooperation between mutant p53 and oncogenic Ras has been shown to activate RhoA and induce cell motility *in vitro* (26). In line with this, mutant p53 has also been shown to induce the expression of the RhoA-specific guanine nucleotide exchange factor GEF-H1, a regulator of RhoA activity at the leading edge and rear of the cell during motility in a large number of cancer types, including the pancreas (27, 28).

As the $Kras^{G12D}$/ $p53^{R172H}$ mouse model (22) recapitulates human pancreatic tumourigenesis in terms of histopathology, disease progression and metastatic profile (29, 30), it provides an excellent system to examine for the first time the precise spatial regulation of RhoA activity in an *in vivo* model of invasive pancreatic cancer. In order to investigate the activity of RhoA, we initially established cells from invasive pancreatic tumors of *Pdx1-Cre, LSL-*$KRas^{G12D/+}$*, LSL-*$Trp53^{R172H/+}$ mice and corresponding non-invasive tumor cells from *Pdx1-Cre, LSL-*$KRas^{G12D/+}$*,*$Trp53^{LoxP/+}$ mice (23). From these cells, we generated stable PDAC lines expressing a modified version of the Raichu-RhoA FRET-reporter (4, 31). RhoA activity in individual cells within subcutaneous tumors was then assessed using fluorescence life-time imaging microscopy (FLIM)-FRET in live animals, allowing us to observe this phenomenon in a more physiological and functional context. Importantly, we demonstrate spatially restricted pools of RhoA activity that are specifically sensitive to drug treatment and correlate with invasion *in vivo*. This result, taken

with our assessment of RhoA activity during organotypic invasion, demonstrates significant differences in the sub-cellular spatial regulation of RhoA in three-dimensions compared to standard two-dimensional culture models. Importantly, this work highlights the advantages of live, sub-cellular, animal imaging in the assessment of invasion and metastasis and demonstrates that FLIM-FRET imaging can be adapted for the *in vivo* assessment and quantification of subtle, context-dependent responses to therapeutic intervention in the treatment of cancer.

Materials and Methods

Cell culture

Primary mouse PDACs were derived from tumors harvested from *Pdx1-Cre-GFP, LSL-KRas$^{G12D/+}$, LSL-Trp53$^{R172H/+}$* mice and *Pdx1-Cre-GFP, LSL-KRas$^{G12D/+}$, Trp53$^{LoxP/+}$* mice (23). Cell lines were tested 1 month before experiments and authenticated by morphology, growth curve analysis, and *Mycoplasma* detection in accordance with the ATCC cell line verification test recommendations and cultured as described in detail in *SI Materials and Methods*.

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 nM *in vitro* (33). ROCK inhibitor Y27632 (Calbiochem) was used at 10 μM and cell permeable C3 Transferase (Cytoskeleton) was used at 0.125 μg/ml.

Plasmids

For details of the modified GFP-RFP Raichu-RhoA reporter see *SI Materials and Methods*.

Organotypic invasion assay

Organotypic cultures were set up as described (32).

RhoA activity assay

GTP loading of RhoA was determined using a RhoA activation kit as described in manufacturers protocol (cytoskeleton).

Imaging

All imaging was performed on a Nikon Eclipse TE2000-U inverted microscope with a LaVision Biotec Trim-scope scan head. See *SI Materials and Methods*.

Fluorescence life-time imaging of RhoA FRET reporter *in vitro and in vivo*

For detailed procedure see *SI Materials and Methods*.

Data Analysis

Data was analyzed using the built-in TCSPC fluorescence lifetime analysis functionality of ImSpectorPro (LaVison Biotec, Germany). See details in *SI Materials and Methods*.

Results and Discussion

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

The use of FLIM to measure FRET reporters has rapidly enhanced our understanding of various biological processes by allowing us to obtain a detailed picture of protein behavior

both spatially and temporally *in vitro* (34, 35). In order to study RhoA activity during pancreatic cancer cell invasion *in vivo*, we first established primary invasive pancreatic tumor cell lines from *Pdx1-Cre, LSL-KRas$^{G12D/+}$, LSL-Trp53$^{R172H/+}$* mice and non-invasive pancreatic tumor cell lines from *Pdx1-Cre, LSL-KRas$^{G12D/+}$, Trp53$^{LoxP/+}$* mice (23). Using these cells we then generated stable PDAC cell lines expressing a GFP-RFP variant of the Raichu-RhoA FRET-reporter (Fig. 1A) (4, 31). The resulting invasive mutant p53^{R172H} and non-invasive p53^{fl} PDAC cells expressing the RhoA-reporter with a common initiating Kras$^{G12D/+}$ mutation (22) serve as an excellent genetic and fluorescent model system to directly compare the spatial regulation of RhoA activity in pancreatic cancer.

To examine the maximum dynamic range of the adapted Raichu-RhoA probe in its 'open' (inactive) or 'closed' (active) state we performed fluorescence life-time measurements in both invasive mutant p53^{R172H} and non-invasive p53^{fl} PDAC cells expressing both the dominant negative (T19N) and constitutively active (Q63L) versions of the probe (4). Transient over-expression, in both PDAC cell lines, of the T19N mutant which reduces the affinity of the probe to guanine nucleotides, resulted in a maximum inactive life-time of 2.2-2.3 ns (Fig. 1B). In contrast, transient over-expression of the Q63L GTPase-deficient mutant probe resulted in a maximum active life-time of 1.7-1.8 ns in both PDAC cell lines (Fig. 1B).

To assess the range of fluorescence life-time of the probe with known biochemical inactivation or activation, we serum starved cells for 48 hours to reduce RhoA activity and subjected cells to biochemical stimulation (see supplementary Fig. 1). Low, basal RhoA activity, is represented in the life-time color maps with red/yellow colors, while high RhoA activity is represented as blue colors and areas with a low signal to noise ratio in which a life-time measurement cannot be achieved are black. Stimulation with known RhoA activators such as lysophosphatidic acid (LPA) or calpeptin significantly activated the probe globally giving an average life-time of approximately 1.8 ns and 1.9 ns respectively (see supplementary Fig. 1A-C with accompanying life-time color maps and Rhotekin pull-down assay), while addition of cell-permeable C3 transferase resulted in the probe remaining in the inactive state (see supplementary Fig. 1A) (1, 36). Finally, in line with this, over-expression of a constitutively active RhoGEF, onco-Lbc (37), also significantly activated the RhoA reporter globally within the cell resulting in an average life-time of 1.7-1.8 ns (see supplementary Fig. 1A).

To assess whether fluorescence life-time imaging could be used to rapidly and accurately detect subtle changes in RhoA activity, invasive mutant p53^{R172H} PDAC cells were initially subjected to wound heal assays in which the activity of RhoA in individual cells at the leading edge of the wound was compared to confluent cells at the rear within different time frames. At early time points after wounding, small sub-populations of cells proximal to the wound edge showed significant activation of RhoA activity, as determined by low fluorescence life-time measurements resulting from FRET of the RhoA reporter (Fig. 1C, white arrows indicate active cells with a fluorescence life-time of approximately 1.8-1.9 ns which appear blue on the life-time color map). In contrast, cells at the rear of the wound had a low basal level of activity (Fig. 1D, red arrows and quantified in Fig. 1E). Importantly, using a Rhotekin-based RhoA-GTP pull down assay, the difference in RhoA regulation at the wound edge could not be detected when only one scratch wound was performed (Fig. 1F). Only when multiple wounds where performed was the activation of RhoA apparent in the Rhotekin-based pull down assay (Fig. 1F), demonstrating the advantages of single-cell fluorescence life-time imaging over standard biochemical techniques in which these effects may be masked.

The specific activation of RhoA in cells at the wound edge suggests that a gradient of RhoA activity may exist within the local environment of migrating tumors. In line with this, differential activity or expression of key proteins at the invasive edge of tumors, or in 'hot spots' within a tumor population *in vivo*, have previously been demonstrated using immunohistochemical staining of fixed tumor sections (33, 38). Consistent with this, using FRAP or photoactivation, we have recently demonstrated *in vivo* that E-cadherin-based junctions and plasma membrane dynamics, both of which play vital roles in invasion, are also locally regulated by different environmental cues within the tumor cell population (39). Using FLIM-FRET to assess protein behavior in individual cells, within living animals, during invasion would therefore provide a more detailed understanding of cell activity during this process and may ultimately contribute to the development of novel targeted anti-invasive drug therapies.

Spatial regulation of RhoA activity during three-dimensional organotypic pancreatic cancer invasion

Invasion in complex three-dimensional matrices that mimic *in vivo* conditions, such as organotypic assays, has revealed distinct modes of cell locomotion adopted by cancer cells that are governed by both the surrounding stromal cells and the density and topography of the extracellular matrix (40, 41). To determine whether RhoA plays a role in mutant $p53^{R172H}$-driven pancreatic cancer cell invasion (23, 27, 28) we examined PDAC cell invasion in a three-dimensional organotypic model. We established organotypic cultures of primary human fibroblasts and rat tail fibrillar collagen I (32, 40), which when exposed to an air/liquid interface creates a chemotactic gradient that induces invasion of overlayed cancer cells (see supplementary movie 2, cancer cells in green invading over fibroblasts in red). The invasion of mutant $p53^{R172H}$ or $p53^{fl}$ PDAC cells was assessed over an 8-12 day period, in combination with multiphoton-based second harmonic imaging of the surrounding extracellular matrix (ECM) (see supplementary movies 3-5, cancer cells in green, ECM appears purple). Consistent with our recent *in vivo* data (23), we observed that mutant $p53^{R172H}$ PDAC cells degrade and collectively invade into the matrix, while $p53^{fl}$ PDAC cells remained on top of the matrix surface (Fig. 2A,B). Quantification of the percentage of total PDAC cells in the organotypic assay that invade beyond 30 μm as a ratio of cells on top by H&E staining confirmed that expression of mutant $p53^{R172H}$ confers the capacity for PDAC cells to invade over time, while $p53^{fl}$ cells do not invade (Fig. 2C). Importantly, inhibition of ROCK, the downstream effector of RhoA, using the inhibitor Y27632 significantly reduced the invasion of mutant $p53^{R172H}$ PDAC cells in the organotypic assay at both 8 and 12 days of invasion (Fig. 2C and supplementary Fig 6). Similar results were observed upon treatment with cell-permeable C3 transferase, suggesting that activation of the RhoA pathway plays a critical role in pancreatic cancer cell invasion (Fig. 2C). A similar induction of invasion was observed when the human equivalent of murine $p53^{R172H}$ ($p53^{R175H}$) was expressed in p53 null PDAC cells (Fig. 2D, E and quantified in 2F)

To specifically investigate a role for RhoA during invasion, we accurately measured RhoA activity using FLIM-FRET at day zero and day 8 of organotypic invasion. Fluorescence life-time imaging of $p53^{fl}$ cells on the matrix surface revealed a weak basal activity of RhoA at day zero (Fig. 3D, column 1) and when exposed to a chemotactic gradient for 8 days, failed to activate RhoA above basal levels (Fig. 3A and quantified in 3D, column 2), correlating with the absence of cell invasion (Fig. 2A). Similarly, over a longer time course of up to 21 days (see supplementary Fig. 7), these cells did not invade, consistent with *in vivo* data (23). In contrast, fluorescence life-time imaging of mutant $p53^{R172H}$ PDAC cells on the matrix surface revealed an initial weak basal activity of RhoA at day zero (Fig. 3D, column 3), however when exposed to a chemotactic gradient for 8 days, cells on the matrix surface exhibit significant RhoA activation (Fig. 3B and quantified in 3D, column 4).

Multiphoton-based FLIM-FRET provides the potential to image RhoA activity at greater penetration depths (42), however previous detection efficiencies have been a limiting factor in the use of FLIM-FRET for three-dimensional investigations. To overcome this limitation, we have utilized a multi-channel TCSPC PMT as a non-descanned detector (see Materials and Methods) allowing for accurate detection and measurement of fluorescence life-times at a depth of ~ 150 μm within an organotypic matrix (Fig. 2B, red arrows depict examples of cells analyzed by FLIM-FRET at depth). Analysis of RhoA activity up to 150 μm within the matrix (Fig. 3C), demonstrated that actively invading mutant $p53^{R172H}$ cells have an enhanced average activity of RhoA compared to those present on the matrix surface (quantified in Fig. 3D, column 5). A similar increase in RhoA activity at ~ 150 μm was also observed over a longer time course of up to 12 days of invasion (see supplementary Fig. 8).

Interestingly, in the majority of invading mutant $p53^{R172H}$ cells we observed a discrete fraction of active RhoA at the pole of the cells (Fig. 3C, white arrow and quantified in 3E). Significantly, we did not observe this sub-cellular spatial regulation of RhoA activity within cells in two-dimensional culture at the rear or front of migrating cells (Fig. 1C,D). This suggests that in a three-dimensional context, RhoA may be spatially regulated at the poles of mutant $p53^{R172H}$ cells to drive protrusion.

Fluorescence life-time imaging of RhoA activity in a live animal microenvironment

Our ability to use fluorescence life-time imaging at depth to assess RhoA activity in three-dimensions and the added contextual detail this provides, prompted us to investigate whether we can assess RhoA activity under more native physiological conditions in live animals. Imaging invasion in a live animal setting is inherently difficult due to sample instability, autofluorescence, poor tissue penetration and light scattering (40, 43, 44). Recent advances in multiphoton microscopy as described here, however, have improved both the resolution and imaging depth thereby providing a powerful tool for directly observing key events in the invasive and metastatic process *in situ*. PDAC cells were therefore grown as subcutaneous tumors in nude mice and the ability of multiphoton-based FLIM to measure activity of the RhoA reporter within tumor tissue was examined (see Materials and Methods). The added sensitivity of a multi-channel TCSPC PMT detector allowed us to detect the activity of RhoA up to ~150 μm within solid tumor tissue, allowing cells completely surrounded by ECM, stroma and vasculature to be readily assessed *in vivo*.

The fluorescence life-time of the RhoA reporter in PDAC cells was quantified *in vivo* at different sites within the tumor. Consistent with our data showing that loss, as compared with mutation, of p53 in the pancreatic mouse model results in non-invasive PDAC cells (supplementary Fig. 9 and (23)), we observed low RhoA activity in $p53^{fl}$ cells *in vivo* (Fig. 4A). In contrast, we found a geometrically distinct sub-cellular fraction of active RhoA at the poles of invasive mutant $p53^{R172H}$ PDAC cells *in vivo* (Fig. 4*B* white arrows and inset compared to Fig. 4A white arrows and inset). Quantification of the sub-cellular distribution within the cell body and poles confirmed that the spatial regulation of RhoA activity was only exhibited by invasive mutant $p53^{R172H}$ PDAC cells and not $p53^{fl}$ cells (Fig. 4C). This result suggests that the fraction of active RhoA at the poles of cells *in vivo* may contribute to invasion. This observation is consistent with recent work in zebra fish, by Kardash and colleagues, where RhoA was shown to drive protrusion in polarized germ-cells at the leading edge during embryonic development (9).

In vivo spatial regulation of RhoA activity in invasive mutant $p53^{R172H}$ PDAC cells upon treatment with the anti-invasive drug dasatinib

Using a genetically engineered GFP pancreatic cancer model *Pdx1-Cre-GFP, LSL-*$KRas^{G12D/+}$*, LSL-*$Trp53^{R172H/+}$ to image invasion and metastasis *in vivo* (33) we have

recently shown that therapeutic intervention with dasatinib, a clinically approved anti-invasive Src inhibitor, significantly impairs metastatic burden in these mice (33). This, together with previous work by our group showing the reciprocal relationship between RhoGTPases and Src kinase activity during polarized cell motility (45) prompted us to assess the effect of dasatinib treatment on RhoA activity in the invasive mutant p53 PDACs both in organotypic assays and in live animals. Dasatinib treatment in the organotypic assay significantly reduced both murine $p53^{R172H}$ and human $p53^{R175H}$-driven PDAC cell invasion (Fig. 5A and B), consistent with our previous finding *in vivo* (33). We therefore went on to investigate the effect of dasatinib on invasive mutant $p53^{R172H}$ PDACs in a live animal setting. Mice bearing subcutaneous PDAC tumors were treated with 10 mg/kg of dasatinib (33, 39) for 3 days prior to fluorescence life-time imaging of RhoA activity in conjunction with second harmonic imaging of the host ECM (Fig. 6A and C shows cells in green and ECM by second harmonic imaging in purple; supplementary Fig. 10 shows dasatinib-mediated inhibition of Src activity *in vivo* by immunoblotting). Strikingly, while the polarized and protrusive morphology of mutant $p53^{R172H}$ PDAC cells remained present upon dasatinib treatment (compare green fluorescence images in Fig. 6A and C, white arrows), the sub-cellular pool of RhoA activity at the front and rear of cells, as determined using FLIM, was inactivated (compare fluorescence life-time images Fig. 6B and D, white arrows and insets). Quantification confirmed that while basal RhoA activity within the cell body of mutant $p53^{R172H}$ PDAC cells was not affected, dasatinib treatment specifically inactivated RhoA activity at the poles of cells *in vivo* (Figure 6E). Whether this polar RhoA activity is driving contraction, protrusion or both *in vivo* is currently unknown. This however could be addressed in the future by simultaneously imaging protrusion and contraction in live animals with real-time FLIM-FRET (5, 11). Moreover, it is also possible that this spatially localized inhibition may affect cell adhesion/interaction with the surrounding ECM components *in vivo* as both impairment of RhoA and Src have previously been shown to play key roles in adhesion dynamics (1, 8, 45, 46). This could therefore partially explain the reduced invasive and metastatic efficiency found in dasatinib treated mice and therefore warrants further investigation in the future (33).

As we have shown, fluorescence life-time imaging allows the differential regulation of protein activity within a tumor cell population to be individually assessed (Fig. 1). Consistent with this, we find that not all cells *in vivo* treated with dasatinib are inactive (Fig. 6F). Approximately 80% of mutant $p53^{R172H}$ cells, examined using FLIM, exhibited RhoA activity in control mice and upon dasatinib treatment we observe that approximately 20% of cells remain active within the tumors, independent of drug treatment (Fig. 6F). This is likely to be due to critical differences within the solid tumor cell mass, for example due to poor vasculature or limited perfusion and access of the drug to key areas of the tumor (47). In this regard, recent exciting work has addressed the role tumor-associated matrix and stromal tissue plays in pancreatic cancer drug delivery (48). Using the mutant $p53^{R172H}$ mouse model described here, combination therapy to deplete tumor-associated matrix and improve vasculature using the Hedgehog inhibitor IPI-926 was shown to improve the delivery and efficacy of chemotherapeutic drug treatment to solid tumors (48). As the small fraction of active cells that we observe may progress and form micro-metastases after initial therapeutic intervention and/or tumor resection, it would be beneficial to pin-point areas of poor drug delivery within different tumor micro-environments using FLIM-FRET in the future.

The adaptation of two-dimensional fluorescence techniques for intravital imaging has resulted in a new era of context-dependent *in vivo* imaging, providing a greater picture of key biological events *in situ* (39, 42, 43, 49). Here we have demonstrated the first use of FLIM-FRET to monitor molecular dynamics of RhoA in tumors upon therapeutic intervention. In doing so, we have specifically isolated at a sub-cellular level a small yet important pool of RhoA, not observed *in vitro*, that is sensitive to drug treatment and

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correlates with invasion. These differences have implications for cancer research, in which the behavior of key molecules associated with motility, invasion and metastasis *in vitro* are used to reveal basic biological mechanisms which are then extrapolated back into the human disease (39, 50). Such detailed topological analysis *in vivo* enables the detection of subtle changes in protein activity following therapeutic interventions that are intractable to *in vitro* assessment and could improve the current high attrition rate of compounds entering clinical trials in the future (50).

The distinct sub-cellular regulation of RhoA at the poles of invading cells observed here sets a precedent that other key RhoGTPases or proteins involved in invasion may also be tightly regulated in such a manner *in vivo*. The use of this technique for the assessment of other prototypical Rho family GTPases, including Rac and Cdc42, could therefore provide insight into their coordinated regulation *in vivo* not only during invasion and metastasis, but also in the context of other biological processes such as transformation, cell cycle progression, transcriptional activation and response to drug treatment (1, 15, 45). Also, as described earlier, Rho GEFs, GAPs and GDIs act as integrated upstream ‘molecular switches’, critical for control of RhoGTPases, and are commonly mutated or aberrantly regulated in many cancer types including the pancreas (8). The use of FLIM to measure FRET reporters, in conjunction with the assessment of altered Rho-signaling components, could allow us to investigate whether global or site-specific deregulation of RhoGTPase activity contributes to these cancer types.

Finally, we and others have recently shown that mutant p53 drives invasion and metastasis in pancreatic, colon, lung and breast cancer (24, 25) and that altered regulation of cell-ECM interactions via integrins seems to play a central role in driving invasion (24). As RhoA is involved in integrin inside-out and outside-in signaling (46), investigation of RhoA activity in these mutant p53-driven cancers should provide a new level of detail regarding the regulation of RhoA during cell-ECM interactions in the course of invasion *in vivo* as a potential effector of mutant p53.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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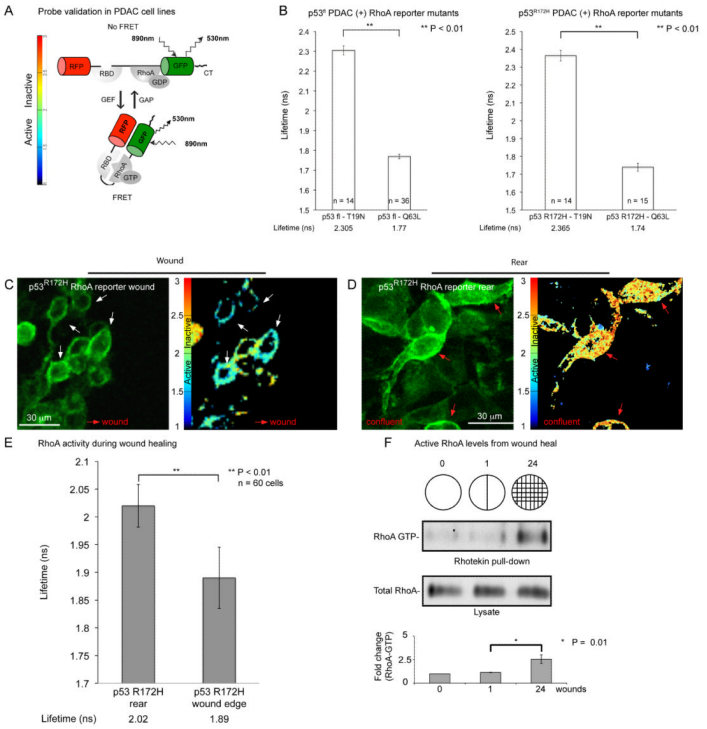

Figure 1.

Fluorescence life-time imaging of RhoA activity within a migrating pancreatic tumor cell population during wound healing. A, Schematic of the adapted GFP-RFP Raichu-RhoA reporter adapted from Yoshizaki et al (4). B, Quantification of life-time measurements in $p53^{fl}$ or $p53^{R172H}$ PDAC cells transiently transfected with dominant negative (T19N) or constitutively active (Q63L) mutants of the Raichu-RhoA reporter. C, D Representative fluorescence image of $p53^{R172H}$ PDAC cells expressing the Raichu-RhoA reporter (green) at the front or rear of a wound with corresponding life-time maps of RhoA activity. White arrows depict active cells. E, Quantification of life-time measurements of RhoA activity for cells at the front or rear of the wound. Cells were classed to be at the front of the wound within the first three cells from the wound border. F, Anti-Rho immunoblot of total cell lysate and GST-Rhotekin 'pulldown' (GTP-Rho) from confluent versus single or multiple wounded $p53^{R172H}$ PDAC cells. Columns, mean; bars, SE. **, P < 0.01

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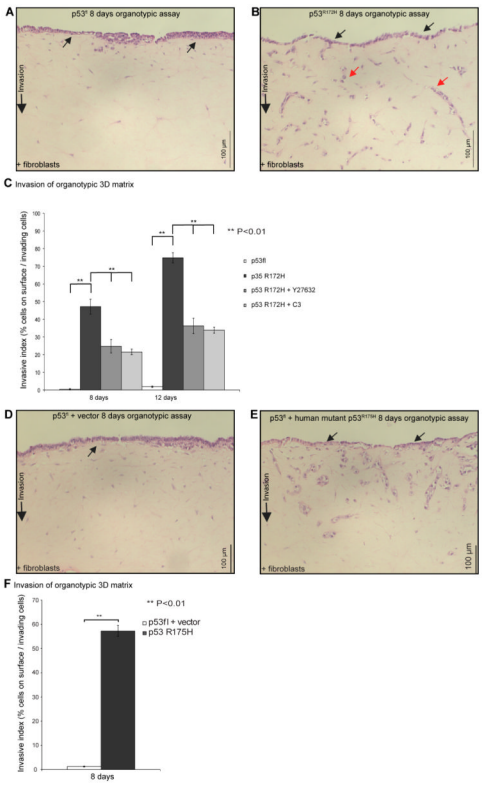

Figure 2.

RhoA activity is required for PDAC invasion. A,B H&E-stained sections of $p53^{fl}$ and $p53^{R172H}$ cells on organotypic matrix. C, Quantification of $p53^{fl}$ and $p53^{R172H}$ PDAC cell invasion ± Y27632 or cell-permeable C3 in the organotypic matrix at 8, 12 days. D,E H&E-stained sections of $p53^{fl}$ cells expressing vector or human mutant $p53^{R175H}$ cultured on organotypic matrix. Quantification of human mutant $p53^{R175H}$-driven PDAC invasion in the organotypic matrix at 8 days. Red arrows depict examples of cells assessed at depth for Fig. 3C. Columns, mean; bars, SE. **, $P < 0.01$

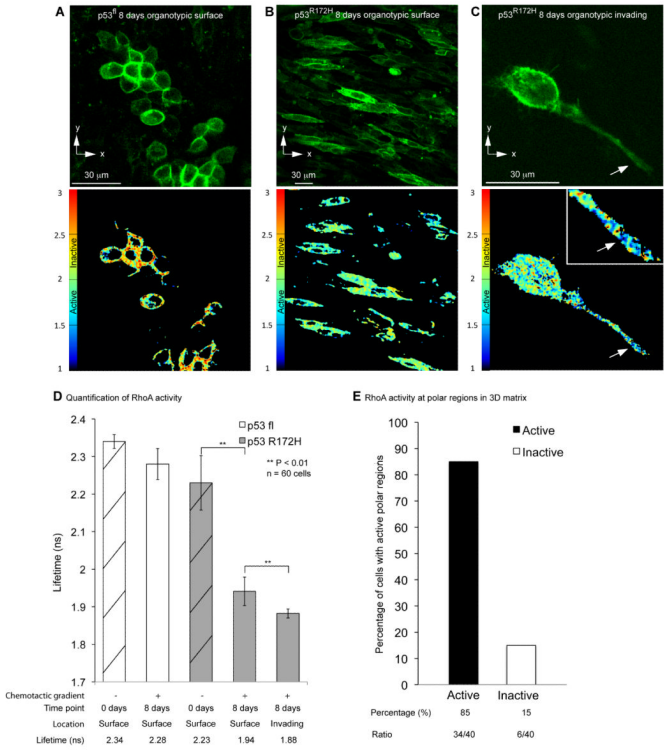

Figure 3.

Spatial regulation of RhoA activity during invasion. A-C, Representative fluorescence images of $p53^{fl}$ and $p53^{R172H}$ cells expressing the Raichu-RhoA reporter (green) on the matrix surface or during invasion with corresponding life-time map of RhoA activity. D, Quantification of life-time measurements of RhoA activity at 0 (dashed columns) and 8 days for cells on and within the matrix surface for both $p53^{fl}$ and $p53^{R172H}$ PDAC cells, respectively. E, Percentage of $p53^{R172H}$ PDAC cells demonstrating distinct RhoA activity at the poles of cells within the three-dimensional matrix. White arrow demonstrates distinct RhoA activity at the pole of the cell. Columns, mean; bars, SE. **, $P < 0.01$

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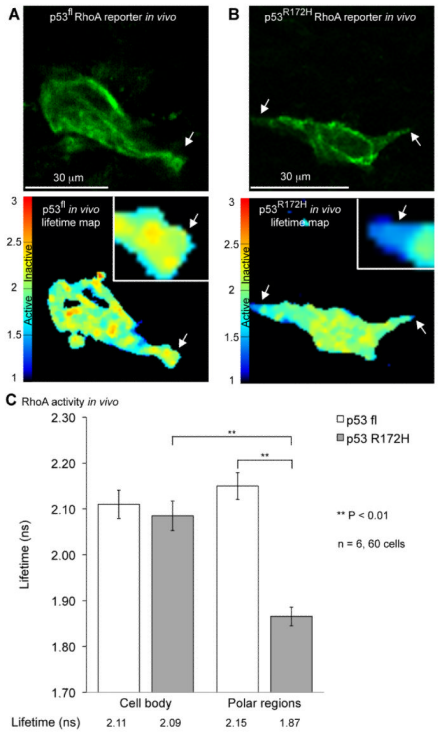

Figure 4.

Spatial regulation of RhoA activity in live animals. A,B Representative *in vivo* fluorescence images of $p53^{fl}$ and $p53^{R172H}$ PDAC cells expressing the Raichu-RhoA reporter (green) with corresponding *in vivo* life-time map of RhoA activity, respectively. C, Quantification of life-time measurements of RhoA activity within the cell body or poles of $p53^{fl}$ and $p53^{R172H}$ PDAC cells in live animals as indicated. Columns, mean; bars, SE. **, $P < 0.01$

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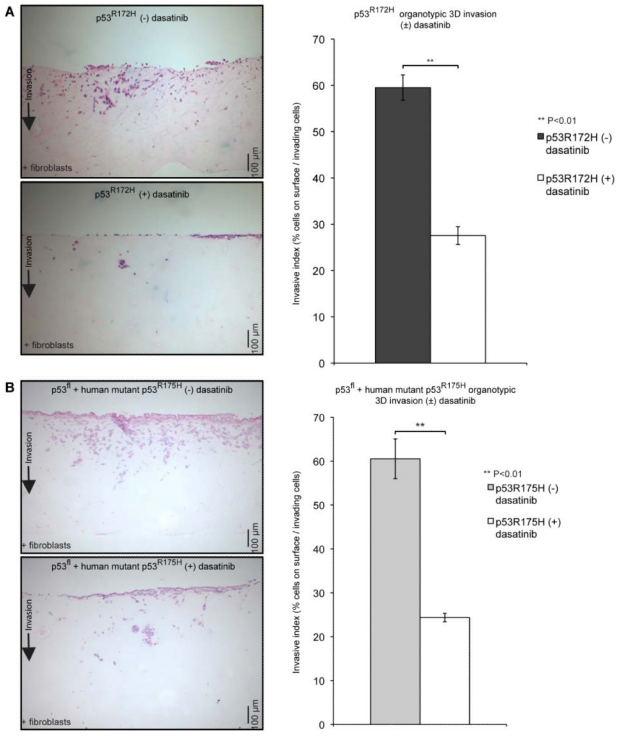

Figure 5.

Dasatinib inhibits mutant p53-driven PDAC cell invasion. A,B H&E-stained sections and quantification of mutant $p53^{R172H}$ or $p53^{R175H}$ -driven PDAC cell invasion ± dasatinib in the organotypic matrix at 8-12 days. Columns, mean; bars, SE. **, P < 0.01

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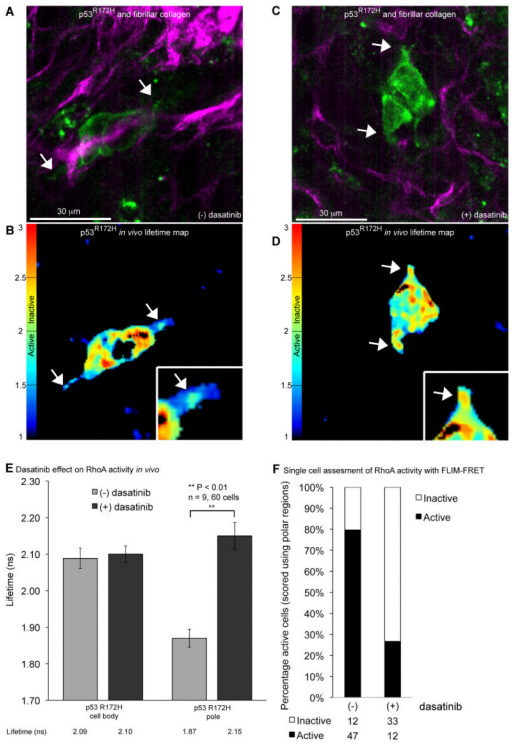

Figure 6.

Spatial regulation of RhoA activity in invasive p53^{R172H} PDAC cells upon dasatinib treatment *in vivo*. A and C, Representative *in vivo* fluorescence images of mutant p53^{R172H} PDAC cells expressing the Raichu-RhoA reporter (green) with SHG signal from host ECM components (purple). B and D, Corresponding *in vivo* life-time maps demonstrating the presence and absence of RhoA activity in sub-cellular polar regions of cells ± dasatinib, respectively. E, Quantification of fluorescence life-time measurements of RhoA activity within the cell body or poles of p53^{R172H} cells ± dasatinib. (F) Distribution of active and inactive polar regions in p53^{R172H} PDAC cells within the tumor tissue ± dasatinib. Columns, mean; bars, SE. **, P < 0.01

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