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.

The small GTPase RhoA has been linked to a wide range of cellular processes such as adhesion, migration, cell cycle progression, apoptosis and mechanotransduction [1]. RhoA is activated by the hydrolysis of GTP and in turn modulates a number of downstream effectors [2]. 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 resolution [3–5] 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 mouse [15] 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 generation [17] (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...
Studying RhoA activity dynamics in a range of tissues

A Conventional single snap-shot biochemical approaches

![GST-Rhotekin pulldown of RhoA-GTP](image)

IP: active RhoA

Total RhoA

GAPDH

Immunofluorescence of RhoA-GTP

mammary

intestine

![mammary](image)

![intestine](image)

B Generation of a new RhoA-FRET biosensor mouse to measure RhoA activity live

![No FRET](image)

![FRET](image)

osteocyte processes

Parallel to force

Perpendicular to force

PDAC

in vivo

mammary
tumour in vivo

neutrophil
migration in vivo

neutrophils
(pre-bleach)

neutrophils
(post-bleach)

Laser ablation

ECM remodelling in response to damage

C Applications and insights gained from the new RhoA-FRET biosensor mouse

| Tissue specific context          | Novelty of approach                                                                 | Key findings                                                                                                                                 |
|----------------------------------|-------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------|
| Osteocyte mechanosensing         | Monitoring of RhoA activity achieved in a native tissue during bone compression, with distinct resolution of RhoA activity within osteocyte processes. | • Demonstration of directionality of RhoA signalling in mechanosensing of osteocytes and activation specifically in the processes of osteocytes during compression |
| Mammary gestation and cancer     | Tissue context specific tracking of RhoA activity in virgin mammary glands, during pregnancy, lactation and involution and the associated remodelling of the mammary glands, visualisation of RhoA activity in mammary tumours. | • RhoA activity is downregulated during phases of gestation and upregulated during involution in the mammary epithelium. • RhoA activity co-option in invasive mammary carcinoma. |
| Neutrophil migration             | Live imaging technique to cause laser ablation associated wound response and to monitor RhoA activity during neutrophil migration. Time-resolved tracking of RhoA activity and tissue remodelling during neutrophil swarming. | • Laser ablation induced neutrophil swarming and associated time-resolved tracking of RhoA activity oscillations in neutrophils migrating towards a tissue wound in vivo, which is of key interest to the field. |
| PDAC progression                 | Full autocrine model of pancreatic cancer recapitulating key features of the histopathology of human disease and monitoring of RhoA activity in progressive stages in PanIN 1, PanIN 2. PDAC and metastasis in a native tissue context during the entirety of the disease, in three different sets of genetic driver-mouse models (GEMM). | • RhoA signalling is downregulated in PDAC progression of both mutant Kras alone and mutant Kras + p53 null and mutant Kras + mutant p53. • RhoA activity is upregulated and co-opted in the invasive mutant p53 model both in the invasive edge and metastasis of the liver. |
| Longitudinal optical window imaging | Timecourse of drug pharmacodynamics of RhoA inhibition in genetic models of pancreatic and mammary carcinomas in the same animal by the use of optical imaging windows. Live tissue specific context dependent drug efficacy evaluation across different models of the same drug. | • RhoA activity can effectively inhibited in vivo and this inhibition can be monitored in a spatiotemporal manner by the use of optical windows with different targeting dynamics of RhoA using the same drug in full autocrine models of pancreatic and mammary carcinomas. • Demonstration of spatiotemporal regulation of RhoA activity in the crypts of the small intestine. |

Figure 1. (For figure legend, see page 3.)
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 cells [23–25]. RhoA and another small GTPase Rac1 are thought to be reciprocally active at the edge of moving cells [26]. 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 mouse [14], 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 recently [33], 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 observed [15] and noted to be similar to those measured for Rac1 in an in vitro chemotaxis assay [14], 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 previously [15] 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 (Qtracker 655), as previously achieved [37] 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 p53R172H driven invasive pancreatic cancer model in vivo [16]. Activation of RhoA via its upstream regulators, such as RhoA GEF-

Figure 1. (see previous page) 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 pull-down 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 μm (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 μm (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/).
H1, has been implicated in the progression and metastasis of pancreatic cancer, amplifying MAPK signalling [38]. Recently a mutant isoform Δ133 of p53 has been linked to pancreatic tumour cell invasion and metastasis via interleukin-6 activation of the JAK-STAT and RhoA-ROCK signalling pathways [39]. 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<sup>G12D</sup> and mutant p53<sup>R172H</sup> driven by Pdx1-Cre (KPC), resulting in invasive pancreatic ductal adenocarcinoma (PDAC) [40,41]. This model closely recapitulates 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 KPIIC (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<sup>R172H/+</sup>) 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.

Figure 2. Spatially defined RhoA activity during the progression of PyMT-driven mammary carcinomas. (A) RhoA-FRET mice crossed to MMTV-polyoma-middle-T antigen (PyMT) mice allow for the tracking of RhoA activity during the progression of invasive mammary carcinoma (n = 1 mouse, 280 cells). (B) RhoA activity is increased at the invasive borders of primary PyMT tumours (white dashed line) compared to tumour core regions (n = 1 mouse, 180 cells). (C) Intravenous injection of a contrast dye (Qtracker655) allows for monitoring of RhoA activity in cancer cells in relation to their proximity to local vasculature (n = 2 mice, 130 cells). Dots, single cells; line, mean; error bars, SD; scale bars, 50 μm.
(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 24 h 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 7 h 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 2 h 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 acid [55–60] as well as characterized the cross-talk of tumour-stroma interactions of patient derived xenografts revealing potential new targets [61]. 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

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 (p53R172H/+) 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 intravally through optical windows (n = 3 mice, 293 cells). Columns, mean; error bars, SEM; *p < 0.05; **p < 0.01; scale bars, 50 μm.
fibrosis in the intestinal tract caused by RhoA-ROCK pathway upregulation in myofibroblasts in Crohn’s disease can be effectively reversed by ROCK inhibition [62]. 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 domain [9] 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.

Disclosure of potential conflicts of interest

The authors report no financial interest or potential conflicts of interest.

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