SMALL GTPASES
2020, VOL. 11, NO. 4, 240–247
https://doi.org/10.1080/21541248.2018.1438024

Taylor & Francis
Taylor & Francis Group

COMMENTARY

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

Max Nobis [a,*], David Herrmann [a,*], Sean C. Warren [a,*], Douglas Strathdee[b], Thomas R. Cox [a], Kurt I. Anderson [c], and Paul Timpson [a]

[a]The Garvan Institute of Medical Research, St Vincent's Clinical School, Faculty of Medicine, University of New South Wales, Sydney, 2010 NSW Australia; [b]Cancer Research UK Beatson Institute, Switchback Road, Bearsden, Glasgow G611BD, UK; [c]Francis Crick Institute, London NW11AT, UK

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.

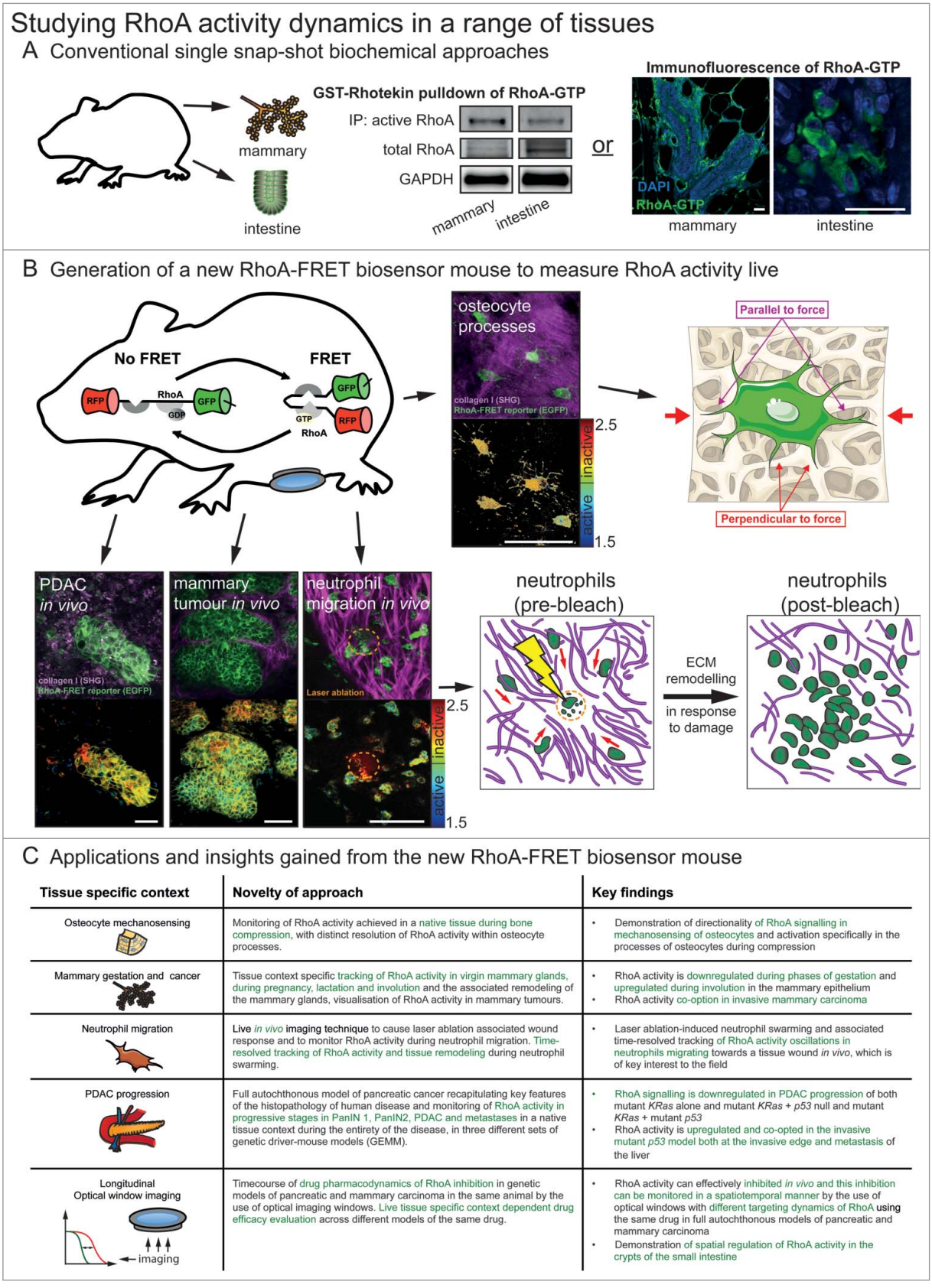
ARTICLE HISTORY
Received 4 December 2017
Accepted 2 February 2018

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

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

CONTACT A/Prof., Paul Timpson p.timpson@garvan.org.au The Garvan Institute of Medical Research, 370 Victoria Street, Darlinghurst, 2010 NSW, Australia; Prof., Kurt I. Anderson kurt.anderson@crick.ac.uk The Francis Crick Institute, 1 Midland Road, London, NW1 1AT, UK.
*Authors contributed equally to the manuscript.



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 remodeling 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 *in vivo* 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 remodeling 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 autochthonous model of pancreatic cancer recapitulating key features of the histopathology of human disease and monitoring of RhoA activity in progressive stages in PanIN 1, PanIN2, PDAC and metastases 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 at 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 carcinoma 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 autochthonous models of pancreatic and mammary carcinoma • Demonstration of spatial 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 $p53^{R172H}$ 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/).

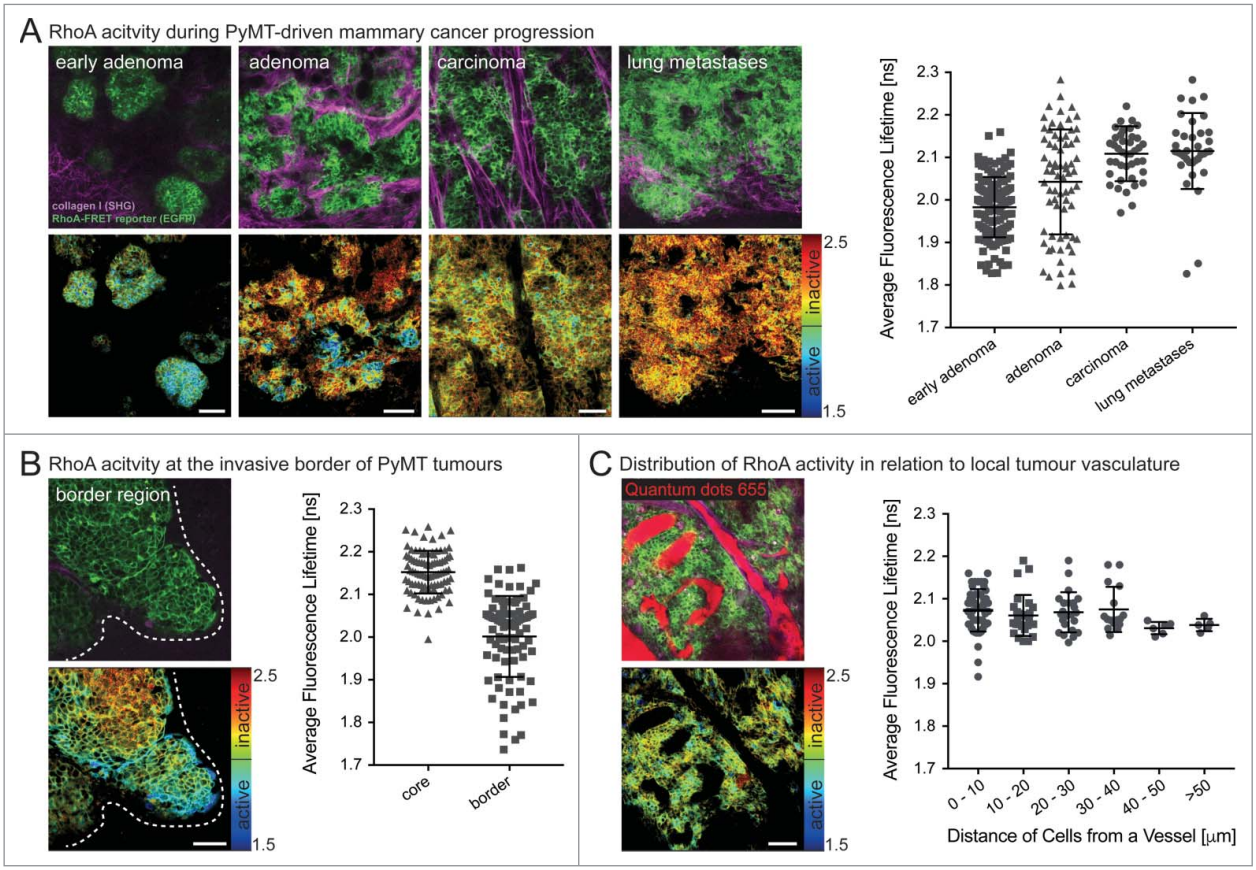


Figure 2. Spatially defined RhoA acitvty during the progression of PyMT-driven mammary carcinomas. (A) RhoA-FRET mice crossed to MMTV-polyoma-middle-T antigen (PyMT) mice allow for the tracking of RhoA activity during the progression of invasive mammary carcinoma (n = 1 mouse, 280 cells). (B) RhoA activity 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 injeciton 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.

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 $KRas^{G12D}$ and mutant $p53^{R172H}$ 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 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

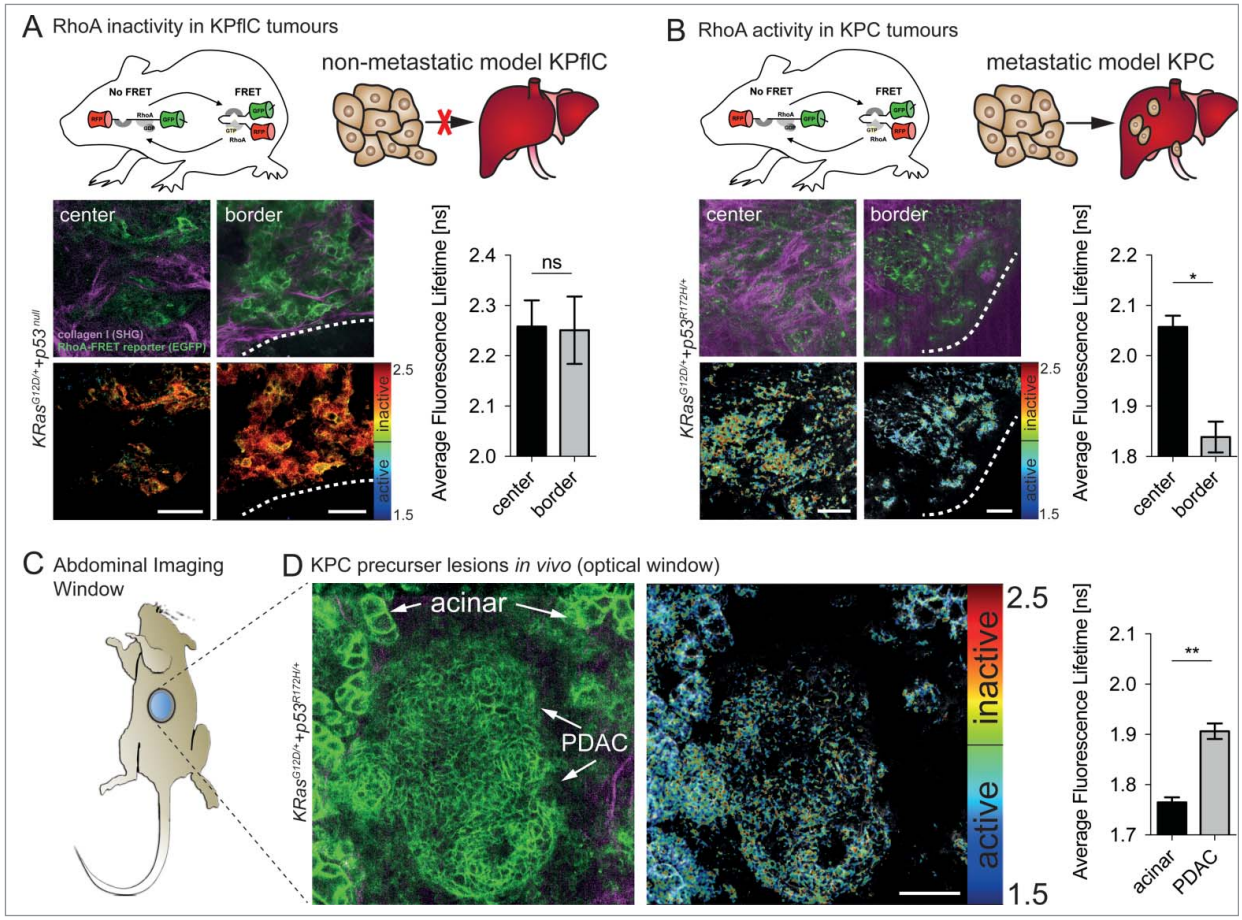


Figure 3. *In vivo* imaging of RhoA activity in the pancreas and KPC tumours reveals spatial activation at the invasive border of mutant p53 driven KPC tumours. (A) RhoA is inactive in non-invasive p53-null PDACs both at the tumour center and borders (white dashed line) (n = 2 mice, 163 cells). (B) RhoA acitvity 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 intravitally through optical windows (n = 3 mice, 293 cells). Columns, mean; error bars, SEM; $^{*}p < 0.05$; $^{**}p < 0.01$; 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

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.

Funding

MN, DH, SCW, TRC and PT were funded by an NHMRC project grant and fellowship, an ARC Future and Len Ainsworth Pancreatic Cancer Fellowship, Cancer Institute New South Wales Early Carrer Fellowship, Cancer Council NSW, Sydney Catalyst, Tour de Cure and a National Breast Cancer Foundation (NBCF) grant. KIA and DS were funded by a CRUK core grant. This project was made possible by an Avner Pancreatic Cancer Foundation Grant.

ORCID

Max Nobis http://orcid.org/0000-0002-1861-1390?
David Herrmann http://orcid.org/0000-0002-9514-7501?
Sean C. Warren http://orcid.org/0000-0002-5253-7147?
Thomas R. Cox http://orcid.org/0000-0001-9294-1745
Kurt I. Anderson http://orcid.org/0000-0002-9324-9598,?
Paul Timpson http://orcid.org/0000-0002-5514-7080

References

[1] Karlsson R, Pedersen ED, Wang Z, et al. Rho GTPase function in tumorigenesis. Biochim Biophys Acta. 2009;1796:91–8. doi:10.1016/j.bbcan.2009.03.003. PMID:19327386.
[2] Hodge RG, Ridley AJ. Regulating Rho GTPases and their regulators. Nat Rev Mol Cell Biol. 2016;17:496–510. doi:10.1038/nrm.2016.67. PMID:27301673.
[3] Conway JRW, Warren SC, Timpson P. Context-dependent intravital imaging of therapeutic response using intramolecular FRET biosensors. Methods. 2017;128:78–94. doi:10.1016/j.ymeth.2017.04.014. PMID:28435000.
[4] Conway JRW, Carragher NO, Timpson P. Developments in preclinical cancer imaging: innovating the discovery of therapeutics. Nat Rev Cancer. 2014;14:314–28. doi:10.1038/nrc3724. PMID:24739578.
[5] Nobis M, Warren SC, Lucas MC, et al. Moving molecular mobility and activity to an intravital imaging setting: implications for cancer modelling and targeting. J Cell Sci. 2018. doi:10.1242/jcs.206995.
[6] Fritz RD, Letzelter M, Reimann A, et al. A versatile toolkit to produce sensitive FRET biosensors to visualize signaling in time and space. Sci Signal. 2013;6:rs12. doi:10.1126/scisignal.2004135. PMID:23882122.
[7] Kardash E, Reichman-Fried M, Maître J-L, et al. A role for Rho GTPases and cell-cell adhesion in single-cell motility in vivo. Nat Cell Biol. 2010;12:47-53-11. doi:10.1038/ncb2003.
[8] Pertz O, Hodgson L, Klemke RL, et al. Spatiotemporal dynamics of RhoA activity in migrating cells. Nature. 2006;440:1069–72. doi:10.1038/nature04665. PMID:16547516.
[9] van Unen J, Reinhard NR, Yin T, Wu YI, et al. Plasma membrane restricted RhoGEF activity is sufficient for RhoA-mediated actin polymerization. Sci Rep. 2015;5:14693. doi:10.1038/srep14693. PMID:26435194.
[10] Yoshizaki H, Ohba Y, Kurokawa K, et al. Activity of Rho-family GTPases during cell division as visualized with FRET-based probes. J Cell Biol. 2003;162:223–32. doi:10.1083/jcb.200212049. PMID:12860967.
[11] Kumagai Y, Naoki H, Nakasyo E, et al. Heterogeneity in ERK activity as visualized by in vivo FRET imaging of mammary tumor cells developed in MMTV-Neu mice. Oncogene. 2015;34:1051–7. doi:10.1038/onc.2014.28. PMID:24632612.
[12] Yamauchi F, Kamioka Y, Yano T, et al. In vivo FRET imaging of tumor endothelial cells highlights a role of low PKA activity in vascular hyperpermeability. Cancer Res. 2016;76:5266–76. doi:10.1158/0008-5472.CAN-15-3534. PMID:27488524.
[13] Hiratsuka T, Fujita Y, Naoki H, et al. Intercellular propagation of extracellular signal-regulated kinase activation revealed by in vivo imaging of mouse skin. Elife. 2015;4:e05178. doi:10.7554/eLife.05178. PMID:25668746.
[14] Johnsson A-KE, Dai Y, Nobis M, et al. The Rac-FRET mouse reveals tight spatiotemporal control of Rac activity in primary cells and tissues. Cell Rep. 2014;6:1153–64. doi:10.1016/j.celrep.2014.02.024. PMID:24630994.
[15] Nobis M, Herrmann D, Warren SC, et al. A RhoA-FRET Biosensor Mouse for Intravital Imaging in Normal Tissue Homeostasis and Disease Contexts. Cell Rep. 2017;21:274–88. doi:10.1016/j.celrep.2017.09.022. PMID:28978480.

[16] Timpson P, McGhee EJ, Morton JP, et al. Spatial regulation of RhoA activity during pancreatic cancer cell invasion driven by mutant p53. Cancer Res. 2011;71:747–57. doi:10.1158/0008-5472.CAN-10-2267. PMID:21266354.
[17] Komatsubara AT, Matsuda M, Aoki K. Quantitative analysis of recombination between YFP and CFP genes of FRET biosensors introduced by lentiviral or retroviral gene transfer. Sci Rep. 2015;5:13283. doi:10.1038/srep13283. PMID:26290434.
[18] Lawson CD, Burridge K. The on-off relationship of Rho and Rac during integrin-mediated adhesion and cell migration. Small GTPases. 2014;5:e27958. doi:10.4161/sgtp.27958. PMID:24607953.
[19] Boyle ST, Kular J, Nobis M, et al. Acute compressive stress activates RHO/ROCK-mediated cellular processes. Small GTPases. 2018. doi:10.1080/21541248.2017.1413496.
[20] Noble BS. The osteocyte lineage. Arch Biochem Biophys. 2008;473:106–11. doi:10.1016/j.abb.2008.04.009. PMID:18424256.
[21] Burra S, Nicolella DP, Francis WL, et al. Dendritic processes of osteocytes are mechanotransducers that induce the opening of hemichannels. Proc Natl Acad Sci U S A. 2010;107:13648–53. doi:10.1073/pnas.1009382107. PMID:20643964.
[22] Thi MM, Suadicani SO, Schaffler MB, et al. Mechanosensory responses of osteocytes to physiological forces occur along processes and not cell body and require $\alpha V\beta 3$ integrin. Proc Natl Acad Sci U S A. 2013;110:21012–7. doi:10.1073/pnas.1321210110. PMID:24324138.
[23] Timpson P, Jones GE, Frame MC, et al. Coordination of cell polarization and migration by the Rho family GTPases requires Src tyrosine kinase activity. Curr Biol. 2001;11:1836–46. doi:10.1016/S0960-9822(01)00583-8. PMID:11728306.
[24] Ridley AJ. Rho GTPase signalling in cell migration. Curr Opin Cell Biol. 2015;36:103–12. doi:10.1016/j.ceb.2015.08.005. PMID:26363959.
[25] O'Connor K, Chen M. Dynamic functions of RhoA in tumor cell migration and invasion. Small GTPases. 2013;4:141–7. doi:10.4161/sgtp.25131. PMID:24025634.
[26] Machacek M, Hodgson L, Welch C, et al. Coordination of Rho GTPase activities during cell protrusion. Nature. 2009;461:99–103. doi:10.1038/nature08242. PMID:19693013.
[27] Byrne KM, Monsefi N, Dawson JC, et al. Bistability in the Rac1, PAK, and RhoA Signaling Network Drives Actin Cytoskeleton Dynamics and Cell Motility Switches. Cell Syst. 2016;2:38–48. doi:10.1016/j.cels.2016.01.003. PMID:27136688.
[28] Hetmanski JHR, Zindy E, Schwartz J-M, et al. A MAPK-Driven Feedback Loop Suppresses Rac Activity to Promote RhoA-Driven Cancer Cell Invasion. PLoS Comput Biol. 2016;12:e1004909. doi:10.1371/journal.pcbi.1004909. PMID:27138333.
[29] McGhee EJ, Morton JP, Von Kriegsheim A, Schwarz JP, et al. FLIM-FRET imaging in vivo reveals 3D-environment spatially regulates RhoGTPase activity during cancer cell invasion. Small GTPases. 2011;2:239–44. doi:10.4161/sgtp.2.4.17275 doi:10.4161/sgtp.2.4.17275. PMID:22145098.
[30] Hirata E, Yukinaga H, Kamioka Y, et al. In vivo fluorescence resonance energy transfer imaging reveals differential activation of Rho-family GTPases in glioblastoma cell invasion. J Cell Sci. 2012;125:858–68. doi:10.1242/jcs.089995. PMID:22399802.
[31] Sahai E, Marshall CJ. RHO-GTPases and cancer. Nat Rev Cancer. 2002;2:133–42. doi:10.1038/nrc725. PMID:12635176.
[32] Friedl P, Alexander S. Cancer invasion and the microenvironment: plasticity and reciprocity. Cell. 2011;147:992–1009. doi:10.1016/j.cell.2011.11.016. PMID:22118458.
[33] Hampton HR, Bailey J, Tomura M, et al. Microbe-dependent lymphatic migration of neutrophils modulates lymphocyte proliferation in lymph nodes. Nat Commun. 2015;6:7139. doi:10.1038/ncomms8139. PMID:25972253.
[34] Bonnans C, Chou J, Werb Z. Remodelling the extracellular matrix in development and disease. Nat Rev Mol Cell Biol. 2014;15:786–801. doi:10.1038/nrm3904. PMID:25415508.
[35] Vennin C, Murphy KJ, Morton JP, et al. Reshaping the tumor stroma: emerging therapies in pancreatic cancer. Gastroenterology. 2017. pii: S0016-5085(17)36738-0. doi:10.1053/j.gastro.2017.11.280. PMID:29287624.
[36] Fischer A, Stuckas H, Gluth M, et al. Impaired tight junction sealing and precocious involution in mammary glands of PKN1 transgenic mice. J Cell Sci. 2007;120:2272–83. doi:10.1242/jcs.03467. PMID:17591691.
[37] Nobis M, McGhee EJ, Morton JP, et al. Intravital FLIM-FRET imaging reveals dasatinib-induced spatial control of Src in pancreatic cancer. Cancer Res. 2013;73:4674–86. doi:10.1158/0008-5472.CAN-12-4545. PMID:23749641.
[38] Cullis J, Meiri D, Sandi MJ, et al. The RhoGEF GEF-H1 is required for oncogenic RAS signaling via KSR-1. Cancer Cell. 2014;25:181–95. doi:10.1016/j.ccr.2014.01.025. PMID:24525234.
[39] Campbell H, Fleming N, Roth I, et al. Δ133p53 isoform promotes tumour invasion and metastasis via interleukin-6 activation of JAK-STAT and RhoA-ROCK signalling. Nat Commun. 2017; accepted. doi:10.1038/s41467-017-02408-0.
[40] Hingorani SR, Wang L, Multani AS, et al. Trp53R172H and KrasG12D cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice. Cancer Cell. 2005;7:469–83. doi:10.1016/j.ccr.2005.04.023. PMID:15894267.
[41] Morton JP, Timpson P, Karim SA, et al. Mutant p53 drives metastasis and overcomes growth arrest/senescence in pancreatic cancer. Proc Natl Acad Sci U S A. 2010;107:246–51. doi:10.1073/pnas.0908428107. PMID:20018721.
[42] Bardeesy N, DePinho RA. Pancreatic cancer biology and genetics. Nat Rev Cancer. 2002;2:897–909. doi:10.1038/nrc949. PMID:12459728.
[43] Biankin A V., Waddell N, Kassahn KS, et al. Pancreatic cancer genomes reveal aberrations in axon guidance pathway genes. Nature. 2012;491:399–405. doi:10.1038/nature11547. PMID:23103869.
[44] Muller PAJ, Caswell PT, Doyle B, et al. Mutant p53 Drives Invasion by Promoting Integrin Recycling. Cell. 2009;139:1327–41. doi:10.1016/j.cell.2009.11.026. PMID:20064378.
[45] Trinidad AG, Muller PAJ, Cuellar J, et al. Interaction of p53 with the CCT complex promotes protein folding and

wild-type p53 activity. Mol Cell. 2013;50:805–17. doi:10.1016/j.molcel.2013.05.002. PMID:23747015.

[46] Vennin C, Chin VT, Warren SC, et al. Transient tissue priming via ROCK inhibition uncouples pancreatic cancer progression, sensitivity to chemotherapy, and metastasis. Sci Transl Med. 2017;9:eaai8504. doi:10.1126/scitranslmed.aai8504. PMID:28381539.

[47] Rath N, Morton JP, Julian L, et al. ROCK signaling promotes collagen remodeling to facilitate invasive pancreatic ductal adenocarcinoma tumor cell growth. EMBO Mol Med. 2017;9:198–218. doi:10.15252/emmm.201606743. PMID:28031255.

[48] Rath N, Olson MF. Rho-associated kinases in tumorigenesis: re-considering ROCK inhibition for cancer therapy. EMBO Rep. 2012;13:900–8. doi:10.1038/embor.2012.127. PMID:22964758.

[49] Whatcott CJ, Ng S, Barrett MT, et al. Inhibition of ROCK1 kinase modulates both tumor cells and stromal fibroblasts in pancreatic cancer. PLoS One. 2017;12:e0183871. doi:10.1371/journal.pone.0183871. PMID:28841710.

[50] Liu S, Goldstein RH, Scepansky EM, et al. Inhibition of Rho-associated kinase signaling prevents breast cancer metastasis to human bone. Cancer Res. 2009;69:8742–51. doi:10.1158/0008-5472.CAN-09-1541. PMID:19887617.

[51] Ritsma L, Steller EJA, Ellenbroek SIJ, et al. Surgical implantation of an abdominal imaging window for intravital microscopy. Nat Protoc. 2013;8:583–94. doi:10.1038/nprot.2013.026. PMID:23429719.

[52] Ritsma L, Steller EJA, Beerling E, et al. Intravital microscopy through an abdominal imaging window reveals a pre-micrometastasis stage during liver metastasis. Sci Transl Med. 2012;4:158ra145. doi:10.1126/scitranslmed.3004394. PMID:23115354.

[53] Wauters E, Sanchez-Arévalo Lobo VJ, Pinho A V., et al. Sirtuin-1 regulates acinar-to-ductal metaplasia and supports cancer cell viability in pancreatic cancer. Cancer Res. 2013;73:2357–67. doi:10.1158/0008-5472.CAN-12-3359. PMID:23370328.

[54] Neesse A, Krug S, Gress TM, et al. Emerging concepts in pancreatic cancer medicine: targeting the tumor stroma. Onco Targets Ther. 2013;7:33–43. doi:10.2147/OTT.S38111. PMID:24379681.

[55] Jiang H, Hegde S, Knolhoff BL, et al. Targeting focal adhesion kinase renders pancreatic cancers responsive to checkpoint immunotherapy. Nat Med. 2016;22:851–60. doi:10.1038/nm.4123. PMID:27376576.

[56] Chakraborty S, Njah K, Pobbati A V., et al. Agrin as a Mechanotransduction Signal Regulating YAP through the Hippo Pathway. Cell Rep. 2017;18:2464–79. doi:10.1016/j.celrep.2017.02.041. PMID:28273460.

[57] Chou A, Froio D, Nagrial AM, et al. Tailored first-line and second-line CDK4-targeting treatment combinations in mouse models of pancreatic cancer. Gut. 2017; pii: gutjnl–2017–315144. doi:10.1136/gutjnl-2017-315144. PMID:29080858.

[58] Yeo D, Phillips P, Baldwin GS, et al. Inhibition of group 1 p21-activated kinases suppresses pancreatic stellate cell activation and increases survival of mice with pancreatic cancer. Int J cancer. 2017;140:2101–11. doi:10.1002/ijc.30615. PMID:28109008.

[59] Wörmann SM, Song L, Ai J, et al. Loss of P53 Function Activates JAK2-STAT3 Signaling to Promote Pancreatic Tumor Growth, Stroma Modification, and Gemcitabine Resistance in Mice and Is Associated With Patient Survival. Gastroenterology. 2016;151:180–193.e12. doi:10.1053/j.gastro.2016.03.010. PMID:27003603.

[60] Hingorani SR, Zheng L, Bullock AJ, et al. HALO 202: Randomized Phase II Study of PEGPH20 Plus Nab-Paclitaxel/Gemcitabine Versus Nab-Paclitaxel/Gemcitabine in Patients With Untreated, Metastatic Pancreatic Ductal Adenocarcinoma. J Clin Oncol. 2018;36:359–66. doi:10.1200/JCO.2017.74.9564.

[61] Nicolle R, Blum Y, Marisa L, et al. Pancreatic Adenocarcinoma Therapeutic Targets Revealed by Tumor-Stroma Cross-Talk Analyses in Patient-Derived Xenografts. Cell Rep. 2017;21:2458–70. doi:10.1016/j.celrep.2017.11.003. PMID:29186684.

[62] Holvoet T, Devriese S, Castermans K, et al. Treatment of Intestinal Fibrosis in Experimental Inflammatory Bowel Disease by the Pleiotropic Actions of a Local Rho Kinase Inhibitor. Gastroenterology. 2017;153:1054–67. doi:10.1053/j.gastro.2017.06.013. PMID:28642198.

[63] Valon L, Marín-Llauradó A, Wyatt T, et al. Optogenetic control of cellular forces and mechanotransduction. Nat Commun. 2017;8:14396. doi:10.1038/ncomms14396. PMID:28186127.