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Shedding new light on RhoA signalling as a drug target *in vivo* using a novel RhoA-FRET biosensor mouse.

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Abstract

The small GTPase RhoA is a master regulator of signalling in cell-extracellular matrix interactions. RhoA signalling is critical to many cellular processes including migration, mechanotransduction, and is often disrupted in carcinogenesis. Investigating RhoA activity in

a native tissue environment is challenging using conventional biochemical methods; we therefore developed a RhoA-FRET biosensor mouse, employing the adaptable nature of intravital imaging to a variety of settings. Mechanotransduction was explored in the context of osteocyte processes embedded in the calvaria responding in a directional manner to compression stress. Further, the migration of neutrophils was examined during *in vivo* “chemotaxis” in wound response. RhoA activity was tightly regulated during tissue remodelling in mammary gestation, as well as during mammary and pancreatic carcinogenesis. Finally, pharmacological inhibition of RhoA was temporally resolved by the use of optical imaging windows in fully developed pancreatic and mammary tumours *in vivo*. The RhoA-FRET mouse therefore constitutes a powerful tool to facilitate development of new inhibitors targeting the RhoA signalling axis.

Key words:

Intravital imaging, Rho-GTPases, pancreatic cancer, breast cancer, extracellular matrix, RhoA, FLIM, FRET, biosensors

The small GTPase RhoA has been linked to a wide range of cellular processes such as adhesion, migration, cell cycle progression, apoptosis and mechanotransduction¹. RhoA is activated by the hydrolysis of GTP and in turn modulates a number of downstream effectors². The rapid, and often fleeting, nature of this process makes it challenging to assess using conventional biochemical methods (Fig. 1A). Förster Resonance Energy Transfer (FRET) biosensors have become a vital tool to study *in vivo* protein interactions with high spatial and temporal resolution³⁻⁵ and several biosensors of RhoA regulation have been demonstrated to date.⁶⁻¹⁰ These FRET-biosensors have played a critical role in the investigation of RhoA signalling dynamics *in vitro*. Here, using fluorescence lifetime microscopy (FLIM) we monitored the changes in FRET when RhoA was activated, however, it should be noted that intensity based approaches to monitor FRET *in vivo* have also been employed recently. This type of analysis can further be performed using this biosensor mouse as previously achieved with other FRET biosensor mice.¹¹⁻¹⁵ To provide a spatio-temporal read-out of RhoA activity in native tissues we created a new RhoA-FRET mouse¹⁵ using a modified version of the Raichu-RhoA biosensor.¹⁰ Here, the original CFP/YFP fluorophore pair was replaced by EGFP and mRFP respectively^{15,16} in order to avoid potential problems with recombination from tandem repeats of related fluorescent protein sequences during mouse generation¹⁷ (Fig 1). In the RhoA-FRET ‘OFF’ mouse, the biosensor construct is flanked by a lox-stop-lox site (LSL), which allows for conditional expression of the reporter using tissue specific Cre recombinases. We then created a RhoA-FRET ‘ON’ mouse that expresses the biosensor constitutively by removing the LSL site with a deleter CMV-Cre.

RhoA has been shown to transduce mechanical stimuli from the surrounding extra-cellular matrix (ECM) via attachment sites, such as integrins, to downstream intracellular signalling pathways e.g.: ROCK.^{18,19} In particular, RhoA has been implicated in the cellular response to mechanical loading in the bone.²⁰ To explore its involvement in mechanotransduction we examined RhoA activity in osteocyte processes in their native environment embedded in canaliculi of the mouse calvaria (Fig. 1B). The application of ~1% lateral compression by an in-house compression apparatus to freshly excised calvaria of Col1a1.3.6-Cre;RhoA-FRET mice revealed activation of RhoA in processes oriented perpendicular to the direction of applied force but not in processes oriented parallel to the force or in uncompressed samples (for further details of the apparatus and compression procedure see Nobis *et al.* 2017 [15]). This illustrates an active role for RhoA in directional signal transduction of mechanostimuli, potentially mediated by the differential shear forces experienced by the dendritic processes in the fluid-filled lacunae upon application of pressure.^{21,22}

RhoA has been shown to be actively involved in the migration of cells at both the leading and retracting edges of cells²³⁻²⁵. RhoA and another small GTPase Rac1 are thought to be reciprocally active at the edge of moving cells²⁶. RhoA and Rac1 are mutually inhibitory and modelling has predicted that the RhoA-Rac1 signalling network can exhibit bistability^{27,28} with stable states associated with different modes of migration such as mesenchymal-like migration, generally driven by Rac1 and amoeboid-like migration, typically driven by RhoA depending on their surrounding microenvironment.^{27,29-32} Having previously explored the oscillatory activation of Rac1 during migration in isolated neutrophils of the Rac-FRET mouse¹⁴, we characterized the activity of RhoA here during chemotaxis *in vivo* in LysM-Cre;RhoA-FRET mice by intravital imaging. Neutrophil egression to and regression from a site of microbial infection have been described recently³³, highlighting the role of neutrophils as primary infection responders. Following enrichment of the local neutrophil population by bacterial particle inoculation of the ear, a resident dendritic cell was laser-ablated creating a damage site to which neutrophils swarm (Fig. 1B). Oscillations of RhoA activity were

observed¹⁵ and noted to be similar to those measured for Rac1 in an *in vitro* chemotaxis assay¹⁴, demonstrating the active role both small GTPases play during coordinated neutrophil migration. Furthermore, active rearrangement of the collagen network by the neutrophils during this acute damage phase was observed, suggesting a role for immune-based ECM remodelling in addition to the known role fibroblasts play in this process.^{34,35} For further and concise overview of the other applications of the biosensor in distinct tissue settings achieved previously¹⁵ see Fig. 1C.

RhoA activity also plays a key role during a variety of tissue remodelling processes and during disease progression such as cancer. In mammary tissue the downstream effector of RhoA PKN1 has been shown to play a role during gestation and lactation.³⁶ Using the conditional RhoA-FRET biosensor mouse crossed to a mammary specific Cre-driver line (MMTV-Cre), we tracked RhoA activity through the gestation cycle. This revealed an initially high RhoA activity during virgin branching morphogenesis, which progressively decreased during alveoli formation in pregnancy and in the mature milk-producing alveoli during lactation. During involution following weaning, RhoA activity was upregulated again as the alveoli break down and the gland returns to the pre-pregnancy state.¹⁵ RhoA activity was further examined during cancerous transformation of the mammary gland in a genetic polyoma-middle-T antigen (PyMT) driven breast cancer model. In this model, RhoA activity was upregulated compared to the wildtype virgin mammary gland during tumourigenesis, pointing to a potential co-option of RhoA in invasive and metastatic breast cancer.¹⁵ RhoA activity can further be tracked in PyMT driven mammary cancer during progressive stages from early adenoma, late adenoma, carcinoma and in metastases of the lung. This revealed a progressive down-regulation of RhoA activity during tumour progression (Fig. 2A). An increase of RhoA activity, however, was revealed at the invasive edges of primary PyMT tumours, again pointing to its potential co-option in the metastatic cascade of this tumour type (Fig. 2B). This is in line with similar discoveries in invasive pancreatic cancer, illustrating the role of RhoA in invasion.^{15,16} Finally, by intravenous injection of the contrast dye

(Qtracker⁶⁵⁵), as previously achieved³⁷ the local tumour vasculature was visualized and RhoA activity in cells mapped within distinct tissue regions in relation to the local vessels (Fig. 2C).

RhoA has also been shown to be activated at both the rear and leading edge of cells in a mutant p53^{R172H} driven invasive pancreatic cancer model *in vivo*.¹⁶ Activation of RhoA via its upstream regulators, such as RhoA GEF-H1, has been implicated in the progression and metastasis of pancreatic cancer, amplifying MAPK signalling.³⁸ Recently a mutant isoform $\Delta 133$ of p53 has been linked to pancreatic tumour cell invasion and metastasis via interleukin-6 activation of the JAK-STAT and RhoA-ROCK signalling pathways³⁹. This led to the utilization of the conditional RhoA-FRET biosensor mouse in tracking RhoA activity in a genetic model of pancreatic cancer with the initiating mutation of *KRas*^{G12D} and mutant p53^{R172H} driven by Pdx1-Cre (KPC), resulting in invasive pancreatic ductal adenocarcinoma (PDAC).^{40,41} This model closely recapitulate the human histopathology, where the initiating *KRAS* mutation is found in up to 90% of patients, while loss of *P53* or mutant *P53* occurs in 50-75% of tumours^{42,43}. Intravital imaging of pancreatic tissue as the disease advanced revealed a progressive inactivation of RhoA from pancreatic intraepithelial neoplasms (PanINs) to fully developed PDAC.¹⁵ In the invasive mutant p53 driven KPC model RhoA activation was observed both at the invasive edges of the primary tumour as well as at distant metastatic sites of the liver, revealing a spatially coordinated switching of activity that may facilitate cancer cell movement.¹⁵ Comparing the invasive fronts of both non-invasive KPflC (p53 null) and invasive KPC tumours revealed that an increase in RhoA activity was confined to mice carrying the mutant gain of function (GOF) p53 allele (p53^{R172H/+}) and, this was absent in mice with non-invasive PDAC driven by loss of p53 (Fig. 3A+B).^{41,44,45}

RhoA is a promising potential therapeutic target as it plays an active role in the progression and invasive potential in both mammary and pancreatic cancer models. Therapeutic intervention targeting RhoA indirectly has been shown previously, particularly targeting downstream effectors of RhoA such as ROCK1 and ROCK2, which are upregulated and associated with poor prognosis in pancreatic cancer.⁴⁶⁻⁵⁰ In our biosensor mouse study, we

tracked RhoA activity using optical windows^{51,52} where we could see a sub-organ resolution distinct difference in RhoA activation states in acinar versus PDAC cells (Fig. 3C+D). This allows for possible future studies on the role of RhoA in acinar to ductal metaplasia (ADM), which is thought to play a partial role in early phases of this disease.⁵³ We administered small molecule drugs which affect the ECM-tumour cell feedback loop, such as Src activity, integrin engagement and EGFR signalling and monitored the effect on RhoA activity. For example, RhoA activity in response to Src inhibition via the administration of the Src/Abl kinase inhibitor dasatinib was monitored over a 24h period.¹⁵ After spontaneous pancreatic tumour development at approximately 125 days in the genetically engineered KPC pancreatic cancer model, mice were engrafted with an abdominal imaging window positioned above the primary tumour. Repeated intravital imaging of the RhoA-FRET biosensor in the pancreatic KPC model upon dasatinib treatment revealed effective inhibition of RhoA activity 7h hours after the final dasatinib administration. In the PyMT-driven mammary cancer model, mammary imaging windows were implanted in the skin above the developing primary tumours after an average of 85 days. In this model the dynamics of RhoA modulation were very different from the pancreatic cancer model with RhoA inhibition observed as little as 2h post-administration.¹⁵ These differences underline the importance of pre-clinical optimisation of drug targeting in the native tissue microenvironment.⁴ The delayed RhoA inhibition in the pancreatic model may be due to delayed drug penetrance of the dense desmoplasia often found in pancreatic cancer.^{35,54} Recent studies in pancreatic cancer have aimed to reduce this fibrosis by targeting FAK, YAP/TAZ, Cdk4, PAK1, JAK/STAT and hyaluronic acid⁵⁵⁻⁶⁰ as well as characterized the cross-talk of tumour-stroma interactions of patient derived xenografts revealing potential new targets⁶¹. The RhoA-FRET biosensor mouse could prove an indispensable tool to optimise targeting of these pathways by providing a live readout of the effect of drug targeting on ECM-cancer cell reciprocity via RhoA activity. More recently, a study investigating Crohn's disease demonstrated that fibrosis in the intestinal tract caused by RhoA-ROCK pathway upregulation in myofibroblasts in Crohn's disease can be effectively reversed by ROCK inhibition⁶². Fine-tuning of this targeting could potentially be

achieved in the future with the RhoA-FRET mouse, which we previously used to observe spatial regulation of RhoA in intestinal crypts by *in vivo* imaging.¹⁵

With the use of the RhoA-FRET biosensor mouse, new insights into several key aspects of this prototypical small GTPase may be obtained in *in vivo* settings ranging from mechanotransduction, migration, ECM remodelling, cancer progression to the spatiotemporal response to drug targeting. This new biosensor mouse therefore lends itself to a wide range of applications exploring the activity of RhoA in native tissue contexts in the future and may reveal new insights into the switch-like rapid behaviour of this small GTPase *in vivo*. Other elegant approaches to imaging RhoA activation have been reported previously, such as using optogenetic activation of RhoA using the CRY2/CIBN light-gated dimerizer system. This allowed for light induced control of traction and tension within cells and their surrounding tissue.⁶³ Future applications in an intravital setting of other RhoA-biosensors that are available, such as cytoplasmic DORA sensors with RhoA binding to a PKN1 domain⁹ or the RhoA-2G biosensor that can report on GDI activity,^{6,8} have the potential to reveal intricate changes of this vital signalling node in normal and disease settings.

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Figure 1. Studying RhoA activity dynamics in a range of tissues. (A) Conventional single snap-shot based biochemical approaches to analysing RhoA activity in two examples tissues of the mammary gland and intestine. These included bead-based pulldown of RhoA-GTP in tissue lysate and a recently developed immunofluorescence of fixed tissue samples using a RhoA-GTP specific antibody.^{15,19} scale bars, 25 μ m (B) With the generation of the new RhoA-FRET biosensor mouse RhoA activity could be monitored live in osteocytes of the calvaria, *in vivo* in pancreatic ductal adenocarcinomas, mammary tumours and during neutrophil migration (RhoA-FRET biosensor, green; collagen-derived second harmonic generation (SHG) signal, magenta) with corresponding fluorescence lifetime imaging microscopy (FLIM) images of RhoA activity (high RhoA activity: blue to green; low RhoA activity: yellow to red). scale bars, 50 μ m (C) A summary of the new insights gained by the use of the new RhoA-FRET biosensor mouse in a variety of tissues and applications. Adapted from Nobis *et al.* 2017, *Cell Reports* and adapted from Servier Medial Art, licensed under the Creative Commons Attribution 3.0 Unported license (<https://creativecommons.org/licenses/by/3.0/>).

Figure 2

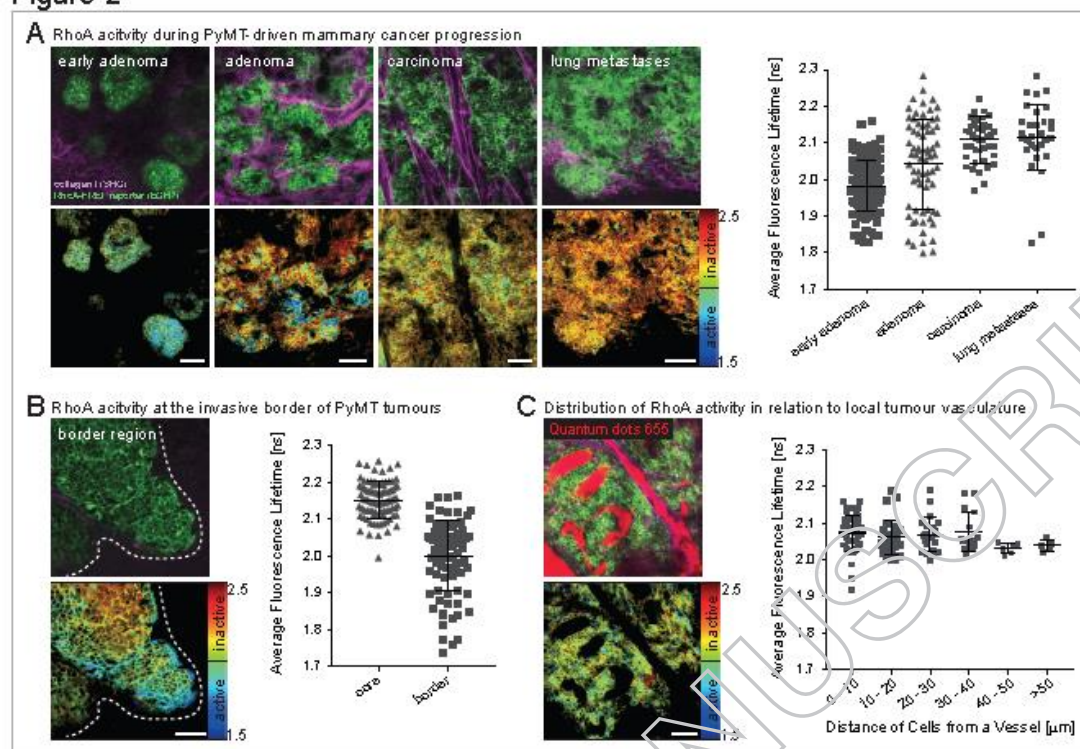


Figure 3. In vivo imaging of RhoA activity in the pancreas and KPC tumours reveals spatial activation at the invasive border of mutant p53 driven KPC tumours. (A) RhoA is inactive in non-invasive p53-null PDACs both at the tumour center and borders (white dashed line) (n = 2 mice, 163 cells). **(B)** RhoA activity is increased at the invasive border of p53 mutant (p53^{R172H/+}) tumours compared to tumour center regions (n = 2 mice, 77 cells). **(C)** Schematic of an abdominal imaging window (AIW) to examine RhoA activity in the pancreas and in primary pancreatic tumours. **(D)** RhoA activity during tumour progression of primary mutant p53 driven PDACs imaged intravitaly through optical windows (n = 3 mice, 293 cells). Columns, mean; error bars, SEM; * p < 0.05; ** p < 0.01; scale bars, 50 μm.

Figure 3

