Mechanisms and functions of the nucleus as a mechano-controller of cell contractility and migration plasticity

PhD thesis in Photonics

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A mio nonno,
Abstract

Living tissues are crowded and dynamic environments, in which signalling molecules and physical forces constantly act on single cells. To ensure correct tissue development and homeostasis, cells function like small processors: they measure and integrate the various mechano-chemical inputs they receive from their surrounding. As an output, cells translate this information into specific signalling pathways controlling their behavior, cell specification or their physical properties, among others. In particular, as tissues are built, when external stresses are applied, or when cells rearrange and move, single cells can undergo dynamic shape deformations. Previous studies showed that large cell deformations in confined environments control cellular contractility by tuning the activity of myosin II motor proteins and can transform various cell types into a novel amoeboid phenotype, termed stable-bleb. Still, how single cells can sense shape changes and, as a consequence, tune myosin II activity and cell behaviour remained unknown.

Here, by combining planar micro-confinement assays with live cell fluorescence microscopy and quantitative image analysis, we performed a systematic study to characterize the response of progenitor stem cells derived from zebrafish embryos to mechanical shape deformations. By quantifying cellular contractility levels in various conditions and by interfering with specific signalling pathway, we then aimed to identify the mechano-sensitive mechanism that allows cells to sense and respond to shape changes. We found that cells can measure different degrees of confinement, which accordingly defines their contractility set-point. We discovered that the nucleus, the biggest cellular organelle, acts as an intracellular mechano-sensor for large cell shape changes. Nucleus deformation induced an unfolding of the inner nuclear membrane, which controls the activity of cytosolic phospholipase A2 (cPLA$_2$) in the nucleus. When active, cPLA$_2$ triggers the release of arachidonic acid that activates myosin II through the Rho/ROCK pathway. As a result, the nucleus allows single cells to accurately and dynamically sense shape deformations and controls cellular contractility and migration plasticity under external force load. This process further equips cells with an "escape reflex mechanism" that allows migration away from confined environments. Moreover, the combination of inner nuclear membrane unfolding and intracellular nucleus positioning, allows cells to sense and distinguish different shape deformations, as anisotropic cell compression versus isotropic swelling, through the same mechano-sensitive pathway. Our data support that the nucleus establishes a functional module for cellular mechano-transduction, enabling cells to sense and interpret different types of shape changes and to dynamically adapt their behavior to mechanical forces in the 3D microenvironment.
Resumen

Los tejidos son estructuras compactas y dinámicas, en las que moléculas de señalización y fuerzas físicas actúan constantemente sobre células individuales. Para garantizar el correcto desarrollo y homeostasis de los tejidos, las células funcionan como pequeños procesadores: miden e integran las diversas señales mecano-químicas que reciben de su entorno. Como resultado, las células traducen esta información en vías de señalización específicas que controlan su comportamiento, la especificación celular o sus propiedades físicas, entre otras. Concretamente, células individuales pueden experimentar deformaciones dinámicas de su morfología durante distintos procesos, ya sea durante la formación de los tejidos, la aplicación de tensiones externas o la reorganización y motilidad celular. Según estudios anteriores, las deformaciones celulares causadas por entornos confinados controlan la contractilidad celular mediante la regulación de la actividad de la proteína miosina II. De este modo, las células adoptan un fenotipo ameboide, denominado stable-bleb. Éste fenómeno se observó en varios tipos celulares pero aún se desconoce cómo las células individuales pueden percibir los cambios de forma y, en consecuencia, ajustar la actividad de la miosina II y su comportamiento.

En este trabajo, hemos combinado el micro-confinamiento planar de células vivas con microscopía fluorescente y el análisis cuantitativo de las imágenes para realizar un estudio sistemático de caracterización de la respuesta celular a deformaciones mecánicas en células embrionarias de pez cebra. Mediante la cuantificación de los niveles de contractilidad celular en diversas condiciones y la interferencia con vías de señalización específicas, pretendemos identificar el mecanismo mecanosensitivo que permite a las células detectar y responder a los cambios de forma. Los resultados nos mostraron que las células pueden medir diferentes grados de confinamiento y en consecuencia ajustar sus contractilidad. Descubrimos que el núcleo, el mayor orgánulo celular, actúa como un mecanosensor intracelular para grandes cambios de forma celular. La deformación del núcleo induce el despliegue de la membrana nuclear interna, que controla la actividad de la fosfolipasa A2 citosólica (cPLA₂) en el núcleo. Cuando se encuentra activo, el cPLA₂ desencadena la liberación de ácido araquidónico que a su vez activa la miosina II a través de Rho/ROCK. Como resultado, el núcleo permite a la célula percibir sus forma y deformaciones dinámicas, y controlar la contractilidad celular y la plasticidad de la migración bajo fuerzas externas. Este proceso, además, dota a las células con un "reflejo de fuga" que permite la migración fuera de entornos confinados. Además, la combinación del despliegue de la membrana nuclear interna con el posicionamiento intracelular del núcleo, permite a las células percibir y distinguir diferentes tipos de...
deformación, tales como la compresión anisotrópica o la hinchazón isotrópica, a través de la misma vía mecanosensitiva. Nuestros datos sugieren que el núcleo establece un módulo funcional para la mecano-transducción celular, permitiendo a las células percibir e interpretar diferentes tipos de deformación y adaptar su comportamiento de manera dinámica frente a las fuerzas mecánicas de su microambiente.
Acknowledgments

Before starting a journey through mechanobiology, I have to acknowledge the people who made this work actually possible and made me enjoy this passionate but crazy journey in science and life.

First of all I want to thank my supervisors Verena Ruprecht and Stefan Wieser for giving me the opportunity of working on this incredible project and even more for their constant support and guidance. When I started my PhD I didn’t know what myosin was, I almost didn’t know cells could move and especially I had no idea I loved squeezing them on top of microscopes. Anyway, you believed in me and guided me through this crazy journey with your incredible passion for science, endless curiosity and strong motivation. These years were intense, but by looking backwards I am really glad I decided to join your groups as the first PhD student and I am happy of the person and scientist I am today, thank you for everything.

Thanks to all the CRG lab members. Thanks for crossing the fish for me, for waiting for the embryos to lay together; thanks for the chocolate shared, chats and laughs; thanks for the constant emotional support and friendship. You are the coolest group of colleagues someone can wish for.
 Thank you Fabio for being "my partner in science" and my biologist of trust. It was fun to have someone to squeeze the cells with and I am glad that person was you. I will miss doing science together.
 Thanks Senda for the incredible help during the last years and for answering to every stupid biological question I had. The entire lab would be lost without you.

Thanks to the best students ever, Ivan and Mariona, for teaching me so much. I am honored I worked with you and so proud of the persons and scientists you are becoming.

Thanks Juanma for teaching me everything about the confiner and PDMS and for all the scientific discussions and fun we had.

Thanks to all SLN members, current and past, for our fun and overly crowded lunch breaks, for the technical and emotional support. In particular, thanks to Maria and Jordi, for helping me everyday, especially when the microscope was literally on fire, beeping or fully dead. Without the two of you, today I would probably still be restarting the SP5, over and over again. Thanks Marina for being a good and supportive friend and vermut partner, I hope everyone has a next-door friend and colleague like you
during their PhD.

Thanks to all collaborators, especially Frederic for starting the challenging tweezer-nuclei collaboration together, for the hard work and for the fun we had while pushing nuclei even when things were not working.

Thanks to the amazing staff of all ICFO facilities: bio-lab, electrical and mechanical workshops. You are the ones who solved most of my daily problems.

Thanks to my parents for being my greatest fan and for your unconditional support. You taught me the most important things in life and made me become the person I am today.

Thanks to all my friends and colleagues I shared this last five years with, you made me have fun during the most stressful days.

Finally, I have to thank all the zebrafish embryos who never became fish, used to get cells to squeeze on top of microscopes. Without you, this thesis would not have been physically possible.

Last, but not least, thank you - the reader - for reading this long thesis. I hope I will convince you that squeezing cells is really cool or at least show you how much I loved it, enjoy the read.
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Part I

Introduction
Chapter 1

Mechanical forces in biology

The understanding of how biological complexity can arise from a single fertilized cell to form an embryo has been - and still is - challenging a broad range of scientists. Our current knowledge sets its basis on the pioneering work by D'Arcy Wentworth Thompson published in 1917 On Growth and Form and on the paper of Alan Turing, The Chemical Basis of Morphogenesis from 1952. Both works established the idea that biochemistry and physics (mechanics) together control tissue morphogenesis.

The interconnection in between mechanics and biochemistry relies on the ability of cells to (1) actively change shape and generate forces through the actomyosin cortex, and (2) to sense the physical information of their environment and transduce it into signalling pathways that control a cells' state. At this interconnection the interdisciplinary field of mechanobiology emerged, connecting concepts and methods from physics with biology in order to understand how forces control biological processes from the molecular to the organism level. In this work we will specifically focus on how mechanical forces shape cellular process.

In the developing embryo, gradients of morphogens, which can be described by reaction-diffusion equations, dictate cell fate specification and can explain the appearance of periodic patterns in nature [1, 2]. At the same time, intrinsic and extrinsic forces play a role in shaping the organism [3, 4]. Referring to single cells, intrinsic forces are those originated within the cell itself as its shape changes because of cell division or polarization, for example. Cells can also experience extrinsic forces, that can arise within tissues as due to crowding or tissue-scale rearrangements. In both cases, when a cell or an organelle within a cell is subjected to a force, its shape will change. Hence, to understand how forces shape cellular processes, we need to understand how cells sense their own shape and deformations.

The next Chapters will introduce the main question of this thesis: "How do single cells sense shape deformations?". The actomyosin cortex will be described in Chapter 2. This is the structure that gives rigidity to cells and tissues, allows for shape changes and force generation and defines their mechanical properties. Different types
and mechanisms of cell migration will be briefly introduced in Chapter 3. Then, an introduction to cellular mechanosensation and mechanotransduction, with specific examples of mechanosensor elements and involved processes, will be given in Chapter 4. Finally, the general aim, specific questions and structure of this thesis is presented.
Chapter 2

The actomyosin cytoskeleton

2.1 Actin and Myosin

The cytoskeleton, composed by actin, microtubules and intermediate filaments, is the structural scaffold of a cell and controls its mechanical properties [5]. Filaments can build dynamic interconnected ensembles and networks that define a cell’s shape, providing rigidity and allowing for dynamic shape changes. The cytoskeleton spans the entire volume of the cells, connecting the cell membrane with the interior, organizing the cytoplasm and allowing for cytoplasmic transport through molecular motors [5].

The actomyosin cortex, a thin network located below the cell’s membrane and bound to it, in particular, enables cells to actively generate forces and move. The cortex can in fact extend, thanks to net actin polymerization, and contract, thanks to myosin II motor proteins. Actomyosin networks are contractile meshes with a typical mesh size of $\leq 100\text{nm}$ [6], a value that can vary depending on the local composition of the cortex or cell line [7]. Its thickness is reported to be $\sim 200\text{nm}$ in mitotic Hela cells [8] and interface dendritic cells [9]. Fluctuations in the lateral and transversal dimensions are associated with myosin II activity that can induce local mechanical instabilities as a consequence of local anisotropies in cell contractility and can generate cortical flow [9,10]. Globally, the actomyosin cortex can be described as a 2D thin active viscoelastic gel [11]. Active gel theory can explain large-scale actomyosin flows [11], such as the ones observed in development [11] or at the leading edge of migrating cells [12]. Flows arise from tension gradients [11] or an asymmetry in cortical actomyosin density [13] and depend on network turnover rate and geometry [14]. At steady-state, the actomyosin network is highly dynamic [15] and the rapid turnover of its components balances the diffusive flux [14]. The actin filaments continuously depolymerize and polymerize, at a timescale that ranges from tens of seconds to minutes in cells [7,9] and in reconstituted networks in vitro [14,15]. The turnover is what limits the ability of the network to store energy and defines its viscoelastic nature [7,11]. As a consequence, forces applied over timescales longer than the turnover time are dissipated [11], as shown during micropipette aspiration experiments [16]. The stress-relaxation timescale defines a threshold (in time) above which mechanical perturbations induce flows and below which the
cortex responds elastically stores energy [11]. However, the exact molecular players that define the network turnover time are not yet fully known [7].

**Actin** is the most abundant protein in eukaryotic cells and it is highly conserved across species [17]. Actin monomers (or globular actin, G-actin) can assemble into a helical polymer filament (4 – 7 nm in thickness) commonly named F-actin (filamentous actin). An ensemble of filamentous structures gives rise to the cell cortex as well as stress fibers or cellular protrusions that allow cells to migrate or to engulf pathogens or particles [17–19]. Filaments have a plus end, where globular actin monomers are added, and a minus end, where primarily de-polymerization takes place. Dynamic actin turnover and its cortical organization are tightly controlled by a diverse class of more than 100 actin binding and regulatory proteins [17] and define the morphology and biomechanical properties of a cell. For example, actin can establish spiky structures, such as the ones observed in filopodia, mediated by formins, which promote filament elongation, as shown in Figure 2.1 A. Branched networks are the result of actin nucleation by the promoting factor ARP2/3, that branches growing actin filaments inducing a 70° angle in between them, favouring the appearance of thin and fan-shaped lamellipodial structures (Figure 2.1 B) [20].

**Myosin(s)** are a super-family of actin-dependent motor proteins that can be divided into various classes (in human myosins are encoded by 38 genes divided into 12 classes), shared in all eukaryotes [21]. All myosins share a motor domain, which is the most conserved domain among the protein family, that can bind to actin filaments and generate movement via ATP hydrolysis [22–25]. The chemical energy of ATP is converted into mechanical force to walk along, slide or produce tension on actin filaments [23,24]. This simple process is at the base of muscle contraction and has a central role in non-muscular cells for intracellular organization, cell division and migration [23–25], among others.

The duration of the ATPase cycle and the structure of the tail domain of the protein, that can vary across the different classes, define the individual function and behavior of each class of motors [21,23]. In particular, the duration of the ATPase cycle controls for how long myosin is bound to actin (duty ratio). For example, myosin II has a
Chapter 2. The actomyosin cytoskeleton

low duty ratio and each motor spends a short time associated with actin [21, 23]. Conversely, myosin V has a long duty ratio and can move on actin filaments for many subsequent steps without detaching [23]. The tail domain controls the binding of the motor to other proteins and therefore controls specific motor protein function and localization in the cell [21]. As a consequence, myosin II monomers dimerize while myosin V or VI do not, but bind to specific adaptor proteins and are often associated with cargoes [23]. Because of their sliding behavior and their specific tails, these motors act as processive transporters and can move melanosomes or endosomes inside the cell [21]. Also, myosin I has several additional actin-binding sites in its tail and can contribute to actin filament nucleation and can directly bind to lipids in the plasma membrane where it can function as a strain sensor [21, 23].

The myosin-2 subfamily, usually referred as "conventional myosins", is the largest class of myosins and it includes both muscular (cardiac, skeletal and smooth muscle myosins) and non-muscle myosin II (NM-II) [22, 24]. The regulation and organization of NM-II with respect to actin determines cellular shape changes and allows for force generation during cell division, in adhesions and during cell migration [24, 25].

2.1.1 Non-muscle myosin II: structure and kinetics

NM-II is expressed in all cell types and tissues where it dynamically associates with actin filaments as a cross-linker and can generate contractile forces [24]. NM-II has a hexameric molecular structure characterized by two heavy chains (230kDa), two regulatory light chains (RLC) of 20kDa, controlling motor activity by phosphorylation, and two essential light chains (17kDa) that stabilize the structure of the heavy chain [24], as shown in Figure 2.2. The globular head domains, that contain both the actin and ATP binding sites, are followed by neck regions where the two light chains bind (Figure 2.2) [24].

The activation of myosin II is regulated by different kinases such as the Rho-associated protein kinase (ROCK), discussed in Chapter 2.2, or the Myosin light chain kinase (MLCK) that controls the phosphorylation state of the RLC on Ser19 (serine 19) and Thr18 [24]. These two phosphorylation sites are commonly used as reporters for myosin activity, such as when performing immuno-labeling against phospho-myosin. Additional motor protein phosphorylations can happen either in the RLC or in the heavy chain and further increase the ATPases activity [21]. Protein phosphorylations are reversible and de-phosphorylations down-regulate myosin II activity [24]. In particular, the myosin light chain phosphatase 1 (MYPT1) mediates the de-phosphorylation of Ser19 and Thr18, opposite to ROCK or MLCK [21, 24]. Unphosphorylated myosin molecules fold into a closed conformation where the two heads interact with each other and the tails interact with the heads to close the molecule in a ring-shape [24]. RLC phosphorylation disrupts these interactions and induces the elongated conformation shown in Figure 2.2, and stimulates ATPase activity promoting filament assembly [21]. Activated myosin II monomers self assemble into anti-parallel structures via the self association of the rod-like helical coiled-coil domain, named bipolar thick filaments (BTFs), as shown in Figure 2.2. BTFs in vitro have been shown to be composed by
～30 molecules and to have an average length of ～300 nm [26] and can be therefore visualized using standard optical microscopy (as their size is above diffraction limit). BTFs constitute the functional unit of myosin II motors and can exert pulling forces on actin filaments.

Figure 2.2: Structure of competent NM-II and BTFs assembly: (top) Competent NM-II has a hexameric structure characterized by two globular head domains that contain ATP and actin binding domains, two essential and two regulatory light chains and one heavy chain. NM-II assembles into anti-parallel structures named bipolar thick filaments (BTFs) that are the functional unit of NM-II capable of exerting contractile forces on actin networks. BTFs in vitro have an average length of ～300 nm and are composed by ～30 NM-II molecules.

NM-II ATPase-dependent motor cycling and the force generation process can be simplified and divided into six steps, each represented by a panel in Figure 2.3. At the beginning of a new cycle/end of the previous cycle, NM-II lacks the ATP in the ATP binding domain and is weakly bound to actin (panel 1). The binding of ATP leads to a conformational change in the actin-binding domain that reduces myosin II affinity to actin. The myosin II head detaches from the filament (panel 2). As a consequence, the arm region (in between the head domain and the heavy chain) bends and moves myosin II in the direction of the (+) end of the filament (panel 3). When ATP is hydrolyzed (panel 2-3), ADP and the phosphate still remain bound to myosin II. The head stabilizes a weak binding to the filament (panel 4), producing a small conformational change that helps the release of the phosphate (panel 5). This further increases the binding affinity of myosin II onto actin: myosin II generates a force on the actin filament (power stroke, magenta arrows in panel 5-6) causing its sliding towards the (−) end. When ADP is released, myosin II is still bound onto the filament until a new
ATP binds and detaches it (panel 6-1). The interaction in between myosin II and actin generates a directional movement for which the motor pushes actin filaments towards their $(-)$ end. If the filament is attached, myosin moves progressively on the actin filament towards its $(+)$ end. This simple mechanism allows a single BTF to break and compact actin filaments \textit{in vitro} \cite{27}. In cells and tissues (similarly to sarcomeres in muscle cells), contractile pulling forces arises when BTFs are bound to anti-parallel actin filaments \cite{25}. This force arises because each motor domain pushes the actin filament towards its $(-)$ (or moves towards its $(+)$ end). By looking at the sketch in Figure 2.3, these movements would result in the contraction of the actin mesh. The time during which myosin II is bound to actin in the force generating state, corresponding to the duration of the steps shown in panels 4-6 of Figure 2.3, is the duty ratio. Together with the rate of ATP hydrolysis, this defines the dynamics of the motor, as it will be discussed in the next paragraph for the specific myosin isoforms.

An interesting property is that myosin II has a homo-cooperativity behavior that results in the cooperative binding to actin \cite{16}. This was suggested to occur because of a local conformational change in the actin filament upon the binding of a NM-II motor head domain that facilitates the binding of new myosin II motor proteins \cite{28}. In particular, myosin II binding to actin (as well as other actin binding proteins such as $\alpha$-actinin) is mechanosensitive, meaning that myosin II binds on actin upon stress, as shown in micro-pipette aspiration experiments in \textit{Dictyostelium} \cite{29}.

\textbf{NM-II isoforms} In mammalian cells there are three genes that encode three heavy chains, defining three different isoforms of NM-II namely: NM-IIA (myh9), NM-IIB (mh10) and NM-IIC (myh14). NM-IIC is expressed only in a few differentiated tissues such as skeletal muscles where it has the highest expression or brain, heart and liver among others \cite{30}. NM-IIA and IIB are expressed in most cell types and tissues and are essential during development \cite{31}. The expression of the two isoforms is regulated in a tissue and cell-specific manner \cite{30} and, because of their different molecular properties, could impact the cell’s cortical tension, mechanics or behavior, among others \cite{31–33}. NM-IIA and IIB, but not IIC, can form heterotypic filaments, meaning BTFs in which two isoform can co-exist \cite{21,32}.

From a molecular point of view, NM-IIB has a slow ADP release, therefore it spends a high portion of its kinetic cycle attached to actin (longer duty ratio) in comparison to other myosins, while requiring less energy \cite{31,34}. NM-IIA has a higher ADP release rate, thus it spends only a small fraction of its ATPase cycle in the actin-bound state (shorter duty ratio) and therefore it propels actin filaments faster \cite{35}. As a consequence, the two different isoforms behave differently \textit{in vitro} or in living cells. \textit{In vitro} studies based on reconstituted actomyosin cortex systems showed that NM-IIB moves processively along actin filaments while, in the same condition, NM-IIA can not \cite{36}. In dividing cells, NM-IIA has been shown to generate cortex tension, while NM-IIB stabilizes the cortex and as a consequence, the relative composition of heterotypic filaments, can tune cellular tension \cite{32}. In migrating cells, NM-IIA is required for adhesion formation and cell rear retraction, while NM-IIB is involved in establishing cell polarity and orienting the nucleus \cite{33}. During cell blebbing, NM-IIA is the essential isoform to drive bleb retraction \cite{37}. All together, these findings support
Figure 2.3: Molecular mechanism of myosin ATPase cycle: (top) BTF bound to actin filaments and zoomed view on the binding of one myosin II motor head to a single actin filament. (bottom) Panels 1 to 6 show one myosin II ATPase cycle. (1) The myosin II head is bound to an actin filament and an ATP molecule associates to the empty ATP binding domain. (2) Once ATP binds, the affinity of the binding in between the motor head and the actin is reduced so that the two proteins detach. When ATP is hydrolyzed (3), the head forms a weak binding onto actin (4). As the phosphate is released, myosin generates a force onto the actin filament causing its sliding (power stroke) towards the (−) end. Sketch inspired by https://www.mechanobio.info/.

The idea that NM-II localization and the relative A/B stoichiometry can be used to tune mechanical cell properties and dynamic cell behaviour.
2.2 Small GTPases and the Rho/ROCK pathway

As discussed in the previous section, the actomyosin cortex is the structural scaffold of a cell. Thanks to the molecular properties of its key components, actin and myosin II, it allows cells to exert protrusive and contractile forces, respectively. As discussed before, actin elongation and the formation of various actin structures is regulated by proteins such as the ARP2/3 complex or formins and myosin II activity. Overall, force generation processes that occur from the sub-cellular to the tissue-scale level, are controlled by hundreds of regulatory proteins.

2.2.1 GTPases

GTPases are enzymes that function as molecular switches as they can alternate in between two conformational states: a GTP (guanosine triphosphate)-bound active state and a GDP (guanosine diphosphate)-bound inactive state, by hydrolyzing GTP to GDP. Only when active, GTPases can interact with specific effectors and initiate signal transduction pathways, as shown in Figure 2.4 specifically for Rho GTPases. Therefore the GTP/GDP cycling behaves like an on/off switch mechanism, that is used in a multitude of biological processes and mammals express hundreds of GTPases [18,38]. Their activity is precisely regulated by various activators, guanine nucleotide exchange factors (GEFs), that catalyse the exchange of GDP for GTP, and inactivators, GTPase-activating proteins (GAPs), that increase the GTP hydrolysis rate [38]. This on/off switching involves also the cycling between the plasma membrane and the cytosol, regulated by GDP dissociation inhibitors (GDIs) that extract Rho-GDP from membranes [38] (Figure 2.4).

Among all GTPases, the Ras superfamily refers to a class of small monomeric enzymes particularly known and studied because of their role in controlling cell dynamics. The Ras superfamily can be divided into five subgroups according to their specific function. Ras GTPases control cell adhesion, Integrin activation, cell growth and cell differentiation [39]. The Ras homology (Rho) subgroup is the key regulator of the actomyosin network, controlling its assembly in different structures [18,38]. The Ras superfamily includes also other three families with specific functions [40]. Rab GTPases regulate membrane trafficking and vesicle formation [41], Arf GTPases lipid transport and plasma membrane signalling [40] and Ran GTPases nucleo-cytoplasmic transport and nuclear envelope formation [42].

2.2.2 Rho GTPases

In humans there are 20 Rho GTPases, that can be further divided into subgroups with specific functions depending on their effectors, thus they generate different cytoskeletal structures [18,43,44]. The most studied and conserved Rho GTPases across species are:

- Rho GTPases composed by RhoA, RhoB and RhoC (in humans). RhoA activates NM-II activity leading to contractile structures such as stress fibers through its
2.2. Small GTPases and the Rho/ROCK pathway

effectors ROCK and by inhibiting the MYPT1 [18,43]. In humans, RhoB and C are also expressed, even though they are less studied. RhoB regulates adhesions [45] and RhoC has been shown to activate ROCK and control cancer invasion [45,46].

- Rac activity leads to branched actin networks, like those observed in lamellipodial structures. The most known effectors are the WAVE/Scar protein, part of the WASP family, that can activate the ARP2/3 complex (Figure 2.1) and PAK, that activates filamin (an actin binding protein), controls focal adhesions and inhibits myosin II activity [43,44]. In humans there are three Rac GTPases that appear to have similar functions with Rac1 being ubiquitously expressed while Rac2 is hematopoietic-specific and Rac3 is expressed in few tissues such as the brain [47].

- Cdc42 induces elongated actin structures like the ones of filopodia via activation of mDia formins (Figure 2.1) and is important for maintaining directionality during cell migration through its interaction with the Par complex [18,44].

The spatiotemporal regulation of Rho GTPases in cells or tissues controls their shape during morphogenesis or movement [18]. For example, when a single cell migrates following a chemotactic gradient: Rac localizes at the front to promote branched actin polymerization and adhesions assembly, Cdc42 stabilizes Rac and directs the movement along the gradient, and Rho is responsible for rear retraction [18,43]. At the same time, different Rho GTPases can interact with each other and evidences of their crosstalk were reported [44].

**Figure 2.4:** The (Rho) GTPase on-off cycle. Rho GTPases, such as all GTPases, switch in between an inactive (GDP-bound) and an active (GTP-bound) state. The activation is regulated by different GEFs (guanine nucleotide exchange factors) that facilitate the exchange of GDP for GTP; the inactivation is mediated by GAPs (GTPase-activating proteins) that inactivate Rho GTPases by GTP hydrolysis; GDI (GDP dissociation inhibitors) can further regulate the spatial localization of Rho GTPase by sequestering it from the plasma membrane into the cytosol upon inactivation. Only when Rho GTPases are in the active (GTP-bound) state, they can interact with their effector molecules, such as ROCK in the case of Rho, that further controls NM II activity and BTF assembly.
2.2.3 The Rho/ROCK pathway

GTP-bound RhoA or RhoC trigger the activation of its effectors, namely Rho-associated protein kinases (ROCK) 1 and 2 [46]. The two ROCK homologs are differentially expressed in a tissue-specific way, play different roles in a multitude of cell lines as shown by knock-down experiments and are thought to have different sub-cellular localization [46].

Active ROCK generates contractile force by increasing the phosphorylation of the myosin RLC on Ser19 and Thr18, and inhibiting MYPT1 activity [21, 24, 48], as discussed before. As a consequence, ROCK induces BTF assembly and growth and generates contraction of both muscle and non-muscle cells [21, 24]. Rho/ROCK localization and activity are spatio-temporally regulated to tune contractile forces from the sub-cellular level to the entire organism [25, 49]. As a consequence, the Rho/ROCK pathway controls cell and tissue stiffness, differentiation dynamics and embryonic cellular movement during morphogenesis [50, 51], is essential to establish cell polarity and defines the migration mode of a cell [52–55].
2.2. Small GTPases and the Rho/ROCK pathway
Chapter 3

Cell migration and the microenvironment

The ability of cells to migrate is a fundamental biological feature with important physiological functions. Cells display migratory capacities from the early stages of development [55, 56] as well as in adult tissues where immune cells migrate to engulf pathogens or bacteria, as well as cancer cells that migrate during metastasis. Cell motility requires different steps. First of all, a cell must polarize and define a front-rear axis of polarization [57]. This is a symmetry-breaking step and results from changes in the architecture of the actomyosin cytoskeleton and the re-organization of proteins, organelles and lipids in the plasma membrane (PM). Cell polarity is established and maintained through a set of positive feedback loops involving Rho GTPases, kinases, PM tension, integrins and microtubules, among others, and defines the front-rear axis of the cell [52, 56–62]. As will be discussed shortly, the polarity of proteins such as Rho GTPases controls cellular shape and the migration phenotype [43, 52, 57]. Various studies showed that protein segregation into membrane domains such as rafts could play a role in maintaining polarity [58, 63, 64] as well as endocytosis and vesicle trafficking [65]. However, how the spatio-temporal redistribution in the plasma membrane can be maintained over time, withstanding the entropy-driven equilibration by diffusion and exo/endocytic events, is not fully understood. Once a cell is polarized, in order to move, it must generate a propulsion force and transmit it to the environment via integrin-adhesions [57, 66] or frictional coupling of cortical flows and surface topographies [66–69]. This is achieved thanks to the properties of the actomyosin cortex that can protrude and contract, thereby generating active force. In particular, retrograde cortical actin flow defines both cell speed and persistence independently of the migration phenotype of the cell [59, 70]. However, the detailed mechanisms of cell polarization and migration depend on the specific phenotype and will be discussed in more details in the following subsection.
3.1 Different ways to move

3.1.1 Mesenchymal and amoeboid migration

Single cell or collective cell migration can be categorized into two exchangeable modes: mesenchymal and amoeboid. The classification is based on differences in cell shape, cortical organization, cell-matrix interaction and force generation [59, 66, 71]. Here, single cell modes will be described, but collective migration shares similar physical principles [72] and amoeboid collective migration was also recently proposed [73].

Mesenchymal migrating cells are cells such as fish keratocytes, fibroblasts or cancer cells that migrate with high substrate adhesions, actin-based protrusions at the cell front such as filopodia or lamellipodia and myosin-II mediated retraction at the rear, as shown in Figure 3.1. These cells move forward thanks to actin polymerization at the front, achieved by the localized activity of actin regulators as PI3K, PIP3, WASP, WAVE [43] or RAC [64]. When protrusions grow, new focal adhesions are formed at the cell front. Myosin II mainly localizes at the cell rear along with its activators such as Rho/ROCK [43, 57]. This allows cell retraction and detachment of cell adhesions. Mesenchymal cells exert high traction forces on their substrate (in the range of $\sim 100\, \text{Pa}$) and they move with typical velocities in the order of $\sim 0.1\, \mu\text{m}/\text{min}$ [66].

Amoeboid migrating cells refer to a broad class of eukaryotic cells such as immune cells, Dictyostelium or zebrafish progenitor stem cells which rapidly change their shape as commonly observed in expansion and retraction cycles during amoeba locomotion [74]. These cells can migrate with low cell-substrate adhesions and using either actin-based protrusions (lammelipodia or filopodia) or contractile protrusions such as pseudopods or blebs (cortex-depleted rounded membrane protrusion) [6, 74]. Amoeboid phenotypes are often observed in 3D environments in vivo and in vitro such as in between parallel plates, as shown in Figure 3.2 C. Cortical retrograde flow drives amoeboid migration by friction coupling to the substrate, with very low pushing forces (at the order of $\sim 1\, \text{Pa}$)
transmitted to the environment [66,68], similarly to the chimneying technique to climb parallel surfaces [6,69,75]. These cells therefore move via a propulsion mechanism that allows them to migrate up to 100 times faster than mesenchymal cells, with velocities of $\sim 1 - 10 \mu m/min$ [66,75]. The exact front/back localization of small GTPases in these cells is not known.

**Figure 3.2: Amoeboid mode of migration** (A-B) Typical phenotypes of amoeboid-migrating cells with either actin-based protrusions at the cell front (A) or contractile membrane-based protrusion such as blebs (B). (C) Bleb-based migration in 3D: the cortical retrograde flow drives cell migration via unspecific friction with the environment.

### 3.1.2 Tuning cellular contractility and stable-bleb migration

As briefly discussed in Chapter 2.2, small GTPases control the dynamics and the type of cell protrusions [18]. For example, as discussed above, Rac promotes branched actin protrusions, as observed at the leading edge of mesenchymal migrating cells. At the same time, high levels of ROCK activity are found at the invasive fronts of primary tumors such as melanoma or breast cancers where ROCK promotes tumor cell survival and extravasation [53]. These highly contractile cells show fast amoeboid migration (associated with high levels of Rho/ROCK activity) [76]. In fact, activation or Rho/ROCK can induce bleb-based migration [54] in both non-motile or mesenchymally-migrating progenitor stem cells derived from zebrafish embryos.

In general, increasing levels of cellular contractility are associated with the appearance or an increase of cellular blebs [54]. Blebs are round membrane protrusions, also referred to as membrane blisters, that can drive migration in 3D [6]. Blebs are initially depleted of the cell cortex and can originate from a local detachment of the cortex from the membrane or from a local rupture of the cortex itself (as shown in panel (i) of Figure 3.3) [6,77]. In both cases, the hydrostatic pressure in the cytosol drives bleb growth by propelling fluid into the bleb [6], while the cell keeps its volume constant [77] (panel (ii) of Figure 3.3). Increasing/decreasing osmolarity leads to bigger/smaller blebs, supporting the role of internal pressure in driving bleb expansion [77]. When the cortex is re-assembled by the diffusion and flow of cortical material into the bleb protrusion, the bleb retracts (panel (iii) of Figure 3.3). Increasing membrane rigidity and cell-cortex attachment inhibits blebbing, while local contractions of the actomyosin cortex induce blebbing as they locally detach the cortex from the PM [77]. In particular, because myosin II activity drives cortical fluctuations [9], increasing cellular contractility can intensify blebbing [54].
3.1. Different ways to move

Blastomeres (cells from blastula stage embryos) derived from zebrafish show a myosin II dependent blebbing behavior when cultured in vitro (Figure 3.4 A-C), as well as inside the living embryo (see Chapter 6). Up-regulation of the Rho/ROCK pathway leads to myosin II activation and its recruitment at the cell cortex, leading to an increase in bleb size and frequency in a dose-dependent manner [54] (Figure 3.4 C). An activation of the Rho/Rock pathway can be achieved by treating cells with Lysophosphatidic Acid (LPA) or by over-expressing a constitutively active form of RhoA (ca-RhoA). When cell contractility reaches a critical level, it was shown that cells can fail to retract a bleb and spontaneously polarize [54] as shown in Figure 3.4 B-C and depicted in Figure 3.5. In this scenario, described in Figure 3.5, as the cortical components flow into the newly formed bleb to reassemble the cortex, the bleb keeps growing because of the high intracellular pressure. Therefore the cortex fails to reassemble at the tip of the bleb and the bleb is not retracted. This results in a density gradient in the cell cortex that generates cortical retrograde flow, which further prevents bleb retraction [54]. Because of this, the tip of the bleb is depleted of cortical components, while the contracting rear is further enriched in myosin II [54]. Cell polarity is thus stabilized by a positive feedback loop in which cortical flows increase myosin II at the cell rear which in turn powers the cortical flow. This leads to a cortical density gradient and the typical "pear like" shape of stable-bleb cell (Figure 3.4 B-C and 3.5) is established. The retrograde flow further gives propulsion to the cells by frictional coupling of the flow to the environment [66,68].
Figure 3.4: Cortical contractility triggers spontaneous stable-bleb cell polarization (A) Fluorescence image of a blebbing non-polarized progenitor stem cell cultured in suspension expressing (from left to right) GPI-RFP (plasma membrane), LifeAct-GFP (actin), merge of these two channels and bright field (BF). (A’) BF time lapse of a blebbing non-polarized (non-motile) progenitor stem cell cultured in suspension. (B) Fluorescence image of a stable-bleb polarized cell cultured in confinement expressing (from left to right) GPI-RFP, LifeAct-GFP, merge of these two channels and BF. (B’) BF time lapse of a stable-bleb motile polarized cell cultured in confinement. (C) Fluorescence image of a cell expressing Myl12.1-eGFP: blebbing non-polarized progenitor stem cells cultured in control condition (serum-free media, top-left), blebbing non-polarized cultured in the presence of 1μM LPA (top-right), blebbing non-polarized cultured in the presence of 10μM LPA (bottom-left) and polarized stable-bleb cell cultured in the presence of 1μM LPA (bottom-right). Magenta asterisks point at cellular blebs, white asterisks point at stable-bleb front (indicative of migration direction). Scale bar 20μm. Figure adapted from [54].
3.1. Different ways to move

Figure 3.5: Bleb cycle and stable-bleb cell polarization For each panel, the cell behavior is shown in the first row and a zoom-in into bleb dynamics is shown in the second row as indicated by the dashed box. Time goes from left to right (qualitative). (Top panel) Low/intermediate contractility: retracting bleb. When a bleb is formed, the membrane detaches from the underlying cortex. The bleb initially lacks cortical components, which re-accumulate at the bleb membrane over time (wave-arrow). Once the cortex is re-assembled, the bleb retracts. This cycle occurs continuously and multiple blebs can be observed at the same time, especially for intermediate/higher contractility levels. (Lower panel) High contractility: stable-bleb cell polarization. When a bleb is initiated, the high intracellular pressure drives excessive bleb growth and a cortical density gradient is established from the cell body to the bleb front (with low density in the bleb tip). This density gradient leads to a mechanical instability in the cortical layer and the spontaneous emergence of retrograde cortical flows which prevent bleb retraction (orange arrow). Cell symmetry is broken and the cell is now polarized. The flow gives propulsion to the cell, allowing for cell movement and initiating a fast amoeboid migration mode (magenta arrow), termed stable-bleb migration.
3.1.3 Mechano-reciprocity in cell migration

There is a reciprocal relation in between the microenvironment and the migration strategy used by cells. This means that on one side cells can actively modify their environment as they migrate, while physical properties of the microenvironment can also modulate single or collective cell migration phenotypes [71,78–80].

Cells actively remodel their environment while migrating: mesenchymal- migrating cells have high proteolysis capabilities and can degrade the extracellular matrix [80], as shown in Figure 3.6. In this way, cells create their own migration tracks and can open a path for more cells to follow, as observed for mesenchymal cells in cancer metastasis [81].

The physical environment controls cell migration: Physical properties such as the stiffness of the substrate, adhesiveness, topology, matrix porosity (relative to cell size), matrix organization (aligned or bundled) or the specific geometry of a tissue such as tubular blood vessels or aligned nerve or muscle fibers can also regulate the migration strategy of a cell or of a group of cells [78,81,82]. This relies on the fact that cells can sense the mechanical properties of their surroundings and respond to changes in these by adapting their cortical organization and, as a consequence, their migration strategy. The full physical characterization of the microenvironment together with the mechanical state of the cell, such as its contractility level, can predict, the migration phenotype of a cancer cell [82]. Importantly, these strategies cannot be explained by 2D migration assays and the distinction between 2D and 3D environments is critical [74,78,83].

Concluding, cells can actively change their surroundings as they migrate and the physical properties of the microenvironment control migration phenotypes. This relies on the mechanosensing and mechanotransducing ability of cells, discussed in the following Chapter. As they migrate, cells can constantly sense their 3D surrounding and adapt their migration strategy by tuning the assembly, turnover and structure of the actomyosin network [81].

3.2 Migration in confined environments

In vivo tissues are dense environments and in order to migrate, cells need to find or create a path. Mesenchymal cells commonly use the strategy of degrading the extracellular matrix to generate a path for movement [80,81]. Cells can also use alternative strategies such as using their nuclei as pistons to increase the intracellular pressure in the front protrusion and expand it [84]. Amoeboid cells usually have low proteolytic capability, but can still migrate through very dense environments by squeezing themselves through tight spaces, using their nucleus as a ruler to choose the path of least resistance [85] (Figure 3.6). The nucleus, being the largest organelle of a cell, is the main physical obstacle for a cell during migration [83,86]. To migrate, nucleus positioning is regulated in a cell type-specific way and depends on the migration mode and the 2D/3D environment [86]. For example, amoeboid cells have their nucleus in front of the microtubule organizing centre, and this facilitates the choice of their migration
3.2. Migration in confined environments

Figure 3.6: Mesenchymal and amoeboid migration in 3D. (A) Mesenchymal migrating cells perform adhesion-dependent migration in 3D, based on actin protrusions at the cell front. These cells create their own migration path by degrading the extracellular matrix (ECM). (B) Amoeboid cells perform adhesion-independent and bleb-based migration in 3D. These cells have low proteolysis activity and choose the path with larger pore size to prevent nuclear damage. Green arrows point at the direction of migration. These are representative phenotypes and others are also observed.

Path [85]. Nuclear mechanical properties also affect the capabilities of single cells to squeeze themselves through tight spaces [72, 87, 88]. As a consequence, cancer cells with softer nuclei can more easily overcome the constraints in crowded tissues and thus have higher metastatic capabilities [89].

3.2.1 Biomimetic environments

The tissue microenvironment has been shown to affect the migration mode of cells (as discussed in Chapter 3). Visualizing how cells migrate in vivo and in general understanding how they respond to mechanical challenges is not trivial. In a living organism, a multitude of processes occurs at the same time and it is particularly complicated to dissect mechanical and chemical contributions that dictate cell behavior. This requires a specific interference with defined signalling pathways or tight control over physical properties of the cell surrounding. Over the last decade, an extensive effort has been put into the establishment of assays that allow to reconstitute 3D tissue environments in vitro via 2D and 3D biomimetic assays that are easy to handle, tuneable and compatible with high/super-resolution microscopy [90, 91].

Biomimetic environments represent bottom-up in vitro approaches to address how cells behave or migrate in different mechanical environments and respond to changes in their surrounding in a controlled and systematic manner. Key parameters such as the geometry of the environment, the degree of confinement or the rigidity/adhesiveness of the surface can be easily tuned. At the same time any kind of chemical interference or media exchange can be performed in order to interfere with signalling and effector proteins of interest. These systems are also compatible with standard or super-resolution fluorescence microscopy as they can be assembled on conventional imaging plates and therefore overcome limitations of in vivo assays (i.e. deep tissue imaging, animal handling, opaque tissues, toxic effects etc.). These approaches do not substitute in vivo studies, but add a complementary methodological approach to obtain a mechanistic understanding of physiological processes observed in vivo.
In 2D, micro-pillar and gel-substrates made of polyacrylamide or polydimethylsiloxane (PDMS) of different stiffness have been widely used to quantify the traction forces that cells exert on their environment and ECM. These methods contributed to the understanding of how cells respond and adapt to mechanical changes of their surrounding such as its stiffness [92–94]. Micro-patterning is another approach to change the geometry of the area in which a cell can adhere [95] or to create patterned lines along which cells can polarize and migrate [70,96].

Figure 3.7: Exemplary biomimetic environments based on micro-channels Caption continues on the next page.

In 3D assays, a collagen matrix can be reconstituted to study 3D cell migration and force generation [80]. However, the local geometry of the collagen cannot be precisely controlled. Biomimetic 3D tuneable systems have been developed thanks to the use of photo-lithography to fabricate negative molds and the use of PDMS to obtain chambers [97,101]. The use of photo-lithography ensures high precision and allows to design versatile chambers, in principle of any desired shape, and the PDMS is bio-compatible and permits CO$_2$ exchange. Among different biomimetic tools, micro-channels have
been widely used to study immune cell migration in a systematic way [97] and to show, for example, that dendritic cells can pass through tiny (micrometric) constrictions (Figure 3.7 A-B) or choose the migration path that better accommodates their nucleus [85] (Figure 3.7 C). When moving through constrictions, the ARP2/3 complex facilitates the formation of an actin shell around the cell’s nucleus and facilitates the passage through narrow constrictions [98] (Figure 3.7 B). Mazes with increasing complexity (Figure 3.7 D) have been recently used to show that both Dictyostelium and cancer cells can navigate over large distances in complex environments. This is achieved because cells are capable of self-generating a gradient that allows for robust chemotaxis and, as an outcome, cells can "sense the space ahead of themselves" in environments they have not explored before [99].

When cells cannot choose a path larger than their size, they can experience high levels of deformations, as when migrating in dense or crowded 3D tissues or as they move through tight tissue spaces or extravasation from blood vessels [80,102]. These deformations can strongly perturb cellular shape and subcellular organization and can even lead to cell fragmentation, nuclear deformation and to DNA damage [100,103]. Cell deformation can be addressed in vitro using PDMS spacers or planar confinement assays. PDMS spacers, as shown in Figure 3.7 E, have been used to specifically address how nuclear deformation can lead to nuclear envelope (NE) rupture, as observed in vivo [100]. This in vitro approach allowed for a more comprehensive description of the process of NE rupture, DNA damage and NE repair and to the understanding that its frequency scales with the degree of confinement [100]. Planar confinement assays [101] have been used to systematically study how single cells migrate in microenvironments of different heights and how cells respond to variable degrees of deformation. This led to the identification of a novel migratory amoeboid phenotype, described in the next section [54,75].
3.2.2 The impact of mechanical confinement on cellular behavior

Cells can adapt their migration strategy to changes in the tissue microenvironment [74, 78, 81–83]. In particular, cells can switch from a mesenchymal to an amoeboid phenotype, thus performing mesenchyma-to-amoeboid transition (MAT), when their proteolysis activity is impaired. By doing so, cells squeeze themselves within dense tissues instead of creating their own path [80]. Both MAT or the transformation from a non-motile to the stable-bleb motile amoeboid phenotype are also observed in various cells types when cultured in confined spaces [54, 75, 83]. To understand how cell deformation in confined spaces affects cell behavior and migration modes, planar micro-confinement assays have been developed [101]. In these assays, cells can be cultured in between two parallel surfaces of defined adhesiveness separated by a distance that corresponds to the cell’s confinement height. This approach showed that the MAT can occur by switching from an adhesive 2D culture condition to a non-adhesive and confined environment [54, 75]. High cell deformation in confined environments was shown to regulate cortical contractility and induce amoeboid motility in non-motile or slow-mesenchymal cells [54, 75].

Figure 3.8: Mechanical confinement induces stable-bleb transformation

(A) Fluorescence images of progenitor stem cells derived from zebrafish embryos expressing Myl12.1-eGFP (from left to right): non-polarized cell in suspension, non-polarized cell in confinement (note an increased myosin II accumulation at the cortex) and stable-bleb polarized cell in confinement. White asterisks marks cellular blebs and magenta asterisk points at the stable-bleb (cell front). Panel adapted from [54]. (B) Phase contrast (PC) and fluorescence images of RPE1 and HeLa cells stained with DNA-Hoechst (nucleus) confined at 3μm in non-adhesive conditions transform into amoeboid motile cells with the A1 and A2 (stable-bleb) phenotypic modes, respectively. Figure adapted from [75]. Scale bar 20μm.

Ruprecht et al. [54] showed that progenitor stem cells derived from zebrafish embryos accumulate myosin II at the cortex when cells are mechanically deformed in confinement.
and this can trigger stable-bleb cell polarization. This polarization phenotype under confinement is similar when cells are treated with LPA, as discussed before, arguing for an increase in myosin II-dependent contractility upon cell deformation in confinement. Pluripotent cells that are non-motile in 2D and mesendoderm progenitor cells that show an actin-based protrusion migration on adhesive substrates, both transform to stable-bleb cells when confined. Furthermore, this amoeboid switch is not a specific feature of primary embryonic cells, but the mechanosensitive switch in migration behaviour upon cell compression is highly conserved across species and cell types [75]. Liu et al. performed a systematic study to address how adhesion and mechanical confinement, at different heights, affect cell behavior and in particular the MAT. More than 20 cell lines have been used and tested: cancer cells such as HeLa, human fibroblast cells or immune cells such as NHDF (normal human dermal fibroblasts) that showed a mesenchymal migration phenotype on 2D adhesive substrates were observed to undergo a MAT when confined. Interestingly, HeLa cells or leukocytes showed stable-bleb motility (referred as A2 phenotype in this study) when confined, while fibroblasts showed another amoeboid phenotype based on actin protrusions at the cell front (named A1 phenotype in this study). The specific amoeboid phenotype of cells depends on the cells’ contractility level: highly contractile cells transform to a stable-bleb phenotype, while lower contractile cells would migrate with the A1 mode. Interestingly, and in agreement with the results of Ruprecht et al., Liu et al. showed that the confinement itself tunes cellular contractility. As a consequence, larger cell deformation (that corresponds to a lower confinement height), increases cell contractility and the ratio of A2/A1 phenotype.

These two studies suggest the presence of a conserved, yet unknown, mechanosensitive pathway that enables cells to (1) sense physical shape deformation under mechanical confinement and (2) translate cell deformations into the regulation of cellular contractility, controlling adaptive cell behavior to mechanical stress.

The same amoeboid transformation under confinement has been recently observed also in choanoflagellates (the closest living relatives of animals) [104], in human melanoma cells [105] as well as in cell clusters [73], suggesting that cellular contractility is regulated at the single cell and cell ensemble level upon mechanical stress and supporting that amoeboid motility under confinement is a conserved mechanism. This opens up the main questions of this thesis: How do single cells sense their own shape and accordingly control myosin II activity and their dynamic migration behaviour?
Chapter 4

Sensing and responding to mechanical forces

4.1 Mechatrosensation and mechanotransduction

The previous sections showed how the cellular microenvironment can affect the migration phenotype of cells [75,80] or induce motility [54]. Mechanical forces that induce cell shape changes can influence a multitude of dynamics such as division [106–108], adhesion and stress fibers formation [92,93], transcription [94] or the mechanical cell properties such as its stiffness [109,110]. All of these processes rely on the ability of cells to sense mechanical forces and transduce this information into specific signalling pathways that control cell behaviour.

Given a mechanical cue as an input signal, cellular force sensing can be divided into two subsequent steps:

1. Mechanosensation: the cell has to sense and quantitatively measure mechanical forces. This step can be achieved thanks to specific proteins or whole organelles that have mechanosensitive capabilities.

2. Mechanotransduction: mechanical forces are commonly translated into biochemical signals to trigger changes in, for example, cell contractility, gene expression or protein activities that further control cell and tissue dynamics or cell fate.

This process, shown in Figure 4.1, can also include multi-level positive or negative feedback loops, leading to adaptive cell responses. Correct mechano-responses are required for tissue integrity and homeostasis and play an important role in embryo development and various diseases.

Mechanosensitive processes are observed from the early stages of embryo development as well as in adult tissues, both at the tissue-scale or single cell level. Forces arise within tissues during growth and division [106,113], as a result of large-scale cell movements as occurring during development [4] or collective cell migration [114] or as the result of
Mechanosensing from the cell surface

Figure 4.1: Mechanosensation and mechanotransduction: Cellular force sensing is divided into two subsequent steps. First, the cell must sense a physical force thanks to mechanosensitive proteins (single proteins or protein complexes), lipid membranes or whole organelles. Mechanosensitive elements typically bear a structural domain that changes its conformation under mechanical load, e.g. leading to protein unfolding [93] or changes in membrane tension that can activate mechanosensitive ion channels [107,111]. In general, these mechanosensitive structural changes activate biochemical signalling cascades that allow the transduction (of the force) into specific molecular and cellular changes such as the regulation of cell contractility [29,112], transcription [93] or the cell’s state [107]. This process can function with positive or negative feedback loops.

externally applied forces [54,75,115]. In general terms, tissues and cells respond and adapt to these forces depending on the type of the force and on the time-scale at which the force is applied [116], using specific mechanosensitive pathways. Intuitively, the first point of connection in between a cell and its microenvironment is the plasma membrane, that is in fact rich in mechanosensing elements: Adhesion molecules and their binding partners such as talin and vinculin [93], stretch-activated ion channels [111], membrane invaginations such as caveolae [117] or the PM itself [118]. As a counterpart to the cell surface, intracellular structures as the nucleus provide another mechano-sensing hub for large cell deformations [119,120]. In the following, these two categories will be described, providing some examples of mechanosensing elements and the involved mechanotransduction pathways.

4.2 Mechanosensing from the cell surface

The cell surface, being the outermost part of a cell, is the first connection in between the cell and its tissue microenvironment. PM tension itself can regulate cell shape or cell migration [118] or act through mechanosensitive pathways [61]. In fact, various mechanosensitive elements reside in the PM and they usually get activated by tension [118]. For example, stretch can disassemble caveolae, which are small membrane invaginations [117], or activate mechanosensitive ion channels [111,121]. These stretch-activated ion channels get physically opened upon force application and as a consequence they gate the influx of ions into cells [111]. Therefore, they function as mechano-transducers of forces as they translate a mechanical force into a biochemical signal. These channels mediate the sensation of touch, shear stress, cell spreading or osmotic swelling [111,121], among others. In particular, the Piezo family of channels, that induces calcium influx into cells when activated, has been shown to sense single cell spreading and to control stress fiber formation [112] as well as to sense tissue
crowding and tension controlling cell extrusion and division rates [122], among others. Importantly, the ion channel structure and ion conductivity have been characterized and shown to be membrane tension-dependent [123].

Adhesion complexes represent another example of widely studied mechanosensors that reside in the PM and have cytosolic protein domains relevant for mechanotransduction. They physically couple the cell’s cortex to the extracellular space or other cells and are responsible for sensing the rigidity of the cell’s microenvironment [92, 93, 124]. Because of the mechanosensitive properties of its components, namely talin and vinculin, adhesion-based mechanosensing controls cell fate [125], stress fiber formations [92], the transcription of specific factors [93, 94] and directional cell migration [126, 127].

4.2.1 Mechanosensitive ion channels ensure epithelial homeostasis

Epithelial tissues commonly act as a barrier in between the organism and its environment as in the case of the surface epithelium in early stage zebrafish or mammalian embryos [113] (see Chapter 6) or in the adult organism considering the epidermis or colon tissue [115, 122]. Epithelia are subjected to mechanical stress as tissues grow and remodel or in the presence of external forces [128]. To fulfil their role of protection barrier, epithelial tissues must maintain their integrity and homeostasis. To do so, a key property is that they can actively remodel their junctions. As a consequence, epithelial can respond to forces showing both plastic and fluid behavior or by locally tuning cell division and extrusion rates [122, 128].

Cell density is a key parameter that needs to be tightly regulated to maintain tissue integrity. Homeostatic cell numbers are achieved thanks to two mechanosensitive mechanisms: extruding cells when a tissue is over-crowded [122] and an increase in cell division rate when a connected tissue is underpopulated and under tension [107]. Interestingly, both processes are mediated by the mechanosensitive ion channel Piezo1 in both mammalian cells and in zebrafish. Live cells are extruded during the process of delamination [129], in which cells lose adhesive strength at their junctional contacts with neighbors and get squeezed out of the tissue. Notably, both live cells and apoptotic cells can be extruded by the activation of Rho upon cell crowding [122]. In the absence of Piezo1, cells are not extruded and epithelial masses are observed [122], suggesting that density control prevents the formation of excess tissue. Under tension, Piezo1 gets activated and increases intracellular calcium, leading to the phosphorylation of a kinase, ERK1/2, that stimulates cell division [107]. Therefore Piezo1 acts as an homeostatic density sensor that induces cell extrusion in high density regions [122] and cell division in low density areas [107].

A similar process of tension-induced cell division happens also during epiboly progression in zebrafish. At this stage of development, the enveloping cell layer (EVL) spreads over the entire embryo as it is pulled by a contracting actomyosin ring [113] that generates a gradient in cortical tension across the epithelial tissue [130] (see Chapter 6). While the tissue spreads, cells flatten and increase their surface area as cell division rates are low [106]. However, the cells that divide, orient their division axis in the direction of
30 4.2. Mechanosensing from the cell surface

the tension anisotropy within the epithelial tissue by a rotation of the spindle [106]. This process, that requires myosin II activity, facilitates EVL spreading and ensures tissue integrity as it reduces tension anisotropy within the epithelial tissue [106,113]. This is a clear example of a negative feedback loop: the anisotropy in tension orients cell division to reduce the anisotropy itself for maintaining tissue integrity.

Epithelial tissues such as the skin are often prone to large-scale and dynamic mechanical stresses such as repetitive stretch. While in deformed cancer cells DNA damage is often observed [100], epithelial sheets were shown to have a robust strategy that guarantees tissue integrity and protects the cell’s genome [115]. This is achieved in two steps: a fast mechanoresponse that leads to a calcium-dependent nuclear softening mediated by Piezo1 (located in the endoplasmic reticulum) that induces the reduction of lamin-associated heterochromatin [115]; and, second, cell-cell adhesion drives a slower supracellular re-alignment of the actin network that reduces the mechanical stress felt by the nuclei over longer timescales [115].

4.2.2 Single cells can sense the stiffness of their environment

Single cells sense and respond to the rigidity of their microenvironment and this is known to affect, for example, cell fate specification [125] or cell migration [92], including single or collective cell durotaxis [114,126]. Rigidity sensing is mediated by Integrin-based adhesion complexes that commonly refer to an ensemble of proteins that are part of focal adhesions (FAs) [124] such as integrins, talin and vinculin [93,94].

As cells spread, they build up traction forces on their substrate, with higher forces being observed for stiffer substrates [92]. The most studied class of adhesion molecules that allow cells to bind to the ECM are integrins, representing specific transmembrane receptors [124]. Integrins are coupled to the actomyosin cortex via talin and vinculin and form focal adhesions with the function of hundreds of other regulatory proteins [124].

FAs allow to transmit the force from the ECM to the actomyosin cortex as cells spread or migrate. In particular, talin has been shown to be a mechanosensitive protein as it unfolds above a certain threshold of force application [93]. When talin unfolds, vinculin can bind to FAs, leading to a reinforcement and stabilization of cell adhesion sites [93] and stress fibers formations [92]. In particular, through its binding with actin, vinculin promotes nascent FAs formation but inhibits FAs maturation by reducing the actin flow in mature FAs [131]. When mature adhesions are formed, cells exert traction forces on their substrates or on the ECM [92,93] and increase their contact area. At the same time, the force transmitted from FAs to the actin cortex leads to cell and nucleus flattening [94]. This has been shown to stretch the nuclear envelope and open nuclear pore complexes, thereby increasing Yes-associated protein (YAP) translocation from the cytoplasm into the nucleus [94]. YAP is a transcription factor with important roles in tissue growth and cancer [132] and its localization correlates with transcriptional activity controlling cell states.

Together, substrate rigidity and adhesion-based mechanosensing can control YAP localization and transcription through a talin-mediated mechanosensing process that drives adhesion growth and stress fiber formation [92,93]. Recent studies further reported that other factors can tune the mechanosensitive cell responses and YAP localization.
In particular, Piezo2 and microtubules acetylation promote stress fiber formations regulating RhoA activity [112,133]. Stress fiber formation further induces nuclear flattening and the activation of a nuclear mechano-transduction pathway [94], described in the following Section.

4.3 The nucleus as a mechanosensor

The nucleus is the largest organelle in the cell, it contains the cell’s genetic information and molecular and biophysical mechanisms in the nucleus control protein expression levels and associated cellular dynamics and fate decisions, among others [119,134]. Together with the PM, the nucleus gains increasing attention as a major cellular mechanosensor and mechanotransducer [119,120]. The nucleus can be subjected to different forces, either directly or through the cytoskeleton [135]. The nucleus is specifically involved in sensing larger-scale cell deformations as small shape changes would mainly affect the cell surface, but not the nucleus [120]. To understand how cells can sense nuclear deformation, a knowledge of the nuclear structure is required and will be discussed in the following.

4.3.1 Nuclear structure and mechanics

The structure of the nucleus is composed of different layers that, one after the other, wrap the nuclear content, the DNA, as shown in Figure 4.2. The specific architecture and shape of the nucleus can vary across tissues and cell types as it is regulated by the cytoskeleton organization and genetically by the composition of the nuclear envelope [136–140]. Moreover, the nucleus to cell size can also vary during development or differentiation [137,138]. Specific nuclear shapes appear to correlate with specific cellular functions, such as neutrophils that have lobulated nuclei to facilitate their rapid migration within tissues [137], and abnormalities in nuclear shape are often associated with disease [138,139,141].

From a mechanical point of view, the nucleus is known to be the stiffest organelle in the cell [142–144]. For example, the elastic modulus of endothelial nuclei, measured by compressing the cells in between glass micro-plates, is $5000N/m^2$, which is 10 times higher than the elastic modulus of the cytoplasm, $500N/m^2$ [145]. This study also reported that the elastic modulus of chemically-isolated nuclei is higher than the ones measured inside the cell [145], suggesting that nucleus isolation protocols affect its mechanical properties. Nuclear mechanical properties depend on various factors, but are governed by chromatin organization and lamin A/C expression levels [141,142,146] as well as other proteins of the nuclear envelope that can impact cell behavior [89]. The main factors that determine nuclear structure and mechanics will be described here, without reference to a specific cell type.

The nuclear envelope (NE), which is build up by a double lipid bilayer, separates the interior of the nucleus from the cytoplasm, preventing free diffusion of macromolecules in between the cytosol and the nuclear interior. The outer nuclear membrane (ONM), that is the outermost lipid bilayer, is continuous with the endoplasmic reticulum (ER).
The ONM is also contiguous with the inner nuclear membrane (INM), the lipid bilayer facing the inner side of the nucleus (Figure 4.2). The nuclear pore complexes (NPCs) pierce the NE and bridge the INM and ONM (see dedicated paragraph). Hundreds of LEM domain proteins associate with the NE, such as LAP1 and LAP2, lamin-B receptor, emerin or SUN, that connect the lipid bilayers of the nuclear envelope with the underlying lamin network or the cytoskeleton [120].

The NE is not a smooth surface, but shows membrane invaginations, formed by either the INM alone (defined as type I) or the INM and ONM (type II) and both types can co-exist in the same nucleus [147,148] (Figure 4.2). These invaginations can contain various folds and branches that can span the entire width of the nucleus [148]. Because of the complex morphological structure of this intra-nuclear network of membranes, the term nucleoplasmic reticulum (NR) is widely used (Figure 4.2). The NR has been observed in normal physiological states of diverse animal and plant cells, and various tumor cell types also showed abundant NR structures [148]. Still, there is not a clear consensus of NR function(s). Similarly to caveolae in the PM [117], nuclear envelope invaginations can serve as membrane reservoirs and nuclear envelope stretching upon cell swelling was observed [149]. One study performed in HeLa cells showed that type II invaginations behave like channels through which molecules of up to 70kDa can penetrate (while molecules only up to 30kDa can freely through NPCs [150]) and that NR can be used for calcium transport and release [151]. There are more evidences that the NR act as an inositol trisphosphate (IP3) receptor-mediated calcium store that enables to rapidly tune nuclear calcium levels [148,152]. Nuclear invaginations are known to be genetically regulated by various proteins [136,153] and by lipid synthesis and composition of the NE [154]. For example, the over-expression of the lamin-B receptor has been shown to increase nuclear envelope folds [155]. NE wrinkles are also known to be regulated by the cell’s environment, with more wrinkled nuclei being observed in 2D versus 3D environments [156]. This difference further tunes the mechanosensitive ability of cells in translocating YAP to the nucleus [156], as discussed for 2D adherent cells [93]. Nuclear stretch and the unfolding of NE invaginations can act as a mechano-transducer [157] as shown during cell adhesion [94] and tissue inflammation [149,158], as will be discussed shortly.

The nuclear pore complexes (NPCs) are protein complexes composed by multiple copies of approx. 30 proteins called nucleoporins. NPCs control molecular import and export in between the cytoplasm and the nucleus [150]. Structurally, NPCs appear like rings that pierce the NE. Similarly to ion channels in the PM, the central region of the NPC can dilate or shrink as a result of changes in the NE tension [159]. As a consequence, this changes the nucleus-cytoplasmic localization of large proteins or transcription factors, such as YAP, under stress [94,160]. Importantly, both the protein size and its mechanical stability control the nuclear translocation across the NPCs under stress [94,160]. Because of this, as discussed in the next section, the NPC is an important mechanotransduction element of the nucleus.

Lamins Underneath the INM is the nuclear lamina: a dense filamentous network of Lamins, type-V intermediate filaments that constitute the nuclear structural scaffold. Lamins can be divided into two groups. B-type Lamins are ubiquitously expressed and
bound to the nuclear envelope (Figure 4.2), while lamin A/C expression is regulated in a tissue-specific manner and only appears in differentiated cells during development [142]. Lamin A/C levels define nuclear stiffness, while lamin B defines nuclear softening and its down-regulation can lead to nuclear blebbing [161]. As a consequence of the increase in lamin A expression during differentiation, nuclei can increase their stiffness up to six times [134]. Stem cells usually lack lamin A/C and thus they have a softer and more plastic nucleus compared to differentiated cells [142]. Lamin A/C expression controls also the stiffening response of the nucleus during large cell deformations and nuclear surface area changes under external force loads [162,163]. Defects in the lamin network, which typically arise from specific mutations in the lamin genes (as observed in patients affected by laminopathies), lead to altered nuclear structure and mechanics and impact gene expression and cell fitness [119,141].

**Chromatin** The $\sim 2m$ double-strand of negatively charged human DNA is packed inside nuclei of a size of $\sim 10-20\mu m$ thanks to four histone proteins (H2A, H2B, H3, H4). The DNA is wrapped around complexes made of 8 histones (two of each protein type), connecting multiple units of these structures (Figure 4.2). This creates the chromatin fiber, that is further folded into chromosomes, packed inside the nucleus and partially attached to the lamina network [120]. Chromatin can be divided into two categories, according to its compaction and transcriptional activity. Euchromatin refers to less packed regions of the DNA where transcription mainly takes place, while heterochromatin refers to highly condensed DNA regions, mostly located closer to the nuclear envelope, with lower transcriptional activity (as sketched in Figure 4.2). The ratio in between euchromatin/heterochromatin modulates nuclear stiffness [162], with more euchromatin leading to nuclear softening such as occurring in epithelial tissues under stretch [115]. External forces, in fact, can lead to chromatin condensation in a myosin II-dependent way [164]. In general, chromatin defines the elastic nuclear response to small deformation [162,165] and controls nuclear volume under external force load [163].

**The LINC complex** The nucleus is connected to the cytoskeleton via different proteins, such as SUN or nesprins, part of the linker of nucleus and cytoskeleton (LINC) complex (Figure 4.2). The LINC complex establishes a physical connection between the nucleus (lamins and chromatin) with the cell cytoskeleton (actin and microtubules) and intermediate filaments [119]. Therefore it mechanically couples the two compartments, allowing for force transmission from the extracellular region or the cytoplasm, through the cytoskeleton, to the chromatin and/or the lamin network [146]. As a consequence, the LINC complex is essential for correct nuclear movement and positioning during cell polarization [146] and controls nuclear shape and organization [139].
4.3. The nucleus as a mechanosensor

Figure 4.2: Nuclear structure The nuclear envelope has a multi-layered structure. A double lipid bilayer establishes the inner and outer nuclear membrane (INM and ONM) and separates the nucleoplasm from the cytoplasm. The INM and ONM are continuous and fuse in the proximity of Nuclear Pore Complexes (NPCs). The ONM is also continuous with the ER and, together with the INM, form various folds and structures, referred to as the nucleoplasmic reticulum (NR). Invaginations can be of type I or II if they are composed by INM alone or by both the INM and ONM, respectively. Underneath the INM is a dense network of the nuclear lamina. Its outermost layer close to to the INM is composed by lamin B that is present in all cell types and tissues. Below it there is a second layer of lamin A/C, found in differentiated cells. Going further inside the nucleus we find chromatin, a fiber-like structure that contains the DNA. The DNA is wrapped around histone sub-units (made of 8 proteins, two of each type) that allow for a high density packing of the negatively charged DNA double strand. Typically, a more dense chromatin (heterochromatin) is positioned closer to the INM and a less dense chromatin, which is transcriptionally active, named euchromatin, resides in the central region of the nucleus. The nuclear membranes and the lamina network are coupled with the cytoskeleton through the LINC complex, depicted in the zoomed view. Other proteins can reside in the nuclear membranes such as Lap2β (depicted in green) that is localized at the INM. The functional role of each compartment is indicated on the right side. Sketch adapted from F. Pezzano (CRG), inspired from [120].

4.3.2 Nuclear mechanotransduction

Evidences of the mechano-sensitivity of the cell nucleus emerged from different fields and was shown to control transcriptional dynamics [94], cell division [166], immune cell migration [85] or inflammatory responses [149], among others [119,120].

Isolated nuclei can respond to mechanical forces applied on the LINC complex and stiffen under external force load [167]. This in vitro study showed that applying forces on nesprin-1 induces Src kinases activation that mediates emerin phosphorylation [167]. Interestingly, when fibroblasts spread on rigid substrates, thereby increase their contrac-
tility, emerin also gets phosphorylated. This further induces lamin A/C recruitment at
the LINC complex [167], through a mechanism that still needs to be elucidated. Using
this pathway, nuclei can rapidly (within seconds) regulate their mechanical properties
in response to external forces that directly act on them or generated by the cells [167].
As a consequence, the connection in between the nucleus and the cortex is reinforced
upon external force, similarly to the growth of focal adhesions on stiff substrates [157].

Inner nuclear membrane stretch has been shown to mediate cellular mechano-transduction
in various processes [157]. As discussed in the previous section, when a cell is cultured
on stiff substrates, focal adhesions are build up through a mechanosensitive response
mediated by talin [93], generating traction forces on the substrates and the formation
of stress fibers [92]. This further induces cell and nuclear flattening [93]. Flattened nuclei
have a stretched NE [140] with reduced invaginations [156]. Similarly to ion channels
in the PM that open upon stretch, NPCs can dynamically open and close as a result
of changes in NE tension [159]. NPC opening controls the translocation of molecules
across the NE, such as observed for the transcription factor YAP that localizes from
the cytoplasm into the nucleus of flattened adherent cells [94]. The translocation of
proteins into or out of the nucleus further depends on the size and mechanical stability
of single proteins and is responsible for the localization and activity of transcription
factors regulating cellular states [160]. Interestingly, uncoupling the nucleus from the
cortex by inhibiting nesprin, prevents NE unfolding and YAP translocation to the nu-
cleus [156], as also shown for emerin [167] or LINC complex inhibition [94]. YAP
localization appears to strictly correlate on NE wrinkling, probably a readout of NE
tension [156] and therefore NPCs opening [159].

NE stretch was further shown to activate a mechanotransduction pathway during in-
flammation in zebrafish [149,168]. When a tissue is damaged, cells and their nuclei
close to the wounding region swell because of the hypotonicity of the external me-
dia (water). Nuclear swelling and the associated increase in inner nuclear membrane
stretch, in the presence of high $Ca^{2+}$ levels, triggers the activation of a lipase, named
cytosolic phospholipase A2 (cPLA$_2$) (Figure 4.3). When active, cPLA$_2$ preferentially
cleaves the fatty acid arachidonic acid (AA) from lipids [169,170]. AA is responsible
for the production of pro-inflammatory signals such as eicosanoids that act chemo-
attratcants for the recruitment of leukocytes towards wounding sites [149,171], as
shown in Figure 4.3.

**Cytosolic phospholipase A2** is a calcium-dependent lipase [169], expressed in ze-
brafish from early development [172]. In zebrafish, cPLA$_2$ is encoded by the gene
pla2g4aa (see Appendix A.1) [168]. Because of a genome duplication that happened in
zebrafish, also the gene pla2g4ab encodes for the same protein. However, it is signifi-
cantly less and not ubiquitously expressed compared to pla2g4aa (VastDb database).
AA is produced by cPLA$_2$ activity at the INM and it is cleaved from the sn-2 position
of phosphatidylcholine (PC), phosphatidylethanolamine and phosphatidylinositol [169],
which are enriched in the ER and NE [154]. cPLA$_2$ has a high affinity for PC as a
substrate and high specificity for AA, which represents its metabolite product [169].
It is upstream of the cyclooxygenase (COX) and lipoxygenase (LOX) pathways and thereby controls the production of prostaglandins and eicosanoids, respectively, which are important in cell motility [173], development [174] and immune cells response [175], among others. Knock out of cPLA₂ in mouse affects macrophages, impacts brain injuries, allergic responses and the reproductive system in female mice [176]. This protein is also essential for ovulation in adult female zebrafish [177] and various studies also reported a role for cPLA₂ in cancer [170]. For example, it is up-regulated in breast cancer, where its expression levels correlate with higher metastatic capabilities and it was implicated in mediating an epithelial-to-mesenchymal transition in a PI3k/Akt-dependent way [178].

Figure 4.3: Nuclear mechanotransduction in inflammation (A) Sketch of a tissue injury in the zebrafish tail. Zoomed view of a wound experiments from [149], showing tracks of leukocytes migrating towards the wound. Scale bar 100μm. (B) Sketch of the molecular mechanism for cPLA₂ mechanotransduction in inflammation. Control cell (left) and cell swelling next to a damage site (right). Upon cell and nuclear swelling, the INM of the nuclear envelope gets stretched and the C2 domain of cPLA₂ binds to the INM. This triggers the production of eicosanoids, that act as chemo-attractants for leukocytes. Sketch inspired by [149].

4.4 Cell mechanics

As briefly introduced in the previous sections, cells or specific organelles can change their mechanical properties. This can be regulated by genetic programs that control protein expression levels, by signalling pathways or by physical stimuli. For example, during differentiation the cell nucleus gets stiffer, as a consequence of the expression of lamin A/C [134,142,143,164], and cell fate specification regulates cortical tension [51]. Cells or specific organelles can also change their mechanical properties as a consequence of changes in their microenvironment and through the activation of mechanosensitive processes [140]. The cell cortex and its organization is the main element known to control cell stiffness and cortical tension [7] and can affect nuclear shape and mechanics [140,179]. Cortical tension is controlled by myosin II activity, F-actin organization and
membrane-to-cortex attachment [7, 25]. In particular, higher levels of contractility result in increased cell stiffness [25]. As a consequence, when single cells are deformed in confined environments, the recruitment of myosin II motor proteins to the cortex [54, 180] increases cell stiffness [181]. Similarly, also isolated nuclei can increase their stiffness when subject to external force by recruiting lamin A/C to the LINC complex as discussed before [167].

The correct regulation of the mechanical properties of a cell or of its compartments is important for its function and survival. For example, the mechanical properties of dendritic cells, regulated by vimentins, are crucial to ensure their correct migration and prevent DNA damage [110]. Changes in the mechanical properties of the nucleus can also impact its mechanosensitive function [149, 181], important for migration [182] and division [183].

4.4.1 Techniques to measure cellular and subcellular mechanics

Various techniques have been used to measure the mechanical properties of entire cells and tissues [184, 185], or of specific compartments and organelles inside a cell [163, 180, 186, 187]. The advantages and characteristics of specific techniques have been described in [184] for embryonic tissues and in [188] for single cells. While cortical and cellular mechanics have been measured and addressed in various condition, it is still not trivial to measure intracellular or nuclear mechanics, which are known to change during development and cell fate specification or upon physical forces [142–145, 167]. It is even more complicated to address these mechanical properties in confined cells, where changes in NE tension have been shown to associate with mechanosensitive pathways and to be relevant in controlling cellular behavior [94, 181, 182]. The main limitation of techniques such as Atomic Force Microscopy (AFM), micropipette aspiration or glass microplate compression [184, 185] to measure intracellular mechanics of organelles as the nucleus arises from the fact that the cells are probed from the outside, through their plasma membrane and cortex. As a consequence, it is difficult to disentangle the role of the plasma membrane and/or cell cortex from the nucleus (or of any other subcellular compartment of interest). A common approach is to depolymerize actin, however, this could affect nuclear mechanics [189] and does not prevent the activation of mechanosensitive responses at the PM due to the deformation (as mediated by ion channels).

A practical example is given by AFM: AFM allows to measure forces with very high accuracy and characterizing the topology of a sample, and it can be combined with various microscopy techniques or other physiological relevant approaches [185]. In AFM experiments, tips of different shapes can be used to indent a cell (or any material of interest) for measuring its mechanical properties. For example, planar AFM cantilever tips have been used to confine single cells and accurately measure their mechanoresponse [181]. It can also be coupled with high-resolution 3D imaging to probe nuclear deformation during the experimental routine [163, 180]. By using sharp tips, nuclei of adherent cells can be locally indented and their mechanical properties measured in situ [180]. A similar approach also showed that the nucleus is not only the stiffest organelle in the cell, but that the cell cortex enhances its stability, preventing large
nuclear deformations [190]. AFM tips have also been coupled with 6μm beads and used to indent whole cells and their nuclei (up to a final height of 2μm, corresponding to a 30% of the original height of the cell). This approach led to the quantitative description of different contributions that regulate physical properties of the nucleus, as the role of lamin A/C and chromatin to independently resist to changes in surface area and volume respectively [163]. All of these studies have been performed in cells cultured on 2D substrates as the cells need to be accessible from the top to measure their mechanical properties with an AFM. Nuclear mechanics have been also measured with micropipette aspiration [191] or glass microplate compression [145], techniques that, however, face the same limitations as they probe the cells as a whole and from the outside. To overcome these limitations, nuclei can be isolated and their mechanical properties [144, 145, 192] and mechano-transduction capabilities [167] can be studied in vitro using AFM or magnetic tweezers. However, it cannot be ruled out that the isolation protocol itself changes molecular and physical nuclear properties as chemical isolation led to the measure of different elastic modulus of nuclei of endothelial cells [145].

Optical Tweezers (OT) offer a flexible and accurate tool to exert and measure defined forces on biological samples. Since its inception by Arthur Ashkin and colleagues [193], for which he was awarded the Nobel prize in Physics in 2018, optical trapping has become a routine technology to elucidate several aspects in biophysics [194]. A near-infrared laser can be used to easily trap and manipulate objects in 3D. This relies on the fact that light carries both linear and angular momentum and, as a consequence, can exert forces and torques on objects that have a higher refractive index than their surroundings [193]. OTs have paved the way to understanding the mechanics of DNA structure and mechanics [195, 196], protein folding [197] and the dynamics of molecular motors [198, 199]. At the cellular level, OTs have been used to unveil the mechanical properties of the cell cortex and its interaction with the PM [200, 201], as well as the behavior of the cytoplasm [202, 203]. Recently, cellular microrheology with optical tweezers has unraveled the fluidization of the cytoplasm during mitosis [186] and the force loading rate driving talin-dependent mechanosensation [187]. The use of light to measure and exert forces has the key advantage that it is non-invasive and can probe sub-cellular mechanics without globally perturbing the cell, compared to AFM tip probing from the cell surface or equivalent techniques.

4.4.2 Properties of viscoelastic materials

Biological materials such as entire tissues, as well as bio-polymers display viscoelastic rheology, meaning that they share properties of viscous fluids and elastic-stiff materials [204]. These material properties can be defined as follows [142]:

- Elasticity is the ability of a material to restore its original shape after a deformation. Gummy bears are elastic since they instantaneously recover their original shape after being deformed (Figure 4.4 A).

- Plasticity is the property of a material to undergo a non-reversible shape change
after deformation. Liquids, creams or clay are plastic materials: a piece of clay can be shaped into any figure, and the figure won’t naturally go back to its original shape (Figure 4.4 B).

- Stiffness is the resistance of an elastic material to deformation. Higher forces are needed to deform stiffer materials.

- Viscosity is the internal resistance of a fluid to flow. The energy associated to the deformation is dissipated because of the thermal fluctuation of molecules inside the fluid.

In order to interrogate the rheological properties of a material, simultaneous measurement of the strain ($\varepsilon$) and stress ($\sigma$), measured in [Pa], are performed with different kinds of deformation assays that will be discussed below. Two quantities describe the response of a material to experiments [205]: the relaxation modulus ($G(t)$) and the creep compliance ($J(t)$).

- For a step in strain, $\varepsilon_0$, the stress response is governed by the relaxation modulus, $G(t)$, as $\sigma(t) = G(t)\varepsilon_0$.

- For a step in stress, $\sigma_0$, the creep compliance $J(t)$, describes the strain response to a step in stress as $\varepsilon = J(t)\sigma_0$.

In elastic materials the force scales linearly with the deformation, such as in a spring: $\sigma = k\varepsilon$, where $k$ is the spring constant. A typical viscous response (Newtonian fluid) is represented with a dashpot, where $\sigma = \eta d\varepsilon/dt$, and $\eta$ is the viscosity. Viscoelasticity can as well be measured in the frequency domain. When a material is subjected to a sinusoidal strain, $\varepsilon(t) = e^{i\omega t}$, the stress scales with it through the complex modulus, $G^*(\omega) = G'(\omega) + iG''(\omega)$. Here, the storage modulus ($G'(\omega)$) and loss modulus ($G''(\omega)$) correspond to the elastic and viscous (with a phase delay of 90 deg) contributions to material viscoelasticity.

Importantly, all of these observations depend on the timescale of the measurement. The same material can be, for example, elastic on a short timescale and viscous on a long timescale, or vice versa. Non-Newtonian fluids (that can be easily obtained by mixing corn starch and water for example) are a classical example of materials with a two-regime response: they behave like a fluid if we slowly touch or move them and like a solid if we punch them. Similarly, biological tissues are viscoelastic materials and reported to be more elastic at short times and more viscous at long ones [184]. It was reported that tissues have a solid-like behavior at short times ($\sim$ minutes), that allow them to quickly respond to fast deformations, and a fluid-like one at longer timescale, as it can happen during development for larger-scale rearrangements ($\sim$ hours) [184]. Other studies suggested that epithelial monolayers and single cells have liquid-like behavior at the second timescale and solid-like behavior over minutes [206]. These observations suggest that the specific timescale of the liquid/solid behaviors and the exact viscoelastic response in general depends on the system itself. A variety of responses can in fact be observed [204,206,207]. Moreover, the material properties of a single cell also change over time as a result of other cellular processes: for example, the cytosol softens and increases its fluidity during cell division [186]. This happens as
a consequence of the different organizations of actin and microtubule networks during mitosis [186]. Similarly, tissues can regulate their mechanical properties by tuning cell density and adhesions: a tissue with low density and no adhesions typically behaves more fluid-like, while a high density and strong adhesions lead to a more solid-like response [184].

Figure 4.4: Elastic, viscous and viscoelastic materials (A) A gummy bear is an elastic material as by deforming it, it will recover its shape. In elastic materials, the force $\sigma$ is proportional to the deformation $\varepsilon$: $\sigma = k \varepsilon$, with $k$ the elastic constant of the material. For this reason, elastic materials are commonly represented by springs. (B) Honey is an example of a viscous material because when deforming it, it doesn’t recover its original shape. The force is proportional to the derivative of the deformation: $\sigma = \eta d\varepsilon/dt$, where $\eta$ is the viscosity of the material. A viscous material is usually represented by a dashpot.

To define the mechanical properties of a material, a deformation has to be applied during a defined time interval and the force (stress) response is recorded. Different approaches can be used. Material deformation can be performed following a step-function, as shown in Figure 4.5 A. For a purely elastic material, the force curve will follow the displacement (blue line, Figure 4.5 A). For a purely viscous material and a similar material deformation, the force would follow a $\delta$ function and therefore increase instantaneously and then rapidly drop to zero (orange line, Figure 4.5 A). Viscoelastic materials show an intermediate response: the stress increases rapidly and then decreases until reaching a final plateau $\geq 0$, depending if the material has an elastic component that stores the energy associated to deformation over long times (magenta lines, Figure 4.5 A). Here, different experimental approaches, depicted in Figure 4.5, typically used to characterize the material properties of unknown samples will be discussed.

**Triangular ramp** During a symmetric ramp protocol, a trapped object deforms the material using the same inward and outward path, thus showing a triangular displacement in time (Figure 4.5 B). The stress-deformation response for an elastic material would be a line (blue line), since the force follows the displacement without dissipation. For a viscoelastic material, the loading and unloading curves would differ (magenta line, Figure 4.5 B), defining an area that corresponds to the dissipated energy (grey area, Figure 4.5 B). This cycle can be repeated a defined number of times to measure energy dissipation and identify potential adaptation processes to repetitive mechanical stimulation.

**Stress relaxation** Elastic and viscoelastic materials can be distinguished by performing stress relaxation experiments (Figure 4.5 C) where a force is applied to deform a material at defined velocity up to a fixed strain. This would correspond to a scenario
in which the trapped object is rapidly moved to indent the sample and is then kept in that position for a certain time over which the force relaxation will be measured. For a purely elastic material, the stress would follow the strain (blue line), while a viscoelastic material would show a stress peak value, followed by a decrease. In the particular case of the standard solid model, the decrease is exponential [207]. The stress plateau (if present) corresponds to the energy stored in the material (magenta line, Figure 4.5 C). The stress-relaxation curves are often plotted in semi-logarithmic and logarithmic scales to identify their exponential and power law components [207] (see the following Section).

Active micro-rheology allows for quantitative measures of the elastic and viscous material properties at various frequencies. In this approach, a trapped particle is oscillated (with either optical or magnetic tweezers) along a defined path at increasing frequencies [208], thus following a sinusoidal trajectory. In general, performing such an experiment on a biological material leads to a complex response function $\chi^*(\omega)$ which allows to directly derive [186,205,208] the complex modulus $G^*(\omega)$ at each oscillating frequency $\omega$. As mentioned above, this can be decomposed in its real and imaginary parts $G^*(\omega) = G'(\omega) + iG''(\omega)$ modulus where $G'(\omega)$, the storage modulus, corresponds to the elastic energy stored in the material and $G''(\omega)$, or loss modulus, to the energy dissipated [208].

Mathematical models based on a circuit-like integration of elastic and fluid elements are often used to describe material properties. In these models, the elastic component is symbolized by a spring (because it behaves like a Hookean spring) and the dissipative element depicted by a dashpot (Newtonian fluid)$^1$. These objects can be combined in

$^1$Not to confuse with electrical circuits where a resistance dissipates energy and a capacitor stores it, even if the symbols are similar.
series or parallel and complex circuits can be created to model viscoelastic characteristics of any material of interest [205, 207]. In particular, a resistance and a dashpot in series define a Maxwell element and it was demonstrated that any power law can be written as the sum of an infinite number of Maxwell elements [205]. To minimize the number of elements necessary to model biological materials, a fractional spring-pot, based on the use of fractional derivatives\(^2\), has been defined. Depending on a parameter \(0 \leq \beta \leq 1\), spring-pots describe power-law response functions by means of two parameters \((c_\beta \text{ and } \beta)\) and can behave like an elastic element (spring, \(\beta = 0\)) or like a viscous fluid (dashpot, \(\beta = 1\)), or show intermediate regimes [205, 207]. Despite the complexity of fractional calculus, power-law microrheology leads to an easy and straightforward interpretation of the experimental data and it is often used [186, 205, 206]. Importantly, no a-priori knowledge of the material is needed, but its mechanical characteristic can be derived.

\(^2\)In the spring-pot fractional element, the stress is proportional to the \(\beta\)-derivative of the strain:
\[
\sigma(t) = c_\beta \frac{d^\beta \varepsilon(t)}{dt^\beta}
\]
Chapter 5

Objectives and structure of the thesis

5.1 General objective

This thesis aims to understand how cells can dynamically sense changes of their 3D shape and accordingly tune their contractility set-point and migration behaviour. Mechanical confinement has been shown to induce stable-bleb transformation and ameboid motility via activation of myosin II in various systems [54, 75, 104], as discussed in Chapter 3.2. These studies suggested the presence of a conserved, but unknown, mechanosensitive pathway capable of sensing cell shape deformation and transducing this information into the regulation of cellular contractility. The goal of this thesis is to identify this mechanosensing element and to characterize the mechano-transducing pathway, in order to understand how single cells can sense their own shape and dynamical changes in it. To do so, we use progenitor stem cells derived from zebrafish embryos, described together with the experimental procedures in Part II. Then, in Part III the main results are presented.

5.2 Specific questions and structure of the thesis

The Results section (Part III) is divided into four Chapters that address specific questions.

1. Chapter 8 will address cellular behavior in confinement. This is based on a quantitative and systematic study, introduced in Chapter 7. Different confinement heights and conditions will be tested in order to understand how cells respond to various degrees of shape changes and how cells behave in different conditions. This is essential to unveil the main characteristics of the mechanosensitive process and will provide hints to identify the mechanosensitive cascade. Several known mechanosensitive pathways will be tested and evidences of a possible role of the nucleus as the mechanosensor element will be presented.
2. Chapter 9 will introduce the results supporting the main finding of this thesis: the nucleus is the mechanosensitive element that allows cells to measure large cellular shape changes. Mechanotransduction is achieved thanks to a lipase, cytosolic phospholipase A2 (cPLA$_2$), that gets activated because mechanical confinement induces nuclear deformation and inner nuclear unfolding. The role of cPLA$_2$ as the mechano-transducer of cellular shape deformation will be demonstrated with different experiments. Also, the mechano-transduction pathway that induces myosin II activity downstream of cPLA$_2$ activation will be described.

3. Chapter 10 will describe a novel method to measure intracellular and nuclear mechanics. This, allowed for studying the viscoelastic properties of the nucleus under confinement and establish a rheological description of the nucleus with respect to the cytoplasm of suspended cells.


4. Chapter 11 will test if cells can use the same pathway to sense different types of shape changes, exemplified by isotropic swelling under hypotonic shock with respect to the mechanical confinement described in the previous chapters. The experiments presented will lead to the understanding that cells can sense and distinguish different kinds of deformations, by combining the information on INM unfolding and intracellular nuclear positioning. These experiments, together with the ones presented in Chapter 9, present a novel function for the nucleus, that act as a mechano-controller of cell dynamics.


Finally, the obtained data will be discussed and conclusions are given.
Part II

Materials and methods
Chapter 6

Embryonic progenitor stem cells from zebrafish

In order to understand how cells can dynamically sense their 3D shape and adaptively regulate myosin II activity, we performed a quantitative study, that is described here in detail. To do so, we used progenitor stem cells derived from zebrafish embryos as a model system. Zebrafish is a known model system in developmental biology (see next Section), thus it is ideally suited to perform complementary in vivo and in vitro experiments. This dual approach enables to study biological processes via reductionist bottom-up assays, while offering the opportunity to validate findings in the physiological in vivo setting, as presented in this thesis. With respect to stable-bleb transformation, the use of progenitor stem cells has several advantages. First of all, the process is faster than in other cell lines such as HeLa cells, thus allowing for a shorter experimental duration. Also, cells can transform either by mechanical or chemical stimuli and this provides a very useful tool for positive control experiments. Finally, progenitor stem cells of different sizes, cell fate or migratory capabilities can be easily obtained, as discussed shortly.

6.1 Zebrafish early development

Zebrafish, or Danio rerio, is a small (3 – 4cm) freshwater fish, commonly used as a model system in vertebrate developmental biology. The main advantages are: embryos are fertilized and develop externally (ex-utero), their development is fast, they can be obtained at high numbers and embryos are transparent, therefore ideally suited for imaging approaches. In this work, zebrafish is used to obtain primary progenitor stem cells from early developmental stages, between 3 hours post fertilization (hpf) up to 9 – 10hpf. The early developmental stages will be briefly described here. A comprehensive description of zebrafish development is given in [211] and a collection of all zebrafish protocols in [212].

As for all animals, development starts with the fertilization of the oocyte, resulting in
the formation of the zygote. The eggs are released from the mother into the water, where they are fertilized. The egg is formed by one cell located on top of a large yolk cell (Figure 6.1 A). The side where the cell resides is defined as the animal pole of the embryo and the opposite side is defined as the vegetal pole. Approximately 40 minutes after the embryo is fertilized, a phase of synchronous divisions, the cleavage period, starts (0.75 – 2.25hpf, green area of Figure 6.1 A). During this period, the pluripotent stem cells reduce their size at every division step as the number of cells increases, while the total volume of the embryo is maintained [211]. This particular feature of zebrafish development will be employed to obtain cells of different sizes.

Over time, cell divisions start to get asynchronous. At around 2.25hpf, the blastula period starts (2.25 – 5.25hpf, cyan area of Figure 6.1 A). This is the onset of zygotic transcription and the moment at which the epithelial enveloping layer (EVL) starts to form from the outermost cells. The EVL is the first tissue that specifies during development and acts as a protective barrier between the embryonic stem cells (deep cells) and the surrounding environment. The cells of the EVL form tight and stable junctions, as shown in the first panel of Figure 6.1 B. The outer epithelial cells that form a connected tissue appear very different from the cells present in the inner cell mass (the deep cells) that do not form stable contacts with each other, but are loose and embedded in the interstitial fluid (visible from the dark areas of Figure 6.1 B).

At this stage (4hpf), the deep cells appear very dynamic as they bleb inside the early blastula embryos (Figure 6.1 C). These are the primary progenitor stem cells that are dissociated from embryos and used for the in vitro experiments presented in this thesis. During blastula stage, some of the cells at the yolk margin fuse into the yolk, creating a multi-nuclear layer (called the Yolk Syncytial Layer; YSL) that is transcriptionally active and controls signals required for the later steps of cell fate specification and tissue patterning. At the onset of gastrulation, the deep cell mass starts to undergo massive rearrangements. At 4.7hpf, the yolk starts to push towards the animal pole and the cells start spreading in the opposite way (yellow arrows, Figure 6.1 A): this is the onset of gastrulation (5.2 – 10hpf, orange area of Figure 6.1 A). During gastrulation, the inner cell mass specifies into three germ layers: ectoderm, endoderm and mesendoderm. Mesoderm is the first tissue that segregates from the others and cells internalize and migrate towards the animal pole, while the EVL and ectodermal tissue spreads towards the vegetal pole covering the yolk cell (magenta arrow 6hpf, Figure 6.1 A). Mesoderm cells are also migratory in vitro when plated on adhesive substrates such as fibronectin [54]. Interestingly, germ layer specification can be induced homogeneously in the entire embryo in order to obtain a specific cell type and study it in vitro [51]. During gastrulation, the spreading of the EVL tissue is mediated by pulling forces generated by a contracting actomyosin ring in the yolk cell [106,130,211,213]. As the EVL tissue spreads, the epithelial cells flatten and increase their apico-basal area [106,211]. Gastrulation ends at 9hpf when the segmentation period starts [211].
Figure 6.1: Zebrafish early development (A) Various developmental stages are shown as part of the three main periods of early development: zygote (0.25 hpf), cleavage period (0.75 – 2.25 hpf, green area), blastula period (2.25 – 5.25 hpf, cyan area) and gastrula period (5.3 – 9 hpf, orange area). The zygote picture is taken from [211], the other images are obtained from a bright field movie acquired by Q. Tolosa (CRG). Scale bar 250 μm. (B) Confocal images of a zebrafish embryo at sphere stage (4 hpf) expressing myosin II-eGFP at different planes as indicated by colors. The EVL is visible at this stage and can be recognized as epithelial cells form tight junctions, while cells in the embryo interior (deep cells) are loosely packed. Stack acquired by Q. Tolosa (CRG). (C) Time lapse of a zoomed view showing deep cells in a 4 hpf zebrafish embryo expressing myosin II-eGFP. Deep cells are highly dynamic as they bleb (yellow arrows) and show actin protrusion (magenta arrows). Scale bars in (B) and (C) 50 μm.

6.2 Progenitor stem cells

Progenitor cells can be dissociated from embryos at different developmental stages to obtain cells of different sizes (larger versus smaller sizes depending on earlier versus later developmental stages), of different cell type (pluripotent or lineage-committed cells) as well as various morphodynamic states (non-motile versus mesenchymal or epithelial cells).
Preparation

The dissociation protocol consist of a few steps, fully explained in a video protocol in [209]. In order to obtain cells, the embryos are first dechorionated and the outermost shell/membrane that protects the embryos from the outer environment is removed. This is manually done with forceps to avoid the use of chemicals that could affect the outermost tissue of the embryo and on a glass dish to avoid embryo ruptures. Then, the embryos are transferred to a tube (typically 1.5ml) with Dulbecco’s minimum essential medium–nutrient mixture F-12 (DMEM) and manually dissociated by gently shaking it. Finally, a centrifugation step (200g, 3min) allows to separate the remaining yolk from the embryonic cells, with the cells remaining in the pellet. The cells are then re-suspended in fresh DMEM and plated for experiments. The DMEM used for all experiments is a CO₂-independent DMEM-F12, with L-glutamine and 15μM HEPES, without sodium bicarbonate and phenol red (Sigma).

If not indicated otherwise, progenitor stem cells are derived from embryos at sphere stage (4hpf) and plated on non-adhesive pll-PEG coated (see detailed protocol in Chapter 6.3) surfaces in standard (serum free) DMEM culture media. At this time point, the blastula cap of the embryo is composed of more than a thousand of identical pluripotent cells. Because of the size reduction of progenitor stem cells during development and of fate specification programs starting after 5hpf it is important to dissociate progenitor stem cells from the same developmental stage to compare results between independent experiments.

Behavior

Isolated primary progenitor stem cells (4hpf, sphere stage) cultured in standard 2D assays (such as non-coated glass) are low adhesive as they do show poor cell spreading over time compared to standard cell culture lines (e.g. HeLa). Isolated cells show few actin-rich protrusions such as filpodia and lamellipodia, and the frequent formation of membrane blebs [54], similarly to the behavior they have in the live embryo at the same stage (see Figure 6.1). Non-adhesive surface coatings, such as pll-PEG, favor blebbing with respect to actin-protrusion (Figure 6.2 A). This cell behaviour is myosin II-dependent and inhibition of myosin II activity with blebbistatin inhibits cellular blebbing (Figure 6.2 A).

As discussed in Chapter 3, when progenitor stem cells are treated with LPA, a selective activator of myosin II activity through the Rho/ROCK pathway, they transform to stable-bleb polarized cells (Figure 6.2 B) [54]. When myosin II is active, it gets recruited to the cell cortex and cellular contractility increases. As a consequence, blebs get bigger [54]. Above a certain threshold of contractility, cells fail to retract the bleb and it becomes stable in time, leading to cell polarization (Chapter 3). Polarized cells are non-motile in suspension (2D surface) and need to be cultured in 3D environments.

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1The EVL is already differentiated at sphere stage (4hpf). EVL cells are typically only recovered in few amounts during the dissociation procedure since they have a lower cell number in the embryo and their tight adhesion complicates the isolation procedure. If EVLs cells are observed in culture assays, they can easily be identified because of their different shape and they were excluded from the measurements shown in this thesis.
Figure 6.2: Blebbing and migration dynamics in progenitor stem cells (A) Bright field time lapse movie (left) and sketch of progenitor stem cells (right) cultured in 2D non-adhesive conditions in control assays (DMEM, top) or upon treatment with 10μM blebbistatin (bottom) that inhibits myosin II activity and cellular blebbing. Yellow arrows point at cellular blebs. (B) Bright field time lapse movie and sketch of polarized progenitor stem cells upon treatment with 50μM LPA in suspension (top) or polarized cells cultured between two glass cover slips to gently confine cells (middle) and of a confined cell using a 1% agarose block. Magenta asterisk points at the cell front of the polarized cell in suspension; orange arrows indicate the direction of cell migration. All scale bars 20μm.

such as between two surfaces to move [54,66]. This can be easily achieved by gently placing a second glass coverslip or a block of agarose 1% on top of a small volume (few-tens of μl) of cells (Figure 6.2 B). Importantly, as shown in Chapter 3.2 and in [54], mechanical confinement alone is also sufficient to induce stable-bleb transformation and cell migration (Figure 6.2 B), similarly to LPA treatment.

To understand how cells sense shape deformations and to characterize their mechanosensitive response, cell confinements were established that enable to deform cells in a robust and reproducible way. This is not achievable by simply culturing cells in between two coverslips or under agarose as these two methods do not allow to tune the confinement height of cells, a key parameter to address the central question of this work. Also, because of the small volumes used to obtain a highly confined space, the sample can dry very quickly if not properly sealed and this can lead to a change of the confinement over time, apart from affecting the viability of cells. For these reasons, a planar micro-confinement assay was developed, as described in the following Chapter.
6.3 Detailed cell culture protocols

In zebrafish, proteins of interest can be visualized using transgenic lines via mRNA injections of fluorescently-tagged proteins or by standard live cell staining protocols. Interference with specific signalling pathways/proteins is obtained by using chemical inhibitors or by injecting dominant negative RNA or morpholinos.

**Zebrafish maintenance**  Zebrafish were maintained as previously described [212]. Embryos were kept in E3 medium at temperatures between 25° and 31°C before experiments and staged according to morphological criteria [211] and hours post-fertilization (hpf). Wild-type embryos were obtained from the AB strain background. All protocols used have been approved by the Institutional Animal Care and Use Ethic Committee (PRBB– IACUEC) and implemented according to national and European regulations. All experiments were carried out in accordance with the principles of the 3Rs (replacement, reduction, and refinement).

**Transgenic fish lines**  The following transgenic lines were used: Tg(actb2:Lifeact-GFP) [214], Tg(actb2:Myl12.1-eGFP) [215], Tg(actb2:Myl12.1-mcherry) [215], Tg(actb2:Lifeact-GFP)(actb2:Myl12.1-mcherry) Tg(mezzo:EGFP) [54], Tg(actb2:H2B-GFP) [216].

**Injections**  For zebrafish mRNA injections, mRNA was synthesized using the Message mMachine Kit SP6 Kit (Ambion AM1340M). The detailed protocols used for generating the constructs used for experiments are described in Appendix A.2. All injections were performed in 1-cell stage embryos. Concentrations of mRNAs are in pg/e (picograms per embryo) and of morpholinos (MO) in ng/e (nano-grams per embryo). The drop size of the needle was measured prior to each injection round to ensure correct concentrations as described in [209]. All injections of morpholinos, unlabeled mRNA, rescue experiments or induction of specific cell fate have been done on Tg(actb2:Myl12.1-eGFP) or Tg(actb2:Myl12.1-mcherry) lines and supplemented with 100pg/e of Lyn-dTomato to select the injected embryos. The following morpholinos have been used:

<table>
<thead>
<tr>
<th>Name</th>
<th>MO sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>casanova MO</td>
<td>GCATCCGGTGAGATACATGCTGTT</td>
</tr>
<tr>
<td>cPLA₂ MO</td>
<td>AAGCGTCACTTACTATAATGTTGGA</td>
</tr>
<tr>
<td>cPLA₂ab MO</td>
<td>AACCTTCACTTTGAATTTGTGTGAG</td>
</tr>
</tbody>
</table>

The following concentrations of mRNA/MO were used to label the indicated structures/proteins of interest or to interfere with specific signalling molecules:
<table>
<thead>
<tr>
<th>Organelle/protein labeled or function</th>
<th>mRNA/MO name</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>INM</td>
<td>Lap2β-eGFP</td>
<td>80pg/e</td>
</tr>
<tr>
<td>nucleus</td>
<td>H2A-mScarlet</td>
<td>100pg/e</td>
</tr>
<tr>
<td>plasma membrane</td>
<td>Lyn-dTomato</td>
<td>100pg/e</td>
</tr>
<tr>
<td>induce mesendoderm</td>
<td>cyclops mRNA</td>
<td>100pg/e</td>
</tr>
<tr>
<td>induce endoderm</td>
<td>Casanova mRNA</td>
<td>50pg/e</td>
</tr>
<tr>
<td>induce mesoderm</td>
<td>cyclops</td>
<td>100pg/e/2ng/e</td>
</tr>
<tr>
<td>induce ectoderm</td>
<td>Lefty</td>
<td>100pg/e</td>
</tr>
<tr>
<td>visualize cPLA2</td>
<td>cPLA2-eGFP</td>
<td>50pg/e</td>
</tr>
<tr>
<td>rescue cPLA2 activity</td>
<td>cPLA2</td>
<td>200pg/e</td>
</tr>
<tr>
<td>interfere with cPLA2 activity</td>
<td>dn-cPLA2</td>
<td>200pg/e</td>
</tr>
<tr>
<td>block splicing of pla2g4aa (cPLA2α)</td>
<td>cPLA2 MO</td>
<td>2.7ng/e</td>
</tr>
<tr>
<td>block splicing of pla2g4ab (cPLA2α)</td>
<td>cPLA2ab MO</td>
<td>2.7ng/e</td>
</tr>
<tr>
<td>interfere with RhoA activity</td>
<td>dn-RhoA</td>
<td>100pg/e</td>
</tr>
<tr>
<td>measure RhoA activity with FRET</td>
<td>RhoABiosensor</td>
<td>400pg/e</td>
</tr>
<tr>
<td>STIM</td>
<td>STIM-CFP</td>
<td>80pg/e</td>
</tr>
<tr>
<td>ORAI</td>
<td>ORAI-YFP</td>
<td>50pg/e</td>
</tr>
<tr>
<td>Measure intracellular and nuclear calcium</td>
<td>gCamp+NLS-dTomato</td>
<td>100pg/e</td>
</tr>
</tbody>
</table>

**Fluorescence staining** Calbryte520 (AAT Bioquest) was used to study calcium dynamics. The staining kit—Red Fluorescence—Cytopainter (ERTracker, Abcam) or ER-Tracker Green (BODIPY FL Glibenclamide) was used to visualize the endoplasmic reticulum. DNA-Hoechst (Thermo Fisher) was used to stain the cell nucleus. Cells were incubated with 5μM Calbryte520 for 20min, with 1μM ER-tracker for 30min and 1mg/ml of DNA-Hoechst for 7-10min, as reported in the corresponding protocols. After incubation, cells were centrifuged (200g, 3min) and re-suspended in DMEM.

**Reagents and inhibitor treatments** Pharmacological inhibitors were used at the following concentrations: 1μM Pyrrophenone (cPLA2 inhibitor, Merck-Millipore), 10μM BaptA-AM (Cayman), 10μM blebbistatin(-) (Tocris Bioscience), 10μM Y-27632 (Tocris Bioscience), 10μM ML-7 hydrochloride (MLCK inhibitor, Tocris Bioscience), 1—10μM Nocodazole (Sigma), 50nM leptomycin B (Sigma-Aldrich), 1μM ionomycin (Sigma-
Aldrich), 1μM thapsigargin (Thermo Fisher), 10μM GsMTx4 (Tocris), 50μM 2-APB (Biogen-Santa Cruz), 10μM gadolinium chloride (Tocris Bioscience), 2μM actinomycin D (PanReac), 100μM Z-VAD(OMe)-FMK (Abcam), 1μM staurosporine (Abcam), 500nM LatrunculinA (Sigma-Aldrich), 10μM COX inhibitor (SC560, Abcam), 10μM FLAP inhibitor (MK866 sodium salt, Tocris), 1μM MEK/ERK inhibitor (Tametrinib, Tocris Bioscience). Arachidonic acid (single use, Sigma-Aldrich) or Arachidonic Acid-d8 (Cayman chemical) was used at 100μM and 1-oleoyl lysophosphatidic acid (LPA, Tocris Bioscience) at indicated concentrations ranging 50 – 100μM. Measurements were done directly after exposure to MLCK inhibitor, GsMTx4, ionomycin, AA, LPA, LatA; all other inhibitors were pre-incubated for 30 or 60min for Y-27632 prior to experiments.

Osmotic shocks D-Mannitol (Sigma) was diluted in DMEM to obtain a culture medium with an osmolarity of ~ 450 milliosmoles per liter (corresponding to a 1.5x media). Milli-Q water was added to DMEM for hypotonic conditions, 0.5x media corresponds to equal volume of DMEM and water.

Surface coating and imaging plates The following products for surface coatings at the indicated concentration have been used: 0.5mg/ml PLL-PEG (PLL(20)-g[3,5]-PEG(2), Susos) and 0.2mg/ml fibronectin (Sigma-Aldrich). Before PLL-PEG coating, both coverslips and dishes were plasma cleaned. Uncoated or PLL-coated glass dishes #1.5 were purchased from MatTek (MatTek Corporation) and used for all experiments. If not indicated differently, top (confiner) and bottom (glass dish) surfaces are coated with PLL-PEG.

Embryo mounting Related to Figure 6.1 and Figure 11.4 F. Embryos were mounted in 2% low melting point agarose prepared in Danieau’s solution (58mM NaCl, 0.7mM KCl, 0.4mM MgSO4, 0.6mM Ca(NO3)2 and 5mM HEPES) on a Mattek dish and covered with Danieau’s solution. Embryos in Figure 11.4 F (H. Hakkinen (CRG)) were dechorionated and placed in single embryo agarose wells (Adaptive Science Tools) at sphere stage. Each embryo was then injected with an average of 16nl of injection mix containing hypotonic media (DMEM:MilliQ 1:1) with Dextran and Alexa Fluor™ 546 (10kMW, Anionic, ThermoFisher Scientific) to label the interstitial fluid and together with 10μM Ionomycin calcium salt (Sigma-Aldrich). Injections were applied into the extracellular space at the animal pole. Embryos at 24hpf (Figure 7.4) were anaesthetized prior to mounting and then embedded in agarose with tricaine in 0.75% to image bead distributions as described in [212].
Chapter 7

A quantitative approach to study single cell mechanosensing

To address how cells measure shape deformations, we used an in vitro quantitative approach, described in the following. First, we cultured progenitor stem cells derived from zebrafish embryos in defined mechano-chemical environments that mimic specific conditions in 3D tissues. This is achieved by using a planar micro-confinement assay that allows us to control various physical parameters of the cell’s microenvironment such as the confinement height, the surface properties or cell density. This assays is then combined with known microscopy techniques and quantitative image analysis. For example, transmission light microscopes (or bright field) allow to characterize cellular behavior in different confined environments. Confocal or total internal reflection fluorescence (TIRF) microscopy can be used to image specific proteins and their localization or dynamical changes within cells. This method will be first used to characterize the cell mechano-response to shape deformation, specifically to understand how cells behave at different confinements heights. Then, a screening process will be performed to identify the mechanosensing pathway that mediates myosin II activation upon confinement.

Here, the cell confinement methods as well as the detailed imaging acquisition and analysis protocols are described. Finally, optical tweezers will be presented in more detail as this is the technology chosen to measure intracellular mechanics in progenitor stem cells in different 3D environments (results presented in Chapter 10).

7.1 Planar micro-confinement assays

To understand the underlying mechanisms of how cells measure shape deformations, we established a culture assay to deform cells in a controlled and reproducible manner. For this purpose, we adapted a dynamic planar micro-confinement system based on
a PDMS micro-fabrication method that allows to confine and deform cells at various heights [75,101] (Figure 7.1 A). This approach enables to dynamically confine cells and further offers versatile surface coating strategies to change the mechano-chemical properties of the two confinement surfaces. Precise confinement heights are obtained thanks to the high precision of photolithography, used to fabricate negative molds from which the confinement coverslips are produced through a PDMS curing process onto a glass coverslip [101]. Confinement surfaces are made of two glass coverslips. One coverslip contains a layer of PDMS micropillars with a defined height that act as physical spacers between the two surfaces, thereby defining the height at which cells are confined (Figure 7.1 A-B). The lower glass is a standard imaging coverslip or bottom-glass plate. In this approach, the coverslips containing the PDMS pillars are used to confine the cells from the top (Figure 7.1 A), so that this method can be easily combined with inverted microscopes. The glass side of these coverslips naturally stick to PDMS hence it can be either placed on a cylindrical PDMS piston to confine the cells statically or to the central part of a suction cup [101], as shown in Figure 7.1 A. This suction cup creates a closed cell culture chamber and it can be coupled to a pressure controller to dynamically confine the cells (Figure 7.1 A-B). Decreasing the pressure leads to the lowering of the central part of the section cup (the "Piston" of Figure 7.1 A) and to the subsequent confinement of the cells. The lowering of the coverslip and subsequent cell confinement are typically performed within a couple of minutes and can be observed in time, as shown in Figure 7.1 B. As a consequence of this approach, the cells are confined to a height given by the height of the pillars and the cells underneath each pillar are lysed (Figure 7.1 B-C).

Pressure controller The AF1 microfluidic pressure pump (Elveflow) was used to change the pressure for tuning the confinement height. This, can be either controlled manually or through the ESI software. Step sizes of $0.5mBar$ were typically used to gradually change the pressure.

Coverslips and suction cup production Multiple Si molds were produced by photolithography in a clean room (Nanofabrication Laboratory, ICFO) by depositing a SU-8 resin on a silicon wafer. To do so, negative and positive photomasks with the desired geometry (as in [101]) were designed. Confinement coverslips were prepared with polydimethylsiloxane (PDMS) with the following heights: 18, 16, 13, 10, 8.5, and $7\mu m$. Coverslips are produced from these molds as described by [101]. This protocol relies on few steps. (1) Circular #1 glass coverslips with a diameter of $10mm$ are cleaned by sonication in methanol, dried and plasma cleaned. (2) The PDMS is gently mixed using a dilution of the PDMS/cross-linker (A/B) of 1/10 and centrifuged for 3mins at 1000g to remove bubbles. (3) The liquid PDMS is then poured on top of the mold, the coverslips are placed onto it (with the plasma-cleaned side facing the mold) and baked for $\sim 15mins$ at 95° on a hot plate or oven. (4) The coverslips are then detached using a blade, cleaned with isopropanol and can be stored on a clean glass slide. Before the experiments, coverslips are plasma cleaned and coated with pII-PEG and equilibrated in DMEM for at least 30mins.
Figure 7.1: Dynamic planar micro-confinement (A) Sketch and working mechanism of the dynamic planar micro-confiner. Confinement surfaces are composed of two glass coverslips, one containing a PDMS layer with micro-pillars of a defined height used as micro-spacers. Dynamic confinement is achieved by positioning a PDMS suction cup with such a cover slip in its central piston on top of cells, which were plated on a bottom glass dish, and by coupling it to a pressure controller. Initially, when no pressure is applied ($P = 0$), the cover slip does not touch the cells. When a negative pressure ($P < 0$) is generated, a vacuum is created and the cover slip is pressed down by the piston (black arrows) until all the pillars are in contact with the lower glass surface and cells are deformed and confined to a height specified by the dimension of the micro-pillars. Sketches adapted from [101, 210]. (B) Bright field time lapse of progenitor stem cells in 2D culture assays in suspension on a non-adhesive substrate ($t = 0$), being gradually confined (during $\sim 3$ min) and deformed to $7 \mu m$ cell height, with cells showing spontaneous cell polarization and migration. Colored lines show cell tracks associated with migrating stable-bleb cells marked with an asterisk of the same color. Note: the cell marked in black de-polarized and the one in white started division. Scale bar $100 \mu m$. (C) Representative bright field images of progenitor stem cells confined at heights of 13, 10, 7 $\mu m$. Scale bars $20 \mu m$. Asterisks indicate stable-bleb polarization associated with amoeboid cell migration.

In order to fabricate the suction cups, two aluminium rings that act as mold have been produced (Mechanical workshop, ICFO) and the suction cup is produced using a liquid PDMS/linker solution (1/10 ratio), as described in [101].
7.2 Imaging and spectroscopy

Various microscopy techniques have been implemented together in this work to characterize specific aspects of the cellular response to mechanical deformation. For example, bright field (BF) microscopes allowed to observe cell dynamics and behavior at low resolution while fluorescence confocal microscopy was employed to image specific organelles or proteins. Both techniques can be combined with the planar micro-confinement assay just described. Finally, Raman spectroscopy was used to quantify the changes of fatty acid concentrations in our samples.

**Bright field imaging** BF movies were acquired using Leica DMI-LED microscopes equipped with IDS-CMOS cameras (UI-3880LE-M-GL) and a Leica 0.4x C-mount. Air objectives 10x (NA = 0.25) or 20x (NA = 0.40) were used to image cell dynamics. Acquisition was controlled using μManager.

**Confocal fluorescence imaging** Confocal fluorescence images were acquired using a commercial Leica TCS SP5 STED CW or Leica TCS SP8 STED 3X microscope equipped with a white light laser source (Leica Microsystems, Wetzlar, Germany). In both cases a 63x oil objective (NA=1.49, HCX PL APO CS 63.x1.40 oil UV) was used. For imaging GFP-labeled proteins, samples were excited with a 488nm Argon laser using the SP5 microscope. For co-staining with Lyn-dTomato (membrane reporter) or myosin II-mCherry, a HeNe laser at 543nm was used for excitation and consecutive images were acquired. In both color channels fluorescence was collected using single molecule detectors (SMD-HyD) in photon counting mode and transmission light was collected using a forward PMT. The Leica SP8 confocal microscope was used for three color imaging and when DNA-Hoechst staining was used. Myosin II-GFP, ER-tracker Red or mScarlet were excited at 488nm, 587nm, 569nm, respectively, using a tuneable white light laser. The fluorescence light was collected using two backwards HyD detectors in photon counting mode, while the DNA-Hoechst was excited with a 405nm semiconductor laser and fluorescence was recorded using a PMT. The SP8 microscope was used for FRET imaging: CFP was excited with the 405nm laser and YFP at 512nm using the white light laser and the emitted photons were collected using the HyD detector in photon counting modes. FRAP images were recorded using the SP5 and the FRAP wizard, exciting the YFP at 512nm. Transmission light was always collected using a forward PMT. A temperature controller set at $T = 28.5\, ^\circ C$ was used for all experiments. All images used for data quantification, such as for cortical myosin II values, were acquired at the spatial resolution imposed by the Nyquist’s theorem, using the same imaging settings.

**TIRF imaging** ER imaging: Images were acquired using a custom-built total internal reflection (TIRF) microscope equipped with an Andor Zyla 4.2 cMOS camera and a UPLAPO100XOHR objective. ER-tracker Green and LifeAct-eGFP were excited with a 473nm GEM laser, myosin II-mCherry with a 571nm laser by Laser Quantum using an exposure time of 0.5s. The microscope used for the ER tracker imaging was developed...
by J. Ziegler (ICFO) and the microscope for two color imaging by L. Reymond (ICFO-CRG).

**Raman spectroscopy**  For Raman spectroscopy experiments, progenitor cells were dissociated from wild type embryos. For confinement conditions, a drop of cells of $1 - 5 \mu l$ was placed between two quartz coverslips (ESCO products, Oak Ridge, NJ) together with 10 $\mu m$ PS beads instead of using PDMS pillars as PDMS has a broad Raman spectrum. For non-confined cells, a separation of around 100 $\mu m$ between the two coverslips was set by using smaller coverslips as a spacer. A total of at least 25 spectra were obtained from different non-polarized cells for each condition. The Raman system (inVia Renishaw, Apply Innovation, Gloucestershire, U.K.) comprised a 532 nm laser ($\sim 10 mW$) that was focused onto the sample plane using a 60X water immersion Nikon objective (backscattered configuration). The laser spot size was set to 0.8 $\mu m$ allowing for localized Raman measurements. Raman spectra were recorded on a deep depletion charge coupled device (CCD) detector (Renishaw RenCam). The recorded Raman spectrum was digitalized and displayed on a PC using Renishaw WiRE software. The spectra were background subtracted with a custom-written Matlab code (see methods in [217]). First, an exploration of the spectral data set was performed using Principal Component Analysis (PCA). Second, a Multivariate Curve Resolution (MCR) algorithm was performed to extract molecular components from the Raman spectral dataset (spectral profile and abundance in each measured sample). For PCA and MCR analysis, the PLS toolbox in Matlab was used. The analysis of the Raman spectra was done by M. Marro (ICFO).

**Calcium imaging**  (related to Figure 8.7) Progenitor cells were added to a Concanavalin A coated glass bottom dish (0.05mg/mL by incubation at 31°C for 1.5h) and loaded for 20min at 28°C with 5$\mu M$ of FURA-2 plus a nonionic surfactant (0.02% pluronic F-127) dissolved in DMSO. The cells were then washed before initiating the experiment. The fluorescence signal was measured in a standard bath solution containing 140mMNaCl, 2.5mMKCl, 1.2mMCaCl$_2$, 0.5mMMgCl$_2$, 10mM HEPES, and 5mM glucose, and pH7.4 adjusted with NaOH ($\sim 320 - 340mOsm/l$)). The following concentrations of reagents were used: 20$\mu M$ of YODA1 (to activate Piezo1, Tocris Bioscience), 10$\mu M$ GsMTx4 (Tocris). Fluorescence time lapse measurements of intracellular calcium concentrations were obtained using an Olympus IX70 inverted microscope (Hamburg, Germany) with a 40X oil-immersion objective (Olympus). A Polychrome IV monochromator (Till Photonics, Martinsried, Germany) supplied the excitation light (340nm and 380nm), which was directed towards the cells in the field of view by a 505DR dichromatic mirror (Omega Optical, Brattleboro, VT). Fluorescence images were collected by a digital CCD camera (Hamamatsu Photonics, Hamamatsu City, Japan), after their passage through a 535DF emission filter (Omega Optical), using the AquaCosmos software program (Hamamatsu Photonics). Cytosolic calcium concentration was presented as the ratio of emitted fluorescence after excitation at 340nm and 380nm relative to the baseline. This experiment and the associated analysis was performed by the lab of M.A. Valverde (UPF).
7.3 Data quantification and analysis

To analyze imaging data, when possible pre-existing plugins were used. Otherwise, Fiji macros and Matlab (2020a or previous versions, Mathworks) scripts were written and used for the analysis. Data plotting and statistics were done in Matlab. Here a detailed description of the performed analysis is given.

Cortical myosin II and actin accumulation  The relative cortical intensity, of either myosin II or actin, was quantified from confocal images of progenitor stem cells dissociated from the following zebrafish lines: Tg(actb2:Myl12.1-eGFP) or Tg(actb2:Myl12.1-mcherry) for myosin II and Tg(actb2:Lifeact-GFP) for actin. Images were acquired in the equatorial cross-section of cells and only non-polarized progenitor stem cells were used for this analysis. The relative cortical intensity was defined, similarly to [54], as the normalised difference in between the mean fluorescence intensity at the cell cortex ($I_{\text{cortex}}$, defined as peaks of the orange line in Figure 7.2), with respect to the cytoplasmic signal in the bleb which was used as a baseline ($I_{\text{bleb}}$, magenta line), namely: $(I_{\text{cortex}} - I_{\text{bleb}})/I_{\text{bleb}}$. This analysis was performed using a custom-built Matlab script. For each cell, a manual selection of multiple points ($N > 10$) along the cortex was performed. For each point a 9x9 pixel area was defined and the mean of the highest 4 values was taken as $I_{i \text{cortex}}$, with $i$ the manually selected points. The value $I_{\text{cortex}}$ of the cell was defined as the median of the single measures along its cortex. The intensity $I_{\text{bleb}}$ was measured as the mean intensity in a rectangular area defined in the bleb with a homogeneous intensity distribution.

![Figure 7.2: Myosin II and actin cortical accumulation](image)

Cell, bleb, nuclear area and diameter measurements  Areas of entire cells, blebs and nuclei were quantified in Fiji using confocal 2D images in the cross-section of cells. When possible, the area of cells/nuclei were automatically segmented and the
"Analyze Particle" function used. For example, cells expressing myosin II-eGFP can be easily segmented to quantify cell area and cells expressing H2B-eGFP to quantify nuclear volume (see next paragraph). Image segmentation started with a threshold step, fine-tuned by the user. Then, steps to denoise the image, fill empty holes and measurement were automatized using the "Measure" tool of Fiji. To measure cell area/perimeter the "Area"/"Perimeter" options were respectively used. When not possible to use automatic thresholding, the perimeter of cells was selected manually using the "polyline" selection tool in Fiji. In particular, for the relative bleb/cell area, the two regions were defined manually using transmission light movies. The frame that showed the maximum bleb expansion was used. Cell and nuclear diameter (such as in Figure 8.8, 8.9) were measured from myosin II-GFP confocal images. For these analysis only non-polarized progenitor stem cells were used. Considering that the nuclei of suspension and confined progenitor stem cells have aspect ratios of $\sim 1$, their radii were quantified from the cross-sectional cell area assuming a spherical cell shape.

**Nuclear volume and surface** Nuclear volume and outer surface (Figure 9.2 F) were measured from 3D stacks of progenitor stem cells dissociated from the H2B-eGFP transgenic line using the "3D object counter" in Fiji. Single nuclei were segmented prior to quantification.

**Quantification of the fraction of stable-bleb cells** Percentages of polarized cells were computed from bright field movies as the number of polarized cells/total cells. Stable-bleb polarized cells can be easily distinguished from non-polarized cells due to their different morphology (pear shaped versus round/blebbing) and thanks to their different morphodynamic behavior (motile versus non-motile).

**Cell migration analysis** Migrating stable-bleb cells were tracked from bright-field or confocal movies using the "manual tracking" plug-in of Fiji. The cell's nucleus or cell rear were used consistently to track cell positions. Tracks were imported into Matlab for visual representation of trajectories, calculating the mean velocity and performing a mean square displacement analysis.

**Inner nuclear membrane characterization** Inner nuclear membrane properties were analyzed using cross-sectional confocal images of non-polarized progenitor cells expressing Lap2β-eGFP in Python using the Scikit-Image library (https://scikit-image.org/). We first determined the inner and outer outlines using the contour detection function and used a median filter for smoothing. From these data the nucleus area, perimeter and the convex image were determined. The ratio in between the inner area ($A_{in}$) and the associated convex area ($C_{in}$) was defined as the invagination ratio, computed as $IR = 1 - A_{in}/C_{in}$. The program was written by M. Colomer-Rossel.

**Inner nuclear membrane curvature analysis** Nuclear envelope fluctuations and bending analysis was performed on Lap2β-eGFP cross-sectional confocal images of non-polarized progenitor cells. The nuclear envelope was manually tracked in Fiji and the
discrete x-y positions were further post-processed using a custom-written Matlab script (by S. Wieser). Between each pair of discrete x-y positions a cardinal spline function was interpolated (tension=0) passing through all x−y positions. The resulting spline vector was overlaid to the fluorescence image to manually control the match with the nuclear envelope circumference. The spline was further used to calculate the curvature along the line using 2D bending vectors. The histogram of bending vectors was compared between confined cells (7μm) and cells in suspension.

**FRET**  RhoA-Förster resonance energy transfer (FRET) images of non-polarized progenitor stem cells were analyzed with the FRET analyzer plugin in Fiji. Donor, acceptor and FRET images were used by the plugin to compute the FRET index and to obtain the FRET image.

**Quantification of calcium dynamics**  Progenitor cells derived from wild type embryos were stained with Calbryte520 AM, a calcium activity reporter. The cell perimeter was segmented using either myosin II-mCherry, LynTomato or a transmission image and the nucleoplasm was manually selected using these same channels. The mean intensity in the Calbryte channel was measured in Fiji using the "measure tool" for mean intensity and raw integrated density. For gCamp experiments, progenitor cells were dissociated from wild type embryos injected with 100pg of gCamp+NLS-dTomato mRNA. The intensities of both gCamp and NLS-dTomato channels were quantified in Fiji by selecting regions in the cell/nucleus, respectively. Also, the NLS-dTomato channel was used to define a mask to quantify the gCamp intensity in the nucleus. Given the fact that mRNA expression was not fully homogeneous among different cells/embryos, the NLS-dTomato was used to normalize the gCamp signal. Two quantities that take into account this variability were used to measure intracellular calcium levels: (1) the total gCamp intensity normalised by the NLS-dTomato intensity, and (2) the gCamp intensity in the cytoplasm with respect to the nucleus.

**Orai channel localization**  Orai-YFP intensity line profiles (at the basal cell membrane) were exported from Fiji and intensity fluctuations were automatically detected in Matlab using the findchangepts function with N = 2. For cells cultured in 7μm confinement a region with a mean intensity increase in the central region of the intensity profile was automatically detected; for cells cultured at 13μm confinement no change in the intensity could be detected.

**FRAP**  Fluorescence recovery after photo-bleaching (FRAP) experiments were performed at the basal membrane of cells expressing Orai-YFP. Multiple regions of interests (ROIs) in the bleb and under the nucleus were defined and the FRAP protocol was performed using the FRAP Widard (SP5 Leica Confocal). The images were then imported in Fiji and the intensity in each ROI was measured along the entire image sequence. Also, the YFP intensity in a ROI defined in a neighboring cell, where no FRAP protocol was performed, was quantified as a reference for bleaching and used for normalization. The measured intensities where then imported in Matlab. First, all values were normalised for bleaching. Then, the normalised intensity I of each ROI was
plotted with respect to time $t$ and the data were fitted with an exponential function $I(t) = A(1 - e^{-t/\tau}) + C$. In this equation $\tau$ provides the decay time and $1 - (A + C)$ corresponds to the fraction of immobile particles.

**cPLA$_2$ translocation ratio** The cPLA$_2$ translocation ratio from the nucleoplasm to the INM was quantified in progenitor stem cells expressing cPLA$_2$-GFP. In these cells, the cPLA$_2$-INM translocation ratio was defined as the ratio in between the fluorescence intensity at the INM divided by the intensity in the nucleoplasm. The two regions were selected manually and the intensities measured in Fiji. The cells in which this translocation was not visible were defined as cells where no translocation was observed and percentages of the two were given.

**Statistical tests** Statistical significance tests were performed with either (1) the two-sample t-test using the ttest2 function in Matlab when the data followed a normal distribution or (2) a non-parametric Kruskal-Wallis test (Matlab) when the data were not normally distributed. In the case of multiple t-tests we corrected for multiple comparisons using the family wise error rate (FWER). Single pairwise data sets were considered non-significant (n.s.) if $p > 0.01$. The following significance symbols were used for the corresponding p-values: $* p < 0.01$, $** p < 0.001$ and $*** p < 0.0001$, while in multiple comparisons we adapted the p-values using the FWER correction for each range of significance. All p-value indicators and sample sizes (N) were provided in figure legends. Data were obtained from at least three independent experiments except indicated in specific figure legends. Unless indicated otherwise, points and bars represent the mean and standard error of the mean (sem). For the boxplots, the central horizontal line represents the median and the box the interquartile range (with the lower limit the first quartile and the upper limit the third quartile).

### 7.4 Optical tweezers to measure subcellular mechanics

As briefly discussed in Chapter 4.4.1, optical tweezers (OTs) offer a flexible tool to exert and measure forces on biological samples. OTs have been used in a multitude of *in vitro* studies, for example to unveil the mechanical properties of DNA [195, 196], as well as in cellular systems [200, 201]. The use of light to measure mechanics has the key advantage that it is non-invasive as it does not require a mechanical perturbation, such as a cantilever tip in AFM measurements. Given this advantage, OTs have been recently used to measure intracellular mechanics and microrheology (see Chapter 4.4.2). In particular, this approach led to the discovery and characterization of the fluidization of the cytoplasm during mitosis [186] and of the loading-rate dependency of the talin-mechanosensation [187]. These studies suggested that, in principle, optical trapping allows to measure the subcellular mechanics of any compartment of organelles of interest. Moreover, OTs do not require the sample to be accessible from the top to physically interact with it, as required in AFM or micropipette aspiration. Therefore,
Optical tweezers (OTs) are in principle compatible with the use of a cell micro-confiner to specifically study intracellular mechanics in cells cultured in different microenvironments.

There is a demand for a technological approach that enables to measure the mechanics of specific intracellular organelles in a controlled and quantitative manner, compatible with assays in which cells undergo different types of shape changes. Hence, we developed a new method, based on OTs, to quantify mechanical properties of intracellular organelles such as the nucleus in cells in suspension and mechanically deformed cells in confinement [209,210].

As introduced in Chapter 4.4.1, in OT measurements a near-infrared laser is used to trap and manipulate objects in 3D. This relies on the fact that light carries both linear and angular momentum and, as a consequence, can exert forces and torques on objects that have a higher refractive index than their surroundings [193]. The trapping - or restoring - force on an optically-trapped bead is depicted in Figure 7.3 C. In the vicinity of the trap focus, typically $x < 200\text{nm}$, the optical force can be described by a harmonic approximation $F = -kx$ [218], where $k$ ($pN/\mu m$) is known as the trap stiffness. Beyond this linear regime, the force steeply increases non-linearly and reaches an escape value, $F_{\text{esc}}$, which corresponds to the external force necessary to drag the trapped particle out of the trap. Optical trapping forces, typically in the $pN$ (pico Newton) range, can be measured by directly detecting changes in the light momentum [219,220], indicated by the green arrow in Figure 7.3 B. In typical optical tweezers experiments, trapped objects can be moved or rotated in 3D by moving the trap itself, or by using multiple traps. In our setup, trapping was controlled with a pair of acousto-optic deflectors (AOD) addressed at $25kHz$, which enabled fast control of single and multiple time-shared OTs [221]. Therefore, the input variable that needed to be defined in the experiments was the trajectory of the trap (trap position) and the measured output was the force felt by the trap (or by the trapped object) [209]. These are the variables that carry the information about the mechanical properties of any material of interest, as discussed in 4.4.2.

Sample preparation for optical tweezers measurements

OTs cannot be used to trap the entire nucleus since nuclei typically have a lower refractive index than the cytoplasm ($n_{\text{nucleus}} \sim 1.35$, $n_{\text{cyto}} \sim 1.38$) [222]. Instead, OTs can be used to indent the cell nucleus. To increase the indenting force, polystyrene microbeads that have a higher refractive index ($n_{\text{bead}} \sim 1.59$) can be used. To have beads inside progenitor cells, we performed micro-injections of $1\mu m$ polystyrene beads (Sigma, polystyrene, non-fluorescent or red-fluorescent)\(^1\) into 1-cell stage embryos (Figure 7.4 A). Bead injections were performed by standardized routines established for mRNA injections at the 1-cell zygote stage at a 1:5 dilution, and bead injections can be combined with mRNA to express fluorescent reporters of interest when necessary. To visualize bead injection efficiency and bead localizations in the live embryo, fluorescent beads

\(^1\)Beads of different sizes can be injected for specific purposes. Smaller beads could be used for experiments at later stages but permit weaker force generation. Larger beads can generate higher forces but could potentially lead to cell and tissue damage when bead size is comparable to nuclear sizes.
Figure 7.3: Force generation and detection with optical tweezers (A) Sketches to qualitatively show the optical forces acting on a spherical particle. The beam is decomposed in rays (ray optic approximation), in this sketch two rays with optical momentum are shown, \(a\) and \(b\), with \(b > a\). Top: non-focused Gaussian laser beam, the particle is not in the center of the beam. Because of the diffraction of the beams \(a\) and \(b\) by the particle, two forces are generated. The scattering force \(F_{\text{scat}}\) pushes the particle forward, while \(F_{\text{grad}}\) pushes the particle along the gradient. Bottom: When the laser beam is focused by a high-NA lens and the particle is at the focus of the beam, the two forces \(F_{\text{scat}}\) and \(F_{\text{grad}}\) balance each other, generating an equilibrium position of the bead at the laser focus. This is the optical trap. (B) Schematic of the trapping setup, including the optical trapping chamber with the sample, where two glass coverslips are separated by a spin-coated PDMS layer with height \(h \sim 50\mu m\) (suspension) or \(h = 10\mu m\) (confinement). A trapped micro-sphere deforming the cell nucleus upon indentation is shown together with the trap force (\(F_{\text{trap}}\), the force that the trap exerts on the membrane) and the membrane force (\(F_{\text{mem}} = -F_{\text{trap}}\), the force that the membrane exerts back) (black arrows). The change in beam momentum, that allows to measure the force [219], is indicated by the green arrow \(P_{\text{out}}\). (C) Sketch depicting the linear regime near the trap focus where \(F = -kx\). Further away from the trap, the force steepens and reaches an escape value \(F_{\text{esc}}\) that corresponds to the force necessary to drag the trapped particle out of the trap.

were injected together with a plasma membrane reporter and embryos were imaged at 5hpf and 24hpf. At early stages, the micro-beads spread well and showed a homogeneous distribution inside the embryo cap tissue (Figure 7.4 B) and we were able to detect beads in the deep cells as well as in the epithelial enveloping layer (Figure 7.4 C). At 24hpf, beads distributed across tissues in the developing zebrafish embryo without compromising development (Figure 7.4 D) and embryo survival (Figure 7.4 E). Primary cells of bead injected embryos can be isolated by standard protocols (see Methods) and plated in both suspension and confined environments to study the role of cell deformation on nuclear mechanics. Only non-polarized cells with 1-2 beads have been used for the experiments.

To measure the force via the change in photon momentum, all the light scattered from the interaction in between the sample and the OT laser must be captured [219,220]. To do so, the system uses a condenser lens on top of the sample (in the forward direction,
7.4. Optical tweezers to measure subcellular mechanics

Figure 7.4: Microbeads disperse in the embryo during development without affecting survival
(A) Sketch showing the micro-bead injection procedure. (B) Representative confocal (left) and
transmission light (right) image of an embryo at 5 hpf injected with 1 μm red-fluorescent beads
and expressing GPI-GFP (plasma membrane). (C) Z-projection of zoomed views of an embryo
similar to (B) where beads are color-coded according to their Z position. Magenta/orange rep-
resents apical/ventral localization. Beads are found both in the enveloping epithelial layer and
in deep cells of the blastula embryo. (D) Representative confocal z-projection and transmis-
sion light images of a 24 hpf embryo injected with 1 μm red-fluorescent beads and expressing
GPI-GFP (plasma membrane). (E) Percentage of embryo survival for control (Ctrl.) and
embryos injected with 1 μm beads at 4, 6, 24 hpf.

Figure 7.3 B) that requires a second glass coverslip on top of the sample. This sample
chamber, defined by the two glass coverslips, must have a maximum thickness of
less than 2 mm. This exact value is specific for the system used. It is defined by the
working distance of the high-NA collecting lens which needs to capture the entire beam
to precisely carry out the light-momentum measurements. For this purpose, PDMS was
spin-coated on glass plates (Willco Wells, #1.5) at either ~ 50 μm or 10 μm to separate
the lower glass from the top coverslip (1x1 inch) and to image cells in suspension and
under confinement, respectively. As an alternative, 10 μm polystyrene beads can be
mixed with the cells to act as spacers. This method is not dynamic and less accurate
compared to the dynamic micro-confinement assay presented before [101], therefore the
height of the confined chamber was measured prior to each experiment to validate its
accuracy. For suspension measurements, the bottom dish was coated with Concanavalin
A (0.05 mg/ml, 30 min, Sigma-Aldrich) to partially adhere the cells and avoid trapping
and moving the entire cell.

Detailed OT methods The OT platform (SensoCell, Impetux Optics, Spain) consists
of a continuous wave laser (λ = 1064 nm, 5 W nominal output power) steered with a
pair of acousto-optic deflectors and a force detection unit that captures the forward-
scattered light from the optical traps. The OT platform is mounted around an inverted
fluorescence microscope (Nikon Eclipse Ti2) equipped with a spinning disk confocal
microscope (Andor DragonFly 502) placed on an active isolation table (Newport). The
laser is directed onto a microscope objective (MO, 60x/NA = 1.2, water immersion, Nikon) after being expanded with a telescope to fill the MO entrance pupil through the epi-fluorescence port. A short-pass dichroic mirror reflects the IR trapping beam and transmits both the excitation and emission light for fluorescence microscopy, as well as bright-field. The force detection unit of our optical tweezers platform operates by detecting light-momentum changes, after capturing the scattered trapping beam through an NA = 1.4 oil immersion lens with a position-sensitive detector placed at the back focal plane [219]. The OT platform (LightAce, Impetux Optics, Spain) allows to perform custom trap trajectories while providing trap position and measuring the force. For indentation and stress relaxation experiments 300mW power (at the sample plane; 3W output power) was used, while for active microrheology the power was reduced to 50mW, as suggested by Impetux. The specific trajectories used for the experiments are shown in the figures.

Nuclear deformation was imaged during the trapping routine using a Nipkow spinning-disk confocal imaging platform (Andor DragonFly 502). Cells were excited with λ = 405nm (DNA-Hoechst staining) and λ = 488nm (myosin II-eGFP). The two laser lines were transmitted through a multi-band dichroic (405 – 488 – 561 – 637nm, AHF) to simultaneously excite the two fluorophores in the sample, while an IR filter blocks the trapping trapping laser light. After emission, light is directed into a long-pass dichroic (500nm), which enables parallel imaging of the two channels using two back-illuminated sCMOS cameras (Sona, Andor) after passing through emission filters 445/46nm and 521/38nm, respectively. Image acquisition was performed using the Fusion Software and obtained data were post-processed in Fiji. Tracking of nuclear morphodynamics was carried out by fitting a double sigmoid function over the segment along the indentation direction with a custom-written analysis script in Matlab, as described in [209, 210]. Data were further processed with custom-written Matlab scripts to obtain τ and the stored elastic component (Figure 10.4), to fit fractional Kelvin-Voigt model to the data and obtain the associated parameters (Figure 10.5 and 10.6).
7.4. Optical tweezers to measure subcellular mechanics
Part III

Results
8.1 Mechanical confinement controls myosin II activity and amoeboid migration

Mechanical confinement was shown to activate myosin II motor proteins and recruit them at the cell cortex, similarly to LPA treatment [54]. To understand how mechanical confinement controls myosin II activation, we cultured progenitor stem cells at different confinement heights and quantified changes in myosin II cortical accumulation by live cell fluorescence microscopy.

8.1.1 Cells measure their confinement and accordingly tune their contractility level

Lowering the confinement height in discrete steps caused a non-linear enrichment of myosin II at the cell cortex, as shown in Figure 8.1 A-B. Notably, this did not coincide with a similar increase of cortical actin (Figure 8.1 C-D). Lowering the confinement also induced an increase in the relative bleb/cell area ($A_{\text{bleb}}/A_{\text{cell}}$), Figure 8.1 E-F), supporting that cell contractility increased upon confinement. Consistently, inhibition of myosin II by treatment with blebbistatin, prevented the increase of the relative bleb area in confined cells (Figure 8.1 E). These results confirmed that cell shape deformation in confinement defines the level of cortical myosin II in cells, with cells increasing their contractility for larger shape deformations at lower confinement heights.

Interestingly, myosin II activation and recruitment at the cortex occurred rapidly upon confinement ($t_{1/2} < 1\text{ min}$), and could be observed in real time while gradually confining the cells (Figure 8.2 A-B). Furthermore, myosin II localization to the cell cortex was stable over time when cells were confined (Figure 8.2 A-B), and reversible upon confinement release (Figure 8.2 C-D). These results suggested that cells continuously
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![Figure 8.1: Cell deformation under confinement defines the cellular contractility set-point](image)

(A) Relative cortical myosin II accumulation for decreasing confinement height in non-polarized cells. N = 477 cells (suspension, unconfined); N = 56 (18 μm); N = 35 (16 μm); N = 103 (13 μm); N = 131 (10 μm); N = 49 (8.5 μm); N = 348 (7 μm). Significance values are with respect to suspension. (B) Exemplary confocal fluorescence images of control progenitor stem cells in suspension (Susp.) and at the indicated confinement heights expressing Myl12.1-eGFP (myosin II). White arrows point to cellular blebs. (C) Relative cortical actin (LifeAct) accumulation for decreasing confinement height in non-polarized cells. (D) Exemplary confocal fluorescence images of control progenitor stem cells expressing LifeAct-eGFP in suspension (Susp.) and at the indicated confinement heights. (E) Relative bleb area with respect to cell area for non-polarized cells in suspension, at indicated confinement heights, and upon myosin II inhibition (10 μM blebbistatin) for 7 μm confinement. (F) Representative bright field images of progenitor stem cells confined at 10 μm confinement (top) and in suspension (bottom). Dashed magenta and green lines indicate bleb and cell areas respectively. **P < 0.0001, *P < 0.01, n.s. not significant. All scale bars 10 μm.

sense cell shape deformations in confinement and rapidly adapt to it, controlling the set-point of myosin II activity and thereby cell contractility.
Chapter 8. Cellular response to mechanical confinement

8.1.2 Mechanical confinement induces stable-bleb migration

As discussed in Chapter 2.2 and shown before, highly contractile cells spontaneously transformed to stable-bleb polarized cells and became motile [54]. This typically happens when contractile cells generate a large bleb that cannot be retracted, as shown in the time-lapse of Figure 8.3 A. Consistently with the increase in cell contractility and the formation of larger blebs (Figure 8.1), we observed a higher fraction of polarized cells at lower confinement heights (Figure 8.3 B).

Cell polarization and the initiation of motility is a rapid process after mechanical confinement is applied, as the percentage of polarized cells shows a fast exponential increase with a half-time of \( t_{1/2} \sim 4 \text{ min} \) (Figure 8.3 C). Cell polarization was prevented by treating cells with blebbistatin (myosin II inhibitor, Figure 8.3 B-C), supporting that cellular symmetry breaking requires myosin II activity [54]. Stable-bleb cells generally
8.1. Mechanical confinement controls myosin II activity and amoeboid migration

Figure 8.3: Stable-bleb cell transformation under confinement

(A) Representative time-lapse fluorescence images of a progenitor cell expressing myosin II-eGFP undergoing a spontaneous stable-bleb transformation under 7µm confinement. (B) Percentage of stable-bleb polarized cells depending on confinement height and for cells treated with 10µM blebbistatin (myosin II inhibitor) cultured at 7µm confinement height. (C) Percentage of polarized migratory stable-bleb cells over time after applying a 7µm confinement (t = 0) for progenitor stem cells cultured in control conditions (DMEM, blue, N=620) or with 10µM blebbistatin (green, N=486); t1/2 ~ 4min. (D) Exemplary confocal image of the basal cortex of a stable-bleb cell expressing myosin II-eGFP under 7µm confinement and associated kymograph along yellow line. Scale bars: kymograph 10µm (x) and 10s (t). Magenta arrow indicates the direction of the cortical retrograde flow and orange arrow the direction of cell migration in both panels. (E, F) Cell tracks (E) and mean cell velocity (F) for stable-bleb polarized (blue) and blebbing non-polarized (orange) progenitor stem cells cultured in 7µm confinement. Red lines represent mean and standard error of the mean. (G) Representative confocal time lapse images of a blebbing non-polarized cell (top) and stable-bleb polarized (bottom) cell expressing Myl12.1-eGFP (myosin II), Lyn-dTomato (plasma membrane) and DNA-Hoechst (nucleus) cultured in 7µm confinement. Red arrows indicate the direction of migration. Scale bars 20µm. ** P < 0.001, *** P < 0.0001.
show two characteristic hallmarks: 1) a cortical density gradient, with a higher actin and myosin II intensity at the cell rear, while the cell front (the stable-bleb) is depleted of cortical components, and 2) a continuous cortical retrograde flow (magenta arrow, Figure 8.3 D), opposite to the direction of cell migration (orange arrow, Figure 8.3 D), that was shown to power cell propulsion [54, 66]. As expected, stable-bleb cells were motile under confinement and exhibited a fast and persistent migration (Figure 8.3 E-G). Cell movement was persistent but not directional, as cells moved into random directions in confinement (Figure 8.3 E). Non-polarized cells were not migratory, but showed a slow and random movement mediated by frequent bleb protrusions, resulting in a small net displacement compared to polarized cells (Figure 8.3 E-F-G). However, stable-bleb cells showed a directional movement in non-planar confinement when cells were confined between two tilted coverslips and migrated towards the less confined space (Figure 8.4 A). This characteristic migration behaviour is reminiscent of an evasion-reflex mechanism that allows cells to evade from tight spaces. To further support this observation, we used PDMS micro-pillars themselves to confine cells, thereby creating a small region of confined cells - underneath each pillar - surrounded by non-confined cells, as shown in Figure 8.4 B. Confined cells underneath the pillars rapidly acquired high contractility and thus transformed to stable-bleb cells and became motile, migrating away from the confined area (Figure 8.4 B), which ultimately leads to a depletion of cells in highly confined regions over time.

These observations support that shape deformation under confinement controls cell behavior and migration plasticity of single cells. If cells are highly deformed, they rapidly activate myosin II motor proteins and increase their contractility, leading to an extensive re-organization of the cell cortex that allows them to migrate away from confined areas. As cell contractility and polarization occur reversibly, cells can transiently activate amoeboid motility mode and evade from confined spaces and revert back to their initial state when not deformed.
8.1. Mechanical confinement controls myosin II activity and amoeboid migration

Figure 8.4: An evasion reflex mechanism (A) Representative bright field image (left) and zoomed view on cells confined with a tilted coverslip with lower/higher confinement height on the left/right part of the image. Magenta zoomed region: confined cells transform into a stable-bleb migratory phenotype and are polarized, with the bleb front facing the region of lower confinement. Yellow zoomed region: non-polarized blebbing cells gently confined. (B) Representative bright field image (left) and time lapse images of zoomed view (right). Two cells are confined underneath a PDMS micro-pillar (indicated with the magenta line), while the cells outside this area are in suspension and non-polarized. The confined cells rapidly transform to stable-bleb polarized cells (as indicated by the orange arrows) and migrate away from the confined area, loosing their polarization outside the confinement area. Dashed orange line shows cell tracks. Scale bars 100μm.

8.1.3 Dependence of the mechanosensitive cell response on cell fate and initial migration phenotypes

During gastrulation, pluripotent progenitor stem cells specify into three different lineages: ectoderm, mesoderm and endoderm (Figure 8.3 A, see Chapter 6) and acquire distinct mechanical and migratory properties that drive tissue segregation [51]. To test whether different cell types can transform to a stable-bleb phenotype when confined, we obtained cells from different lineages, as discussed in Chapter 6.3. Interestingly, mechanical confinement was sufficient to recruit myosin II at the cortex of non-polarized cells and to induce stable-bleb cell transformation in all cell lineages independent of cell-cell and cell-substrate adhesion, as shown in Figure 8.5 C-D-E. As discussed in Chapter 6, mesoderm progenitor cells show mesenchymal migration both in vivo and in vitro. In vivo, mesoderm is the first tissue in which cells show active migration, driving segregation from the overlying ectoderm tissue during early gastrulation. The same slow and collective migration can be observed by culturing mesoderm-induced cells on a 2D substrate coated with fibronectin in vitro (2D substrate, Figure 8.5 B-C). Strikingly, these cells quickly transformed to an amoeboid stable-bleb mode when confined.
(3D confinement, Figure 8.5 B-C, F-G). Stable-bleb cells showed higher velocities than mesenchymal migrating cells (Figure 8.5 G). These results support that shape deformation under confinement induces myosin II activation and stable-bleb transformation independently of cell fate or preexisting migration programs.

Figure 8.5: Confinement triggers myosin II accumulation and stable-bleb migration in all cell lineages (A) Sketch of the developing zebrafish embryo at sphere stage (4hpf, hours post-fertilization), shield stage (6hpf), and 75% epiboly (8hpf). (B) From left to right, exemplary confocal image of mesodermal cells in vivo expressing Lyn-dTomato (magenta, plasma membrane) and GFP (green) under the mezzo promoter; bright-field images of induced mesendodermal cells in vitro plated on a 2D fibronectin-coated surface (middle) and under 7μm confinement (right). Asterisk indicates stable-bleb. (C) Sketch of cell behavior on 2D/3D environments depending on surface coating and cell fate.(D) Relative cortical myosin II intensity for mesendodermal and ectodermal progenitor cells in suspension/confinement (non-adherent substrate). (E) Exemplary confocal images of stable-bleb polarized (top) and non-polarized (bottom) progenitor cell types expressing myosin II–eGFP under 7μm confinement. From left to right: ectoderm, endoderm, and mesoderm cells. Dashed line and yellow asterisks indicate the stable-bleb cell front, and red arrows indicate the direction of cell migration. (F) Percentage of stable-bleb cells for induced mesendoderm and ectoderm cells on passivated surfaces and mesoderm cells on fibronectin (FN)-coated surfaces for suspension (2D) and 7μm (3D) confinement. N>350 for all conditions. (G) Cell velocity for mesendoderm and ectoderm cells on passivated surfaces and mesoderm cells on FN-coated surfaces in suspension (2D) and 7μm (3D) confinement. Red lines represent mean and standard error of the mean (sem). N: mesoderm on fibronectin in 2D N=14; mesendoderm 3D N=17; ectoderm 3D N=19; mesoderm on fibronectin 3D N=25. Injections to induce the different lineages performed by H. Häkkinen. *** P < 0.0001, not significant (n.s.). Scale bars 10μm.
8.2 Myosin II activation is independent of known mechanosensors

In order to identify the potential mechanosensor element(s) capable of sensing shape deformation and controlling cellular contractility upon confinement, we performed a quantitative imaging-based screening approach. By culturing cells in defined conditions (such as variable surface coatings and cell densities in micro-confinement assays) or by interfering with specific signalling pathway (for example by using chemical inhibitors or genetic interference approaches) and using cortical myosin II levels as a quantitative read-out, we aimed at identifying the element(s) that mediate the cellular mechanoresponse to shape deformation. Inhibition of any step of the mechanosensitive pathway must prevent cortical myosin II enrichment in confinement, while interference with dispensable elements should not affect it. Here we present a first screening study that we performed by testing known mechanosensitive pathways.

The actomyosin cortex  Mechanosensitive properties of the actomyosin cortex were previously reported for micro-pipette aspiration experiments [16,29,223] (as discussed in Section 2). All myosin isoforms, in mammalian cells or Dictyostelium, rapidly accumulate at the deformed cell cortex until a plateau is reached. However, this response is usually transient as the rapid turnover of the cell cortex can lead to a dissipation of mechanical strains imposed by shape deformations [11]. In contrast, our observations support that myosin II accumulation is stable over time during cell deformation in confinement, implying that mechanical cell shape changes cannot be solely sensed by the cortex itself, but rather by a non-dissipative element that can continuously measure mechanical deformation strain.

Apoptosis and transcription  To exclude that stable-bleb transformation is associated with a pro-apoptotic program mediated by caspase activity, or involves the activation of transcription-translation processes, we used a pharmacological inhibition approach. Interfering with caspase activity using a pan-caspase inhibitor (see Figure 8.6 A-D) did not block cortical myosin II accumulation under confinement, supporting that the process is not mediated by a pro-apoptotic programs. Also, myosin II cortical accumulation occurred rapidly (< 1min), suggesting that it was not regulated by a transcription-translation process. Pharmacological inhibition of transcription further did not impede cortical myosin II recruitment under confinement, as shown in Figure 8.6 B-D.

Cell-cell and cell-substrate adhesion  Focal adhesions allow cells to sense the rigidity of their environment, further controlling stress fiber formation and, by inducing nuclear flattening, also YAP localization and associated transcriptional changes [92–94] (as discussed in Chapter 4). To study the role of cell-substrate adhesion molecules in the mechanosensitive response of cells to shape deformation, we cultured cells on different adhesive substrates using Poly-L-Lysine (PLL) or ECM proteins (such as fibronectin) and compared cellular responses when non-adhesive passivated substrates (PLL-PEG)
Figure 8.6: Myosin II cortical accumulation under confinement does not depend on a pro-apoptotic signal, transcription or adhesion molecules. (A) Relative cortical myosin II intensity for control cells or cells treated with a pan-caspase inhibitor (100\(\mu\)M Z-VAD(OMe)-FMK) in suspension and 7\(\mu\)m confinement. (B) Relative cortical myosin II intensity for control cells or cells treated with 10\(\mu\)M Actinomycin D (transcription inhibitor) in suspension and 7\(\mu\)m confinement. (C) Relative cortical myosin II enrichment under 7\(\mu\)m confinement for cells cultured on passivated (PLL-PEG) or adhesive surfaces (PLL, fibronectin). (D) Representative confocal images of progenitor stem cells expressing myosin II-eGFP under 7\(\mu\)m confinement for caspase inhibitor, transcription inhibitor, PLL and Fibronectin coating. (E) Relative cortical myosin II intensity (left) for progenitor stem cells cultured in DMEM (suspension) or under 7\(\mu\)m confinement for isolated single cells (no cell-cell contacts, low density) or cell clusters (high cell density) and exemplary confocal images of progenitor stem cells expressing myosin II-eGFP under the indicated conditions (right). ** P < 0.0001, not significant (n.s.). Scale bars 10\(\mu\)m.

were used. Both cortical myosin II accumulation and amoeboid cell transformation under confinement occurred independently of adhesive substrate coatings (8.6 C-D). To test the role of cell-cell adhesions and, in general cell-contacts, we cultured cells at different densities and classified them into high/low densities depending if they had contacts with neighboring cells or not. Cell density and cell-cell contacts also did not affect cortical myosin II accumulation in suspension nor under confinement, as shown in Figure 8.6 E. Altogether these data suggest that cell surface adhesion molecules are dispensable for the mechanosensitive cell response to deformation under confinement.

**Stretch-activated ion channels** Mechanosensitive ion channels such as the Piezo family have been shown to regulate RhoA/myosin II activity in confined human can-
8.2. Myosin II activation is independent of known mechanosensors

cancer cells [224], as well as stress fiber formation in breast cancer cells [112]. We thus inhibited their activity using both gadolinium trichloride (GdCl$_3$, gadolinium) or GsMTx4. Gadolinium is a general inhibitor of stretch-activated ion channels [225], while GsMTx4 is a selective inhibitor of Piezo1/2, with minor effects on the activity of other mechanosensitive channels [224,225]. Piezo channels are functionally expressed in progenitor cells, as validated by calcium imaging in the presence of Yoda1, a potent and selective activator of Piezo1, that induced an increase of intracellular calcium (Figure 8.7 C). This increase could be inhibited by treating cells with GsMTx4, supporting the functionality of the compound (Figure 8.7 C, experiment performed by the lab of M. A. Valverde, Universitat Pompeu Fabra). Furthermore, treatment with GsMTx4 and Gadolinium did not prevent cortical myosin II accumulation in confinement, as shown in Figure 8.7 A-B, supporting that mechanosensitive ion channels do not play a key role in cellular mechanosensation.

Figure 8.7: Myosin II cortical accumulation under confinement does not depend on mechanosensitive ion channels (A) Relative cortical myosin II intensity of cells in control condition and treated with GsMTx4 or Gadolinium in suspension and 7—10μm confinement. (B) Representative confocal images of progenitor stem cells expressing myosin II-eGFP cultured at 7μm and 10μm confinement in control conditions and treated with 10μM of gadolinium chloride and 10μM GsMTx4, respectively. (C) Normalized calcium signal (left) and representative fluorescence images (right) of isolated progenitor stem cells stimulated with the Piezo1 activator YODA1 (gray, N=39) and in the presence of YODA1 supplemented with GsMTx4 (yellow, N=46). Data represent the mean and standard error of the mean. Data in (C) acquired by the lab of M. A. Valverde (UPF). *** $P < 0.0001$, not significant (n.s.). Scale bars 10μm.
8.3 Nucleus size and integrity determine the myosin II mechano-response

8.3.1 Myosin II accumulation correlates with nuclear deformation

Our data support that mechanical cell shape deformation is sensed by an elastic mechanosensor capable of measuring different degrees of cell deformations. Figure 8.1 A showed that myosin II accumulates at the cortex in a non-linear manner when lowering the confinement height. This led to a progressive cell compression in confinement. Suspension cells were almost spherical and have an average cell and nucleus diameter of \( \sim 25\mu m \) and \( 10\mu m \), respectively. Confined cells deformed and flattened as shown in Figure 8.8 A-B and both the cell and nucleus diameters increase when confinement heights were decreased. To understand how myosin II accumulation at the cortex correlated with the actual changes in cell architecture, we plotted myosin II cortical accumulation with respect to the relative cell and nucleus diameter, normalised to cell and nucleus diameter values in suspension (Figure 8.8 C). Consistent with the measure of their absolute size in suspension, cells started to be deformed from a confinement height of \( 18\mu m \) (the highest tested), as shown by an increase in their relative diameter (Figure 8.8 C). In addition, below \( 13\mu m \) the relative nuclear diameter increased significantly, indicative of nuclear deformation (Figure 8.8 C-D). We thus defined these two values as the cell and nuclear deformation threshold (indicated by the dashed orange lines in panel C). Interestingly, the threshold for myosin II accumulation, defined as the confinement height below which the cortical enrichment of myosin II is significantly different with respect to suspension (see Figure 8.1 A), was also at \( 13\mu m \) (cyan dashed line in panel C). This indicated that cortical myosin II correlated with nuclear deformation but not with cell deformation. Figure 8.8 C illustrates this bi-modal response behavior to shape deformation, with a first region where nuclear deformation is absent and no myosin accumulation is observed (confinement heights \( h > 13\mu m \)) and a second region (\( h \leq 13\mu m \)) where myosin II accumulation scales linearly with nuclear deformation.

To further probe if myosin II accumulation correlates with nuclear deformation we confined cells of different sizes obtained from different developmental stages. During early zebrafish development, there is a first cleavage period (0.75 – 2.25hpf) when cells divide synchronously and decrease their volume in each division cycle, followed by the blastula period during which cells keep on dividing but at a slower rate. As cells divide, both cell and nuclear diameters decrease, as quantified in vivo (Figure 8.9 A) and in vitro, by dissociating cells from embryos at different developmental stages and and/or ectopic induction of cell fate specification in the whole embryo (see Methods) (Figure 8.9 B). Of note, the relative decrease of the nuclear diameter at different developmental stages is significantly smaller than the change in cell size, leading to an increase in the nucleus-to-cell size ratio during early development. As an example, cells at 3.3hpf (high-oblong stage) have a median diameter of \( \sim 33\mu m \) and a nucleus size of \( 12\mu m \) (\( d_{\text{nucleus}}/d_{\text{cell}} = 0.36 \)), while at 4.7hpf cells are \( \sim 20\mu m \) in diameter and the nucleus...
8.3. Nucleus size and integrity determine the myosin II mechano-response

Figure 8.8: Nucleus deformation defines the myosin II response to confinement

(A) Representative confocal images of cell cross-sections (xy) and lateral views (xz) of progenitor cells expressing myosin II-eGFP, Lyn-dTomato (PM, plasma membrane) and stained with DNA-Hoechst in suspension and 7 μm confinement. (B) Representative confocal images (xy) and lateral views (xz) of progenitor cells expressing myosin II-eGFP and stained with DNA-Hoechst and ER-tracker Red cultured in 13 and 7 μm confinement. Scale bars 5 μm. (C) Relative cortical myosin II accumulation with respect to the relative cell (left) or nucleus (right) diameter. Data points represent mean ± standard error of the mean. (D) Double boxplot of relative cortical myosin II enrichment (left axis, gray) and relative nucleus diameter (right axis, teal) for decreasing confinement heights. Statistical test comparing relative nucleus diameters of confined cells with respect to cells in suspension. For panels C-D: N = 144 cells (susp.); N = 44 (18 μm); N = 32 (16 μm); N = 37 (13 μm); N = 45 (10 μm); N = 37 (8.5 μm); N = 50 (7 μm). All thresholds (dashed lines) are defined as the values above which there is a significant change of myosin II accumulation at the cortex with respect to non-deformed cells in suspension (Figure 8.1 A). *** P < 0.0001, not significant (n.s.).

is approximately 9 μm ($d_{\text{nucleus}}/d_{\text{cell}} = 0.46$). We took advantage of this change in nucleus-to-cell size ratio and confined cells dissociated from different stages at defined heights. Confining progenitor cells dissociated from 3.3 and 4 hpf at 16 μm led to a larger cell deformation of bigger cells derived from an earlier developmental stage, but induced a similar nucleus deformation given that nucleus sizes were comparable in both stages. Importantly, a similar cortical myosin II level was measured for cells confined at the same confinement height (leading to a similar nucleus deformation but different cell deformation), as shown in Figure 8.9 C-D. In contrast, when cells derived from stage 3.3 hpf and 4 hpf were compressed from 16 μm down to 13 μm, an increase in cortical myosin II levels was observed in both cells derived from 3.3 hpf and 4 hpf (Figure 8.9 C-D). These two points also showed different cell deformation, but similar
nuclear deformation and myosin II recruitment at the cortex.

![Cellular response to mechanical confinement](image)

**Figure 8.9: Nucleus to cell size determines the myosin II accumulation in confinement** (A) Double axis plot for cell (left, blue axis) and nucleus (right, orange axis) sizes measured in-vivo from 2 to 4hpf. Data acquired by Q. Tolosa. (B) Cell and nucleus size in pluripotent cells dissociated at 3.3 and 4hpf (blastula stages) and induced mesoderm, ectoderm, endoderm cells at 4.7, 8 and 10hpf (during gastrulation). Data from differentiated cells acquired by H. Häkkinen. (C) Relative myosin II cortical enrichment for cells dissociated from embryos at different developmental stages and cultured at different heights with respect to the cell deformation (defined as the cell diameter in suspension divided by the confinement height, left) and nucleus deformation (that is the nuclear diameter relative to the suspension value, right). Data points indicate mean ± sem. 3.3hpf: \( N = 58 \ (16\mu m), \ N = 61 \ (13\mu m) \). 4hpf: \( N = 44 \ (16\mu m), \ N = 60 \ (13\mu m), \ N = 45 \ (10\mu m) \). (D) Representative confocal images of cells expressing myosin II-eGFP dissociated at 3.3hpf (bottom) or 4hpf (top) cultured at 13\( \mu m \) or 16\( \mu m \) confinement. Dashed lines outline cell nuclei. Scale bars 10\( \mu m \) (same length in all pictures to compare cell-nuclei sizes).

Concluding, confining cells derived from sphere stage, or from earlier stages because of their different relative nucleus-to-cell sizes, at different heights and measuring both cell and nuclear deformation, showed that myosin II enrichment at the cortex correlated with nuclear deformation, but not with cell deformation (Figure 8.8 and 8.9). These data suggest that nucleus deformation mediates the mechanosensitive increase in cell contractility upon confinement.
8.3.2 Myosin II accumulation requires nuclear envelope integrity

Previous results showed that cortical myosin II enrichment is stable over time in confinement, with the cortical myosin II level defined by the specific confinement heights. To investigate whether this mechanosensitive response indeed requires the presence of the nucleus we monitored the mechanosensitive response of mitotic cells in confinement. In fact, mitotic cells in confined environments did not accumulate myosin II at the cell cortex, as shown in Figure 8.10 A. To increase the percentage of mitotic cells in our culture assays, we made use of Nocodazole to depolymerize microtubules (MT, blocking the cell cycle during mitosis) and arrest cells in M phase. Again we observed that mitotic cells were not able to recruit myosin II at the cortex in contrast to interphase cells. However, addition of LPA to Nocodazole-treated cells robustly activated myosin II and led to an accumulation of myosin II at the cell cortex and transformed cells into a stable-bleb phenotype (Figure 8.10 B), suggesting that myosin II activation and its ability to bind to the cortex is not impaired by Nocodazole treatment in mitotic cells.

Mitotic cells clearly have very a different bio-chemical and mechanical composition of their cytoplasm [186,226], as well as cortical organization and contractility [32]. When cells enter mitosis, prior to chromosome condensation, their nuclear envelope (NE) breaks down. This can be visualised using the myosin II-eGFP line or any other cytosolic reporter that is excluded from the nucleus in control interphase cells but diffuses into the nucleus upon NE disassembly (Figure 8.10 C) and provides for a readout of this exact moment. To quantify the cortical myosin II levels in relation to NE integrity, we imaged cells over time and quantified cortical myosin II levels before, during and after nuclear envelope breakdown defined as $t = 0$ (visible because of the abrupt diffusion of myosin II into the nucleus) and for the 7.5 min afterwards, when chromosomes were observed to be fully condensed. We could observe an instantaneous and drastic reduction of cortical myosin II upon nucleus envelope breakdown, as shown in Figure 8.10 C. Also the opposite process is true: when cells exit mitosis and the two daughter nuclei are formed, myosin II rapidly accumulates at the cell cortex (Figure 8.10 D). During division, myosin II is needed to cleave the two daughter cells. Thus, when the nuclei re-assembled and myosin gets re-localized to the cortex, the two daughter cells often fail to divide and one cell with two nuclei is formed (Figure 8.10 D). Sometimes, dividing cells also directly transformed to stable-bleb cells (Figure 8.10 D).

These experiments suggest that nuclear envelope integrity is required for myosin II activation and further support that cells continuously sense shape deformation in confinement and accordingly adapt their contractility level.
Figure 8.10: Nucleus integrity is necessary for myosin II mechanoresponse

(A) Left: Relative myosin II cortical enrichment for cells during interphase or mitosis, in control condition or upon treatment with $1\mu M$ of Nocodazole in suspension or $7\mu m$ confinement. Right: representative confocal image of cells expressing myosin II-eGFP confined at $7\mu m$. Yellow asterisk points at a cell during interphase and cyan asterisk at a cell undergoing mitosis. (B) Representative confocal image of non-polarized (top) and stable-bleb polarized (bottom) cell expressing myosin II-eGFP treated with $1\mu M$ Nocodazole and supplemented with $50\mu M$ LPA. (C) Time lapse (top) and relative cortical myosin II accumulation over time (bottom) for $N = 7$ cells expressing myosin II-eGFP and H2A-mCherry entering mitosis under $7\mu m$ confinement. The time $t = 0$ was defined as the time of nuclear envelop breakdown. The white arrows point at the cell cortex with myosin II enrichment ($t = -1min$) or depletion of myosin II ($t = 7.5min$). The nucleus and the condensed chromosomes can be observed in the magenta channel for the indicated time points. (D) Time lapse of representative progenitor stem cells expressing myosin II-eGFP and H2A-mCherry going out from mitosis under $7\mu m$ confinement. Cells are mitotic at $t = 0$ and when the two nuclei start to form again, the cells fail to divide. At $t = 14min$, accumulate myosin II at the cortex (left) or transform to stable-bleb cell (right). Scale bars $10\mu m$. 
8.4 Conclusions

The experiments presented in this Chapter showed that cell shape deformation under mechanical confinement defines the cortical contractility set-point of progenitor stem cells (Figure 8.1). Myosin II motor proteins activate upon confinement, to levels that depend on the deformation itself, in a stable and reversible way (Figure 8.2). Highly contractile cells spontaneously transform to stable-bleb cells and migrate away from the confinement area, reminiscent of an evasion-reflex mechanism (Figure 8.3, 8.4). This process is independent of fate specification as mesoderm, endoderm or ectoderm induced-cells all accumulate myosin II at the cortex and transform to a stable-bleb phenotype when confined (Figure 8.5). Furthermore, mesodermal cells cultured on fibronectin can undergo a mesenchymal-to-amoeoboid transition when confined. Thus, stable-bleb transformation does not depend on initial cell states or a given migration phenotype. A screening approach showed that the mechanosensitive process of myosin II activation in confinement further does not depend on transcriptional changes or a pro-apoptotic pathway mediated by caspase activity and was also independent of mechanosensor elements at the cell surface such as adhesion molecules (cell-cell and substrate) and stretch-activated ion channels (Figure 8.6, 8.7). Instead, we found that myosin II accumulation under confinement correlates with nuclear deformation (Figure 8.8, 8.9) and requires nuclear envelope integrity (Figure 8.10).

These data support that nuclear deformation is a key parameter that correlates with myosin II accumulation at the cell cortex in confinement. The nucleus, being the largest organelle of the cell, further defines an absolute intrinsic scale, suggesting that it acts as an intracellular ruler for measuring shape deformations in each cell. The following chapter will address how the nucleus can sense physical shape deformation and functions as a mechanosensor element, capable of controlling cortical contractility and cellular behavior under mechanical stress conditions that lead to cell shape changes.
Chapter 9

The nucleus gauges mechanical cell deformation

The results presented in Chapter 8.3 showed a correlation between myosin II accumulation and nuclear deformation in confined cells and that nuclear envelope integrity is necessary for cells to measure and adapt to shape changes. To investigate the specific role and possible mechanisms of the nucleus in regulating mechanosensitive cell behaviour and myosin II activity in confinement, we next characterized nuclear architecture in progenitor stem cells in normal and mechanical stress conditions and tested the involvement of possible mechanotransduction pathways.

9.1 Mechanical confinement induces inner nuclear membrane unfolding

To study nuclear envelope architecture in primary progenitor cells and the response of the nucleus to mechanical cell shape deformation, we first visualized the nucleus surface in unconfined and confined cells. Imaging of Lap2β (protein–tagged lamina-associated polypeptide 2)-mScarlet showed its specific localization at the inner nuclear membrane (INM). The ER-tracker green was further used to stain the ER and ONM (Figure 9.1 A-B). This double labeling allowed to distinguish type I invaginations (containing only the INM) and type II invaginations (made by the INM and ONM) [148], as depicted in the sketch of Figure 9.1 A and described in Chapter 4.3. In suspension cells, the nuclear envelope exhibited various membrane invaginations of variable appearance, resembling mainly type II folds as both INM and ONM markers were detected (yellow arrows in Figure 9.1 B). Interestingly, upon cell confinement the nuclear envelope surface increased and these invaginations were stretched-out, as evident in the nuclear cross-section of confined cells (Figure 9.1 B). INM unfolding was previously reported to mediate a mechano-transduction process involved in the inflammatory response upon zebrafish tail fin wounding [149,168], as discussed in Chapter 4.3.
To address how INM folds behave under nucleus deformation, we confined progenitor stem cells expressing Lap2β-eGFP at variable heights. Upon mechanical nucleus deformation in confined cells we observed that these INM folds are gradually lost when lowering the confinement height, as shown in Figure 9.2 A. Notably, INM unfolding was stable over time when cells were deformed in confinement, with confined cells showing an unfolding of their INM up to 1h after confinement was applied (Figure 9.2 A). To quantify the INM unfolding, we defined an invagination ratio $IR$ given by the relative difference in between the nuclear convex area (blue area, $A_{\text{convex}}$) and the area included inside the INM (yellow area, $A_{\text{INM}}$), as $IR = 1 - A_{\text{INM}}/A_{\text{convex}}$ (see Figure 9.2 B, similar to [179], and as described in Chapter 7). By plotting the nuclear area-to-perimeter ratio and the invagination ratio, we could observe a continuous INM unfolding under confinement (Figure 9.2 C). Curvature analysis of unconfined and confined nuclei showed a reduction in nuclear curvature (Figure 9.2 D-E) and a decrease in temporal fluctuations of the nuclear envelope. These changes were not accompanied by changes in nuclear volume or total surface (Figure 9.2 F). Therefore, these data support that the nuclear envelope shows a mechanical stretch response to nucleus deformation, leading to a stable unfolding of the INM surface. Notably, the INM unfolding occurred already when confining cells at heights $\leq 13\mu m$ and a progressive unfolding was observed when lowering the confinement height at discrete steps.
Figure 9.2: Unfolding of the inner nuclear membrane during nucleus deformation in confined cells (A) Exemplary confocal images of cells expressing Lap2β-eGFP under varying cell deformations, with the time after confinement as indicated (left), and a sketch of progressive INM unfolding for decreasing confinement heights (right). (B) Exemplary image analysis protocol to quantify INM folding based on the INM marker Lap2β—eGFP: (i) raw image; (ii) detection of inner and outer contour (magenta and yellow line); (iii) inner area (yellow, $A_{INM}$); (iv) detection of convex area of iii ($A_{convex}$), (blue). The invagination ratio (IR) is defined as 1 minus the ratio in between the yellow and blue area. (C) Nuclear area–to–perimeter ratio (top) and nuclear invagination ratio (bottom) for increasing confinement strength. (D) Curvature analysis of nuclear envelope shape for 20 consecutive frames ($t_{lag} = 10$ s) for unconfined (suspension, top) and $7 \mu m$ confined nuclei (bottom). (E) Histogram of nuclear curvature for unconfined and $7\mu m$ confined nuclei related to (F) with $N = 10$ cells for each condition ($P < 10^{-12}$). (F) Nuclear surface and volume for cells in suspension and variable confinement heights. *** $P < 0.0001$, * $P < 0.01$, n.s. not significant. All scale bars $10\mu m$. The script for the analysis shown in panels B-C was written by M. Colomer-Rosell and the analysis in D-E was done by S. Wieser.

9.2 The nucleus as a mechanosensor of large cell shape deformations

9.2.1 A lipase in the nucleus controls myosin II activity under confinement

Our results indicated that myosin II accumulation in confined cells occurred above a threshold of nuclear deformation. By confining the cells to heights lower than $13\mu m$,
the nucleus started to get deformed, myosin II was recruited to the cell cortex and the
two quantities correlated with each-other (Chapter 8.3). The data shown in Figure
9.2 supported that this threshold corresponded to an unfolding of the INM. We thus
aimed to unveil whether and how physical changes in nuclear envelope folding during
cell deformation can be translated into the regulation of myosin II activity. NE stretch
is known to trigger nuclear mechano-transduction pathways [157], including nuclear
pore opening [159], known to control transcription [93], and the tension-dependent ac-
tivation of cytosolic phospholipase A2 that mediates inflammatory responses [149]. As
myosin II accumulation at the cortex was independent of transcription and translation
programs (Chapter 8.2), we tested the role of cPLA$_2$ as possible a mechanosensor.

Chemical inhibition of cPLA$_2$ activity using 1µM Pyrrophenone led to a significant
decrease of cortical myosin II levels at various degrees of cell deformation (Figure 9.3
A–B). As a consequence, a lower percentage of motile stable-bleb cells was observed
in cPLA$_2$-inhibited cells upon mechanical confinement (Figure 9.3 C). Importantly,
cells treated with Pyrrophenone were capable of transforming into stable-bleb cells
upon addition of LPA (Figure 9.3 C), supporting that myosin II motor proteins can be
activated by alternative pathways and are still competent to bind to the cell cortex of
cPLA$_2$-inhibited cells. Inhibition of cPLA$_2$ activity further blocked cortical myosin II
accumulation and stable-bleb transformation in primary progenitor cells derived from
various developmental stages and different embryonic cell lineages (Figure 9.3 D–E).
These data indicate that cPLA$_2$ activity is at the core of a mechano-transduction
pathway that enables cells to sense shape deformations and to activate myosin II and
an amoeboid migration mode in confined cells.

To further validate the function of cPLA$_2$ as a mechanosensitive signal transducer,
we performed a genetic interference approach using a dominant negative construct
of cPLA$_2$ and morpholino interference. We developed a dominant negative cPLA$_2$
construct (dn-cPLA$_2$) by mutating the sequence in two positions that were previously
reported to affect the protein’s catalytic activity and binding to the INM [227], see
Appendix A.1. Injection of the dn-cPLA$_2$ led to a strong reduction of cortical myosin II
recruitment under mechanical confinement (Figure 9.4 A–B). As discussed in Chap-
ter 4.3 and further described in Appendix A.1, the protein cPLA$_2$ is encoded by the
genes $pla2g4aa$ and $pla2g4ab$ (because of a genome duplication). The first gene,$pla2g4aa$, was the one identified to mediate leukocyte recruitment to wounding sites in
zebrafish [168] and was expressed from early developmental stages in zebrafish, while
the second one, $pla2g4ab$, showed a specific expression in the adult, in the heart and
testicles (VastDB). We tested morpholinos against both genes: using a splicing and
translational-blocking morpholino against $pla2g4aa$ (cPLA$_2$ MO, also validated and
used in [168]), led to a reduction of cortical myosin II, while the translational-blocking
morpholino against $pla2g4ab$ (cPLA$_2$ abMO) did not show an effect. The reduced lev-
els of myosin II accumulation observed for the MO against $pla2g4aa$ (cPLA$_2$ MO) could be rescued
by co-injecting cPLA$_2$ mRNA that was not targeted by the morpholino (Figure 9.4
A–B). This rescue condition (cPLA$_2$ MO+mRNA) led to comparable cortical myosin II
levels as in the control case in confinement. Over-expression of cPLA$_2$, by injecting
the mRNA alone, did not increase cortical myosin II in confinement, indicating that
Figure 9.3: Nucleus deformation in confinement activates a mechanosensitive lipase controlling myosin II activity in different cell lineages (A) Relative cortical myosin II fluorescence intensity upon cPLA2 inhibition by Pyrrophenone in cells in suspension and at the indicated confinement heights. (B) Exemplary confocal images of progenitor cells expressing myosin II–eGFP in (from left to right) suspension and upon 13 or 10 μm confinement in control (top) or upon cPLA2 inhibition (bottom). (C) Percentage of polarized stable-bleb cells and sketch depicting the process for control conditions under 7 μm confinement and in the presence of cPLA2 inhibitor or unconfined (suspension) cells stimulated with 50 μM of LPA. For all conditions, N > 200 cells. (D) Relative cortical myosin II fluorescence intensity upon cPLA2 inhibition for cells dissociated at 3.3 hpf (pluripotent), induced mesendoderm or ectoderm cells in suspension and upon confinement at indicated heights. Injections to induce different cell lineages were performed by H. Häkkinen. (E) Percentage of stable-bleb cells in control (top) and upon cPLA2 inhibition (bottom) for induced mesendoderm and ectoderm cells on a non-adhesive substrate and mesoderm cells cultured on fibronectin at 7 μm confinement. The significance tests in the lower panel refer to the corresponding control case. *** P < 0.0001, * P < 0.01, n.s. not significant. All scale bars 10 μm.

cPLA2 activity was potentially saturated by other factors rather than by its expression level, but over-expression slightly increased the baseline of cellular contractility in cells in suspension (Figure 9.4 A-B).

These results further support that cPLA2 is at the core of a mechanotransduction pathway that allows cells to sense shape deformations and adapt their contractility and migration dynamics. Our data support that the mechanosensitive protein is encoded by the pla2g4aa gene, consistent with previous findings [149, 168].
9.2. The nucleus as a mechanosensor of large cell shape deformations

Figure 9.4: cPLA2 is a mechanosensitive signal transducer at the INM controlling myosin II activity in deformed cells in confinement (A) Relative cortical myosin II intensity in progenitor cells cultured in suspension versus 7μm confinement for control condition (DMEM), treatment with cPLA2 inhibitor, or cells dissociated from embryos injected with dn-cPLA2 mRNA, cPLA2 MO (splicing morpholino targeting transcripts of the gene pla2g4aa) alone or cPLA2 MO + cPLA2 mRNA (rescue), cPLA2 mRNA alone (over expression), cPLA2 abMO (morpholino targeting transcripts of the gene pla2g4ab). (B) Representative confocal snapshots of progenitor cells expressing myosin II-eGFP cultured in suspension or under 7μm confinement at the indicated conditions. The magenta symbols (+−) indicate the conditions in which cells activate (do not activate) myosin II when confined. *** P < 0.0001, n.s. not significant. All scale bars 10μm.

9.2.2 cPLA2 activity is required in the nucleus where it controls AA production

Upon activation, cPLA2 was shown to translocate to intracellular membranes including the NE, ER and Golgi membranes [228], and to translocate from the nucleoplasm to the INM during inflammation [149, 168]. This translocation has been considered as a hallmark of cPLA2 activity, even if the mechanisms regulating its localization have not been fully understood yet [170]. According to its name, cPLA2 was shown to be localized in the cytosol in diverse cell lines when not active, however, previous studies reported that in zebrafish and HeLa cells its localization occurred constitutively in the nucleus [149]. To monitor cPLA2 localization in isolated progenitor cells, we ectopically expressed a GFP-tagged form, cPLA2-GFP (see Chapter 6.3 and Appendix A.2). In cells
isolated from the early stage blastula embryo and cultured in suspension, cPLA$_2$ was consistently localized to the nucleus (Figure 9.5 A), as reported previously [149]. To assess the influence of fluorophore position and type, we generated various constructs by changing the fluorophore localization, the fluorescent protein itself, as well as by adding a linker in between the protein and the fluorophore (see Appendix A.2). All constructs showed a consistent outcome in localization in the nucleoplasm (Figure 9.5 A). Immuno-fluorescence approaches to detect endogenous protein levels failed because no specific antibody could be identified. One of the factors known to control cPLA$_2$ localization is its calcium-dependent binding to lipid membranes [229]. Treatment with BAPTA-AM, however, did not have any effect on the nucleoplasmic protein localization (Figure 9.5 A). We thus modified the cPLA$_2$ mRNA by adding nuclear export signal (NES) and observed that its cytosolic-to-nucleoplasmic ratio increased, suggesting that its localization could be regulated by specific protein sequence(s). In agreement with this, we could find two putative nuclear localization signal (NLS) and one NES in the zebrafish pla2g4aa gene that are conserved in human and might explain these findings (see Appendix A.1).

To directly assess whether cPLA$_2$ is activated upon cell squeezing we expressed cPLA$_2$-GFP in progenitor stem cells and monitored protein localization in confinement. In agreement with previous work [149], we expected to observe cPLA$_2$ translocation to the INM upon stretching of the nuclear envelope. Notably, INM re-localization was only detected in 2% of cells, while the vast majority kept an homogeneous nucleoplasmic localization (Figure 9.5 B). cPLA$_2$ translocation was previously reported to be required for its activity [149, 169, 170, 228]. This inconsistency with our results raised various questions regarding cPLA$_2$ activation under confinement. To address if cPLA$_2$ was indeed required in the nucleus of confined cells, we performed a rescue experiment using the cPLA$_2$-NES-GFP construct as rescue-RNA on the morphant background, in combination with Leptomycin B (Lb) to block nuclear export. In this condition, the NES-construct showed an altered localization, with a higher cytosolic signal, which allowed to test if cPLA$_2$ was required in the nucleus. Treatment of cells with Lb increased the nuclear localization of the NES construct, therefore providing a tunable cPLA$_2$ localization. In this experiment we observed a complete rescue of cortical myosin II in confinement when the rescue experiment was combined with Leptomycin B (Figure 9.5 C-D), supporting that cPLA$_2$ activity was indeed required in the nucleus.

Given the fact that different cPLA$_2$-labeled constructs that we generated did not show INM translocation upon mechanical nucleus stretch, we validated the role of cPLA$_2$ via complementary methods to directly assess the protein’s activity. Arachidonic Acid (AA) is the primary cleavage product generated by the lipase activity of cPLA$_2$ [169], and can quantitatively inform on cPLA$_2$ activity via measuring AA production upon confinement. AA is a polyunsaturated fatty acid and, as most fatty acids, it has clear Raman peaks that allow for its specific detection and identification among other fatty acids or cellular components [230].
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The Raman effect

The Raman effect consists in the inelastic scattering of light and is caused by the excitation of specific vibrational levels in the sample. When a monochromatic light interacts with a species composed by two molecules connected by a chemical bond, it is scattered mainly at the input frequency. A small part of the incident light experiences a shift in frequency that corresponds to the vibrational frequency of the bond, representing a unique signature of a specific molecule. This frequency shift, typically in cm\(^{-1}\), is the parameter that can be measured with Raman spectroscopy and allows for the identification of specific chemical bonds in a sample. Importantly, the Raman effect is a linear process and it is therefore proportional to the density of emitting molecules. Lipids and fatty acids show a high Raman emission thanks to their long acyl chains and their unique bands that have been widely studied and characterized.

By performing Raman spectroscopy in progenitor stem cells and principal component analysis of the recorded spectra (see Methods for the complete protocol, analysis performed by Dr. Monica Marro), we identified a component that included various AA peaks [230], shown in Figure 9.5 E. We thus quantified the score of this component in the cytoplasm of suspension and confined cells (10\(\mu\)m height) and could observe a net increase of AA concentration when cells were deformed in confinement (Figure 9.5 F). This increase was inhibited by treating cells with the cPLA\(_2\) inhibitor Pyrrophenone in confinement (Figure 9.5 G), supporting the specificity of the detected signal. Importantly, we could not detect AA in the nucleus of progenitor stem cells, suggesting that AA is directly released into the cytosol upon cleavage at the INM. Also, not any other component that showed significant differences in between the nuclei of suspension and confined cells could be detected with Raman spectroscopy.

Concluding, these data support that cell shape deformation in confinement induces cPLA\(_2\) activation in the nucleus and is associated with the production of AA that is released into the cytoplasm. This process then controls cortical contractility and amoeboid motility in confinement.

9.2.3 AA activates myosin II via the Rho/ROCK pathway in a cell autonomous way

AA has been widely studied for its role in inflammation where it acts as a precursor of a large family of compounds called eicosanoids, produced by oxygenation. This can be mediated either by the cyclooxygenase (COX) [231] or lipoxygenase (LOX) pathways [232] that convert AA into prostaglandins or leukotrienes, respectively [169]. Importantly, these pathways commonly function via autocrine/paracrine signalling: they require that a cell releases some ligands in its surroundings, that then bind to specific receptors (typically GPCRs) on the cell surface of the same (autocrine) or of other cells (paracrine).

To understand the role of AA in regulating myosin II activity, we first tested whether oxidation of AA is involved in the cellular response by stimulating cells with either AA
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Figure 9.5: cPLA$_2$ is active in the nucleus of confined cells where it triggers AA release

(A) Representative confocal fluorescence images of cells in suspension expressing cPLA$_2$-GFP in control condition (top) or upon treatment with BAPTA-AM (bottom left), expressing cPLA$_2$-linker-GFP (top right) or cPLA$_2$-NES-GFP in control condition (bottom right). (B) Representative confocal fluorescence images of cells expressing cPLA$_2$-GFP under 7μm confinement that show homogeneous nucleoplasmic localization (top, 98% of the cells) or cPLA$_2$ translocation to the INM (bottom, 2% of the cells). (C) Relative cortical myosin II fluorescence intensity for cells dissociated from control (uninjected) embryos or embryos injected with cPLA$_2$ MO, cPLA$_2$ MO + cPLA$_2$-NES-GFP mRNA (RNA) and with or without addition of leptomycin B (Lb). (D) Exemplary confocal fluorescence images of cell expressing myosin II–mCherry (right) and cPLA$_2$-NES-GFP (left) under 7μm confinement with (top) or without (bottom) the addition of Lb. (E) Component of the Raman spectra associated with Arachidonic Acid (AA) used for the quantification of AA production. The Raman peaks indicated in red are specific for AA. (F and G) Scores of Raman components associated with AA measured in the cytosol of cells in (F) suspension (unconfined, N = 24) and confinement (10μm, N = 28) and (G) in control confinement condition (Ctrl, N = 52) or treated with cPLA2 inhibitor (N = 22). Red lines indicate mean and SEM. *** P < 0.0001, * P < 0.01, n.s. not significant. All scale bars 10μm.

or deuterated AA (AAd-8), Figure 9.6 A. Oxidation of deuterated AA is much slower compared to standard AA. Addition of both compounds led to a similar cortical myosin II accumulation, suggesting that un-oxidized AA mediates the process. To specifically test the involvement of the COX/LOX pathways in the regulation of myosin II activity under confinement, we treated progenitor stem cells with specific inhibitors targeting the function of COX and FLAP (LOX activating protein). Results did not reveal a decrease of cortical myosin II levels in confined cells (Figure 9.6 B).

To further assess the contribution of paracrine and autocrine signalling, associated with metabolic products of AA, such as Ltb4 receptor signalling involved in neutrophil
swarming during inflammation [175], we decided to confine cells under a PDMS micro-

pillar. This led to a confinement of cells underneath the pillar at a measured confine-

ment height of approximately $7 \sim 10 \mu m$ (corresponding to the area included inside the

thick magenta half-circle in Figure 9.6 C), surrounded by a region of non-confined cells. We observed that only cells in the confined region accumulated myosin II and trans-

formed into stable-bleb cells (Figure 9.6 C). In this configuration, also pairs of cells were

contacting each other at the confinement boundary, with one cell being confined and the other cell non-confined cell outside the micro-pillar, as shown by the zoomed view and the sketch of Figure 9.6 C. We quantified cortical myosin II levels in these pairs of cells and observes that only confined cells accumulated myosin II at the cortex (Figure 9.6 C), while non-confined cells revealed no increase in comparison to isolated suspen-

sion cells (statistical test, n.s.). Together, these data support that the processing of AA

by LOX/COX enzymes and the activation of autocrine/paracrine signalling pathways downstream of AA production are not required for the mechanosensitive increase in cortical contractility.

AA has also been implicated in the direct regulation of myosin ATPase activity [233] as well as in the activation of RhoA-mediated processes in cells [234,235]. We thus tested the involvement of various regulators of myosin II contractility: myosin light chain kinase (MLCK) inhibition showed no significant effect, while a clear reduction of myosin activity under confinement was observed upon treatment with staurosporine (a general kinase inhibitor) and ROCK inhibition via treatment with Y-27632 (Figure 9.6 E). Genetic interference with a dominant negative version of RhoA (dn-RhoA), that competes with the endogenous protein in binding to RhoGEF but cannot interact with its effector ROCK [236], also inhibited myosin II enrichment upon confinement, as shown in Figure 9.6 E. Interference with MEK/ERK using an inhibitor also did not have an effect on cortical myosin II levels, though cPLA$_2$ was reported to activate this pathway [178,237]. To directly assess the involvement of RhoA/ROCK signalling in cortical myosin II enrichment, we used a RhoA-FRET (Förster resonance energy transfer) sensor [55] which showed an increased cPLA$_2$-dependent RhoA activity under confinement (Figure 9.6 F). A screening for known myosin II regulators identified the

RhoA/ROCK pathway as the mediator of myosin II activity in confined cells. Therefore, single cells are capable of measuring shape changes using their nucleus and regulate cell contractility by the activation of cPLA$_2$, which controls the cell autonomous production of AA and tunes myosin II activity via the Rho/ROCK pathway.
Figure 9.6: cPLA$_2$ and arachidonic acid regulate myosin II activity through the Rho/ROCK pathway

(A) Relative cortical myosin II fluorescence intensity and representative confocal images of control cells or cells treated with 100$\mu$M arachidonic acid (AA) or 100$\mu$M deuterated AA (AAd-8). Scale bars 10$\mu$m. (B) Relative cortical myosin II fluorescence intensity for cells in suspension and 7$\mu$m confinement in control condition or treated with inhibitors of COX (SC560, 10$\mu$M) and FLAP (MK866, 10$\mu$M). (C) Representative stitched confocal view of cells expressing myosin II-eGFP cultured in DMEM and confined below a micro-pillar (approx. height 7$\mu$m), resulting in confined cells underneath the micro-pillar and non-confined cells in close proximity outside the micro-pillar. The magenta line shows the edge of the pillar; the dashed lines indicate the front of stable-bleb polarized/migrating cells and the magenta asterisk a mitotic cell (no cortical myosin II). The zoomed view and sketch show adjacent confined and non-confined cells. Scale bars 50$\mu$m. (E) Relative cortical myosin II intensity for control cells and different chemical inhibitors (Y-27632 10$\mu$M, M-L7, staurosporine, MEK1b/ERK inh.) or genetic interference (dominant negative RhoA, dnRhoA) with myosin II regulators. (F) RhoA-Fret index scatter plot for non-confined (suspension) and confined cells cultured in control (DMEM) conditions or supplemented with cPLA$_2$ inhibitor. Red lines represent mean and sem. From left to right: N=20, N=26, N=19, N=21. *** $P < 0.0001$, ** $P < 0.001$, * $P < 0.01$, n.s. not significant.
The role of calcium in cPLA$_2$ lipase activity

As discussed in the previous sections, cPLA$_2$ is a calcium-dependent lipase. Calcium is needed for the binding of the C2-domain to lipid membranes and it is therefore required for cPLA$_2$ activity [100]. Some studies reported that increased calcium levels were sufficient to induce cPLA$_2$ activation [100], while others reported a necessary but not sufficient role for calcium in the presence of INM stretch [149]. To understand the role of calcium in the cPLA$_2$ mechano-transduction pathway, we performed a screening of known calcium regulators, as shown in Figure 9.7. Depletion of ER calcium stores with Thapsigargin or removal of extracellular calcium by using calcium-free media did not significantly block myosin II accumulation under confinement (Figure 9.7 A). Instead, chelation of intracellular calcium by BAPTA-AM appeared to be necessary for the cellular mechanoresponse (Figure 9.7 A). Treatment with 2-APB, an inhibitor of IP3 receptors as well as of some TRP channels and of STIM-ORAI channel activity, also completely blocked the cortical contractility increase under mechanical shape deformation (Figure 9.7 A). Treatment with BAPTA-AM as well as the cPLA$_2$ inhibitor Pyrrophenone did not prevent myosin II motor proteins to accumulate at the cell cortex upon stimulation with LPA, supporting that these treatments did not affect myosin II activation by complementary pathways and its ability to bind to actin filaments (Figure 9.7 B-C). Furthermore, increasing intracellular Ca$^{2+}$ levels by treating cells with ionomycin neither lead to increase in cellular contractility nor did it trigger AA production in progenitor cells in suspension (Figure 9.7 D-E). These results suggested that high calcium levels were not sufficient for cPLA$_2$ activation, but intracellular calcium is required for cPLA$_2$ lipase activation upon nucleus deformation in confined cells.

Inner nuclear membrane binding of PLA$_2$

As described before, cPLA$_2$ translates nucleus deformation into a signal (AA production) that regulates myosin II activity. Considering that signalling pathways in biology commonly involve feedback loops, we tested the presence of possible feedback mechanisms in the cPLA$_2$/myosin II pathway. Possible feedback mechanisms could act in a reverse way, from myosin II or cPLA$_2$ activity back to INM unfolding. For example, the activity of cPLA$_2$ might affect INM unfolding. When cPLA$_2$ is active it cleaves phosphatidylcholine [169,238]. This could influence the lipid composition of the inner nuclear membrane under mechanical stress and, as a consequence, the folding of the nuclear envelope. Moreover, the increase in contractility in confined cells can also impact on intracellular and nuclear mechanics. Contractility drives nuclear fluctuations [239], correlates with intracellular pressure [77] and cell stiffness also scales with volume [240]. As a consequence, the global contractility of the cell might affect nuclear shape and/or volume and therefore NE architecture. Also, on the long term, mechanical confinement could alter nuclear properties in a way that nuclei adapt to the stress as previously reported in other systems [115].

In order to investigate possible feedback processes that influence INM unfolding, we expressed the Lab2β-eGFP construct in progenitor stem cells treated with Pyrrophenone (cPLA$_2$ inhibitor) or Latrunculin A (LatA) to depolymerize the actin cortex, thus impeding myosin II motor binding to actin filaments and an increase in cell contrac-
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Figure 9.7: Intracellular calcium is required for myosin II accumulation (A) Relative cortical myosin II fluorescence intensity for control cells or for treatments with 1μM Thapsigargin, 50μM 2-APB, 10μM BAPTA-AM, 10μM BAPTA-AM in combination with 1μM Pyrrophenone (cPLA2 inhibitor) or cultured in calcium-free media (Ca2+ free). (B) Representative confocal images of suspension cells expressing myosin II-eGFP cultured with 1μM cPLA2 inhibitor or 10μM BAPTA-AM, or in control conditions supplemented with 50μM LPA, or with 1μM ionomycin. (C) Relative cortical myosin II fluorescence intensity for controls cell or cells treated with 1μM cPLA2 inhibitor or 10μM BAPTA-AM in DMEM (Susp.) or supplemented with 50μM LPA. (D) Relative cortical myosin II fluorescence intensity for controls cell (Iso) and cells treated with 1μM ionomycin (Iso+iono). (E) Normalised Raman score component corresponding to AA for control cells (Iso) or cells treated with 1μM ionomycin (Iso+iono). Red lines represent mean and sem. *** P < 0.0001, * P < 0.01, n.s. not significant. All scale bars 10μm.

Contractility under cell confinement (Figure 9.8 A). The INM of treated cells in confinement was observed to fully unfold similar to the control case (Figure 9.8 A). When cPLA2 was inhibited cell contractility did not increase as the mechano-transduction pathway is shut off. In contrast, we observed that cells treated with LatA could still activate myosin II upon confinement. In fact, these cells presented bright myosin II clusters that dynamically merged and moved the remaining actin micro-filaments on the cell cortex (Figure 9.8 B), resembling asters observed in reconstituted actomyosin networks in vitro [27]. Finally, by imaging the INM of confined cells over longer periods (up to one hour), we did not observe any adaptation process. We observed that the INM unfolding was stable in time under confinement (Figure 9.2 A) and, moreover, when cells reassembled the nuclear envelope after dividing under confinement, the INM appeared already stretched (Figure 9.8 C). This unexpected observation suggests that the "amount" of INM is "limited" by specific factors. This also explains our observation...
9.3 A compensatory mechanism ensures mechanosensitive cell characteristics

9.3.1 Generation of a cPLA$_2$ knock-out zebrafish line

To investigate the role of cPLA$_2$ in development, we generated a zebrafish stable knock-out (KO) line using the Crispr-Cas9 system. In a first approach, we cut the gene...
of interest (pla2g4aa) using CRISPR-Cas9. Different guide mRNAs (gRNAs) were tested and the best outcome was obtained by using the gRNA shown and described in Appendix A.1, that recognizes the sequence in its catalytic domain. Using this approach, the Cas9 protein cleaves the sequence in the position defined by the guide RNA. Then, the cell machinery will fix the DNA double-strand break induced by the RNA guided Cas9 endonuclease activity via non-homologous end joining. As this DNA repair process is not perfect, a STOP codon can be randomly inserted. The embryos in which this happens are the first (F0) KO generation and are identified by PCR-based screening for the expression of the gene targeted by the guide RNA. These embryos are not full KO, but they can be crossed to obtain a KO line. Their in-cross generates the F1 generation of both heterozygous and homozygous embryos (F1), while a second generation of homozygous KO embryos (F2) can be obtained by in-crossing of F1 adult fish. The F1 and F2 generations of KO were validated by western blot of adult fin clip samples and by qPCR at various developmental stages, as shown in Figure 9.9 A-B. All the KO embryos used for the experiments are homozygous and derived from the generation F2.

A first problem encountered was that cPLA2-KO adults showed limited laying since pla2g4aa was previously indicated to be essential for ovulation [177]. For this reason, embryos from KO adults were obtained by in vitro fertilization (IVF) in the following.

To validate the KO in vitro, we confined progenitor cells derived from KO embryos and, unexpectedly, observed that cells were able to polarize and migrate, at percentages comparable with WT cells but with higher experimental variability (Figure 9.10 A-B). By plotting the histogram of the obtained percentages of stable-bleb cells across different experiments, WT data approximately followed a gamma distribution, while KO data were more disperse and almost showed a bi-modal distribution, with some lower data values (Figure 9.10 B). These data came from different crosses with no
apparent correlation. Considering that the KO embryos and adults were thoroughly validated, we tested for potential compensations of the mechanosensitive cPLA\textsubscript{2} pathway. A morpholino interference approach supported that in WT embryos cPLA\textsubscript{2} was encoded by the gene \textit{pla2g4aa}, which was targeted by the Crispr-Cas9 KO approach, and excluded a possible role of \textit{pla2g4ab} (Figure 9.4). We thus tested next if in KO embryos another gene gives rise to a similar function as cPLA\textsubscript{2}.

To do so, we treated cells derived from KO embryos with the cPLA\textsubscript{2} inhibitor Pyrrophe-none. Consistent with previous experiments, we observed a reduction of the fraction of motile cells in both WT and KO samples (Figure 9.10 A).

### 9.3.2 Genetic compensation of lipase expression in cPLA\textsubscript{2} knock-out embryos

The results obtained with the Crispr-Cas9 knock-out approach suggested that another lipase could allow cells to trigger a mechanosensitive activation of myosin II and associated amoeboid migration in the cPLA\textsubscript{2} KO line. Indeed, two recent publications showed that the Crispr-Cas9 KO approach can trigger a process of spontaneous genetic compensation [241, 242]. When Crispr-Cas9 is used to induce a premature stop codon to knock out a gene, the resulting truncated mRNA is commonly fragmented and degraded in the cell. These fragments can possibly bind to a factor (named Upf3a) that makes the complex translocate to the nucleus, where it interacts with another protein (COMPASS) that can modify the histone H3 in order to activate the transcription of gene paralogs [242]. This "nonsense-induced transcriptional compensation" [241] was identified in zebrafish and in mouse cell lines [242]. Even if the entire mechanism or why it occurs remains unclear, these studies were able to provide an explanation for the apparently inconsistent results obtained with our generated cPLA\textsubscript{2}-KO line and suggested further experiments.

To identify possible up-regulated gene paralogs we thus searched for all the lipases present in zebrafish that can release AA. We identified 7 genes, related to \textit{pla2g4aa} (cPLA\textsubscript{2}). One of them, \textit{pla2g4ab}, is a gene duplication, while others are \textit{pla2g-} (\textit{pla2g1b, pla2g6, pla2g7, pla2g12a, pla2g12b and pla2g15}) and four \textit{pla2g4c} genes. Thus, we performed a q-PCR in WT and cPLA\textsubscript{2} KO embryos at various developmental time points to quantify the expression levels of all these genes (Figure 9.10 D-E). Among all the genes tested, we identified four genes that appeared over-expressed in KO embryos with respect to WT embryos at sphere stage: \textit{pla2g6, pla2g7, pla2g15} and \textit{pla2g4c}. These lipases might be up-regulated in KO embryos through mechanisms as previously described [241, 242]. Still, as the qPCR shown in Figure 9.10 E shows some variability in between different KO samples, it is not clear if the compensation is quantitatively similar in all embryos or if the different levels of genetic compensation can explain the higher variability in cell behavior. Interestingly, at 24\textit{hpf} the expression levels in between WT and KO embryos were comparable, suggesting a transient up-regulation of gene paralogs in early embryonic stages.

Notably, the proteins encoded by these gene paralogs can all produce AA from phospholipids, but in a calcium-independent manner. To test the involvement of these
Figure 9.10: cPLA\(_2\) KO: a compensatory mechanism activates a calcium-independent PLA\(_2\) mechanosensitive pathway (A) Percentage of polarized stable-bleb cells derived from WT (left) or cPLA\(_2\) KO (right) embryos at sphere stage and cultured under 7\(\mu\)m confinement in control condition (Ctrl.) or upon cPLA\(_2\) inhibition with 1\(\mu\)M Pyrrophene. \(n \geq 5\), for each experiment \(N > 100\). (B) Histogram of the percentage of stable-bleb cells obtained in independent experiments for progenitor stem cells derived from WT (left) or cPLA\(_2\) KO (right) embryos under 7\(\mu\)m confinement. The distribution of the KO data is broader with a portion of experiments yielding a low fraction of polarized cells. (C) Percentage of polarized stable-bleb cells for progenitor stem cells derived from WT (left) or cPLA\(_2\) KO (right) embryos in control condition (Ctrl.) or upon treatment with 10\(\mu\)M BAPTA-AM. Each line represents a batch of different embryos. (D) Relative expression levels of various pla2g-genes by q-PCR in WT and cPLA\(_2\) KO embryos at sphere stage (4\(hpf\)), shield stage (6\(hpf\)) and at 24\(hpf\). Magenta arrows indicate genes that are over-expressed in KO fish at sphere stage, while levels are similar at 24\(hpf\). (E) Relative expression levels of various pla2g-genes by q-PCR in WT and 3 independent sets of cPLA\(_2\) KO embryos at sphere stage. Magenta arrows indicate genes that are over-expressed in KO samples. Note, pla2g\(4aa\) is the gene encoding for cPLA\(_2\) and pla2g\(4ab\) refers to the gene duplicate. For the plots in D-E: results are expressed in percentages, normalised by eef1\(\alpha\); cDNA concentration was 5ng/\(\mu\)l per well. \(* * * P < 0.0001\), n.s. not significant. Experiments in D-E performed by S. Jiménez-Delgado (CRG).

proteins, we treated progenitor stem cells derived from either WT and KO embryos with BAPTA-AM to chelate intracellular calcium. This treatment reduced the percentage of stable-bleb cells in WT (consistently with the reduction of cortical myosin II under confinement, Figure 9.7), but not in KO embryos. In KO-cells, independently of the fraction of polarized cells in the control case, treatment with BAPTA-AM had no effect. In consequence, we concluded that a compensatory mechanism in the cPLA\(_2\)
KO line leads to the over-activation of genes that encode for calcium-independent lipases that can also allow cells to measure shape changes in confinement.

9.4 Conclusions

In this chapter, we presented a novel role of the nucleus as a mechanosensor of large cell shape deformations in confinement. While previous studies pointed at the important role of the plasma membrane (and embedded proteins therein) for sensing local deformations and forces at the cell surface, the nucleus is suitable to act as a mechanosensor of larger cell deformations. We show that when the nucleus gets deformed, cells are capable of increasing their contractility, and therefore their stiffness, and cells can acquire a fast migration mode that enables cell to move away from confined spaces.

Progenitor stem cells in suspension showed a highly folded nuclear envelope, with both INM and ONM portions present in these invaginations (Figure 9.1). Cell confinement below 13 μm (that corresponds to squeezing the cells to approx. half or less of their height) induced an unfolding of the INM, as observed by expressing the INM marker Lap2β-eGFP (Figure 9.2). The unfolding was gradual by lowering the confinement height, but stable in time under confinement (Figure 9.2). Interestingly, above this deformation threshold, cortical myosin II enrichment correlated with nucleus deformation. INM unfolding was previously shown to activate cPLA2 upon nuclear swelling during tissue damage and led to the production of lipid metabolites that function as chemo-attractants responsible for leukocyte recruitment at wounding sites. We found that cPLA2 translates mechanical cell shape changes into myosin II activity and ameboid motility under confinement in embryonic cells derived from different lineages and developmental stages (Figure 9.3). We demonstrated the necessary role of cPLA2 in controlling myosin II via chemical inhibition using Pyrrophenone as well as by a genetic interference approach (Figure 9.4). For this purpose, we developed a dominant negative construct with deficient catalytic activity and INM binding and used a splicing-blocking morpholino to interfere with its expression (Figure 9.4). Moreover, the morphant phenotype could be completely rescued by injecting cPLA2-mRNA.

In progenitor stem cells, cPLA2 localizes in the nucleus in both suspension and confinement conditions and it is required in the nucleus to activate myosin II upon confinement (Figure 9.5). Our fluorescent cPLA2 probes showed a homogeneous INM translocation in confined cells in the presence of high intracellular calcium levels, and we confirmed cPLA2 activity in confined cells by Raman spectroscopy (Figure 9.5). This method allowed us to directly quantify AA production and its release in the cytosol. AA appears to control myosin II activity via the Rho/ROCK pathway in a cell-autonomous way, independently of oxidation processes associated with eicosanoid production or other signalling metabolites of the COX/LOX pathways (Figure 9.6). Finally, since cPLA2 is a calcium-dependent lipase, we studied the role of intracellular calcium levels. We found intracellular calcium, but not extracellular calcium, to be necessary for proper mechanosensation (Figure 9.7). Notably, increased calcium levels were not sufficient to activate cortical myosin II in the absence of cell deformation. We further showed that cPLA2 inhibition was not affecting INM unfolding, excluding the presence of a lipase-dependent feedback process controlling nuclear envelope architecture in de-
formed cells in confinement (Figure 9.8). Finally, we developed a Crispr-Cas9 cPLA$_2$ KO line that led to unexpected results (Figure 9.10). We identified that a genetic compensation mechanism induced the activation of gene paralogs of $pla2g4aa$. These proteins can release AA from membranes in a calcium-independent manner, as confirmed by chelating intracellular calcium in KO cells using BAPTA-AM, which did not affect the mechanosensitive response in KO cells.

![Figure 9.11: The nucleus gauges mechanical confinement and controls cellular contractility](image)

Increasing cell deformation induces INM unfolding and positively correlates with higher cell contractility. We found that cPLA$_2$ is activated upon nucleus stretch and catalyzes the release of AA that further controls myosin II activity through the Rho/ROCK pathway in a cell-autonomous way.

Concluding, the mechanosensitive cPLA$_2$ pathway allows cells to accurately measure shape deformations (and physical constraints in their environment) and controls cell contractility and migratory behavior under mechanical stress. The same nuclear ruler pathway is conserved in other cell types, such as HeLa or immune cells [181]. These results establish a new role for the nucleus as a mechano-controller of dynamic cell behavior.
Chapter 10

Characterization of intracellular and nuclear mechanics

This project was carried out in collaboration with the group of M. Krieg (ICFO) and in particular with F. Català-Castro and S. Ortiz-Vásquez.

As discussed in the previous chapters, the nucleus can function as a mechanosensor and allows cells to measure large shape changes. Our obtained results suggested that the nucleus has elastic material properties and functions as a non-dissipative sensor of cell deformations, given that the activation of mechanotransduction pathways in the nucleus occurred in a rapid, stable and reversible manner when cell shape changes were transiently imposed (Chapter 8). The nucleus is known to be the stiffest organelle in a cell and various studies showed that its mechanical properties are controlled by the molecular composition of the nuclear envelope together with chromatin organization [142,143], as discussed in Chapter 4.3. However, so far, nuclear mechanics in cells were assessed by methods that probe nucleus material properties from the extracellular space, therefore measuring also contributions from the PM and cortex and limiting experimental data interpretation (as discussed in Chapter 4.4.1). Instead, light offers a non-invasive mechanism to exert and measure forces in biological samples. Therefore we developed a new method, based on optical tweezers (OTs, introduced in Chapter 7.4), to quantify mechanical properties of intracellular organelles and applied it to study the nucleus of single cells in suspension and mechanically deformed cells in confinement [209].
10.1 Optical tweezers to measure nuclear mechanics in live cells

To perform measurements of intracellular nuclear mechanics in live cells we developed a protocol using OTs [209]. This is achieved using a commercial system by Impetux, combined with a spinning disk fluorescence microscope that allows to perform live cell imaging during optical tweezers routines (see Chapter 7.4 for complete protocol).

As discussed in Chapter 7.4, OTs can exert forces on objects that have a higher refractive index than their surroundings and the generated force depends on the difference of these two indices [193]. For this purpose, we injected 1μm polystyrene micro-beads into 1-cell (zygote) stage zebrafish embryos. As a consequence, progenitor stem cells dissociated from these embryos will contain 1μm beads in their cytoplasm. Isolated primary embryonic cells can be plated in suspension or confinement using a PDMS spin-coated layer of either 50μm or 10μm separated by two glass coverslips to culture cells respectively in non-confined or confined conditions (see Figure 7.3 B), provided that the OT setup is not compatible with the use of the dynamic confiner assay (see Chapter 7.4). To avoid excessive cell movement during OTs routines (due to the fact that the trap itself has the strength to trap the entire cell and move it), suspension cells were plated on Concavalin A. Only cells with one or two beads, as shown in Figure 10.1 A, were used for the indicated experiments, while cells without beads or multiple beads were excluded. Nuclei were labeled with DNA-Hoechst in order to track nuclear deformation due to the indenting trap as shown in Figure 10.1 B.

To measure nuclear mechanics we used different approaches, as described here.

Figure 10.1: Indenting the nucleus with OTs (A) Representative transmission light snapshots of progenitor stem cells injected with one or two beads (magenta arrows) in suspension (top) or under 10μm confinement (bottom). (B) Representative fluorescence images of cells expressing myosin II-eGFP and labeled with DNA-Hoechst in suspension (top) and confinement (bottom) and nuclei before and during indentation. The magenta arrows point at the position of nucleus indentation by the trapped bead. (C) Kymograph of an indentation routine of a nucleus labeled with DNA-Hoechst generated along the bead trajectory with a 1 pixel line width. (D) Fit of the kymograph in (C) showing the proximal and distal nuclear boundaries where the nucleus was indented by the bead or its opposite direction, respectively. Scale bars 10μm.
10.1.1 Protocols for cell nucleus indentation and force measurements

To characterize nucleus mechanics, we first used a triangular ramp to indent the nuclei of suspension or confined cells labeled with DNA-Hoechst (Figure 10.1 B) and tracked nucleus deformation over time, as shown in the kymograph and related fits in Figure 10.1 C-D. In all the nucleus-indentation experiments, the proximal side of the nucleus refers to the indented part close to the bead, while the distal side is at the opposite position. Obtained force profiles followed the trap movement: the force appeared to increase when indenting the nucleus and to decrease when the trap was moved backward (Figure 10.2 A-C), supporting that the nucleus is stiffer than the cytoplasm. By performing simultaneous time-lapse imaging during force indentation of the nucleus, we could divide obtained force tracks into two regions where the bead was in contact with the nucleus during deformation or located in the cytoplasm (as indicated by the grey area in the plots of Figure 10.2 B-D). Notably, when approaching the nucleus, the force increased before physically indenting it; this might be due to the dense ER around the nucleus or other cytoskeletal elements. By plotting the force with respect to the trap position, energy dissipation was evident in each cycle (given by the area in between the curves), supporting that the nucleus is a viscoelastic material (Figure 10.2 B-D). However, it is not fully clear if this area, the energy dissipated, decreases upon multiple nuclear indentations, or not. If this was true, the data would support a stiffening of the nucleus upon repetitive deformations. However, as nucleus sometimes moved during the routines, it is hard to exclude that small movements happened during each routine. This would lead to a smaller indentation and smaller dissipated energy, as often observed. In general, by using this approach, we could not extract quantitative information that describe nuclear mechanics or the differences in between the nuclei of suspension and confined cells. The main limitation was that neither the force nor the absolute trap position were fixed parameters and therefore it was complicated to understand the relation in between them. To overcome this limitation and to extract quantitative material characteristics, complementary approaches as microrheology and stress-relaxation measurements were implemented [208], as described in the following.

10.1.2 Measuring nuclear viscoelastic properties in mechanical confinement

Given the limitations of the indentation experiments presented in the previous section, we next performed stress-relaxation routines (as shown in Figure 4.5 C) to characterize the mechanical properties of the nucleus.

This approach generally consists in rapidly deforming a viscoelastic material and then observing the force-relaxation profile (see Chapter 4.4.2). In our experimental setting, this can be achieved by indenting the nucleus using a 1µm polystyrene bead present in the cytoplasm of isolated cells obtained from bead-injected embryos. Setting the bead indentation distance to approx. 2–3µm, we observed a peak force of ~100pN (Figure 10.3 A-B). The trap was kept in the indentation position for 10s before retracting it (Figure 10.3 A-B). During this time, the force followed a biphasic stress relaxation that
10.1. Optical tweezers to measure nuclear mechanics in live cells

Figure 10.2: Force indentation of suspension and confined nuclei. (A) Trap position and recorded force over time for a nuclear indentation routine performed in a cell cultured in suspension. (B) Force-trap plot associated to panel (A) showing a hysteresis cycle. (C) Trap position and recorded force over time for a nuclear indentation routine performed in a cell cultured under $10\mu m$ confinement. (D) Force-trap plot associated to panel (C) showing a hysteresis cycle. The grey areas indicate the regions where the optical trap is indenting the nucleus as segmented from fluorescence time-lapse movies of cells labeled with DNA-Hoechst. The yellow lines correspond to bead indentations and the cyan lines to bead retractions.

The measured force response followed a fractional viscoelastic model defined by a dashpot and a spring-pot in series, in parallel to a spring [207], as depicted in Figure 10.3 B. This model features an initial fast decay, modeled by a power law with exponent $\beta$, followed by a slower exponential decay with a characteristic relaxation time $\tau$ [205]. Explicitly, the force decay can be written as:

$$ F(t) = F_{\text{stored}} + (F_{\text{peak}} - F_{\text{stored}}) t^\beta e^{-t/\tau} $$

(10.1)

In a semi-logarithmic scale and for intermediate times, the force can be fitted with a line, with the slope corresponding to the decay time of the exponential viscous contribution (Figure 10.3 C). The same curve in log-log scale has a linear behavior at short times and the exponent of the power law can be obtained from a linear fit (Figure 10.3 D).

As the cellular response to mechanical shape deformation can be modulated by the mechanical properties of the nucleus (the mechano-sensor), we next evaluated if the nucleus changes its mechanical properties upon cell deformation. For this purpose, we performed stress-relaxations routines in suspension and $10\mu m$ confined cells. The nucleus was labeled with DNA-Hoechst (Figure 10.4 A-B) to track nuclear indentation over time (Figure 10.4 C). We observed that nucleus indentation caused a movement of the distal side of the nucleus, leading to a smaller net indentation at the proximal...
Figure 10.3: Stress relaxation experiment (A) Trap trajectory (top) and recorded force (bottom) obtained by performing a stress-relaxation routine via indenting the nucleus with a trapped bead. (B) Force decay after the peak force is reached and sketch depicting the fractional viscoelastic model used to fit the data. Blue lines: raw data; magenta lines: fit. The plateau at long times represents the stored elastic energy associated with the material resistance. (C) The force decay in semi-log scale can be fitted with a line to obtain the decay time for long times. (D) The force decay in log-log scale can be fitted with a line for short times and the power law exponent can be derived. Grey areas corresponds to the regions of the force decay where the corresponding curve can be observed and fitted.

side of the nucleus, especially in suspended cells. This nucleus movement was corrected in a data post-processing routine to quantify the net indentation (Figure 10.4 A-B-C). In confinement, the nucleus is physically constrained in between the apical and basal PM/cell cortex, a region in which the ER also gets immobilized (Chapter 11.6). This suggests friction between the nucleus and cell surface in confined cells, supported by an absence of nucleus movement in the indentation routine (Figure 10.4 A-B-C). We quantified the exponential decay of different nuclei in suspension and confined cells (Figure 10.4 D) and observed that both the decay time $\tau$ and the stored force were not affected by the confinement (Figure 10.4 E-F). These data support that the nucleus has viscoelastic properties and maintains similar mechanical properties when deformed, supporting its role as an elastic mechanosensor of cell shape deformation.

10.1.3 Active microrheology of the nucleus and the cytoplasm

As introduced in Chapter 4.4.2, microrheology allows for quantitative measures of the elastic and viscous material properties by oscillating a trapped particle at defined frequencies. We next sought to apply microrheology to measure the mechanical properties of the cytoplasm and of the nucleus in a frequency dependent manner. To do so, we
performed rheological routines in the cytoplasm, following a trajectory parallel to the nucleus, or at the nucleus surface, perpendicularly to it (therefore leading to a gentle nucleus indentation), as shown in Figure 10.5 A. Performing such a routine on a purely elastic material would result in a measured force that is in phase with the applied bead displacement (as the force scales linearly with the displacement, Figure 10.5 B). Since for viscous materials, the force is proportional to the derivative of the displacement (and the derivative of a sine is a cosine), a 90° phase-shift is observed (Figure 10.5 B). In general, since biological materials are viscoelastic, an intermediate response is expected. To understand such a response - for each oscillating frequency - is not trivial. The force-response curve is a complex function that can be decomposed in its real and imaginary parts, respectively, yielding the elastic and loss modulus, \( G'(\omega) \) and \( G''(\omega) \) (as discussed in Chapter 4.4.2).

Rheological measurements in the frequency domain in both cytoplasm and at the nuclear envelope showed a two-regime response (Figure 10.5 D-E) that could be modeled with a fractional two-element approach as a modification of the standard Kelvin-Voigt...
model (Figure 10.5 C):

\[ G^*(\omega) = c_\alpha (i2\pi\omega)\alpha + c_\beta (i2\pi\omega)\beta \]  \hspace{1cm} (10.2)

where \( G^*(\omega) \) is the frequency-dependent complex G modulus and \( \alpha \) and \( \beta \) the power law exponents. The so-called fractional Kelvin-Voigt model [205], depicted in Figure 10.5 C, describes a material behaving nearly elastic at short frequencies, with a power-law exponent close to zero \( (\alpha \to 0) \), while it fluidifies at higher frequencies \( (\beta \to 1) \). The advantages are that the complex frequency-dependent measurements are reduced to the fit of four parameters, two per fractional element/power law, and the model can describe in which frequencies the elastic/viscous properties dominate [186, 205]. As shown in Figure 10.5 D-E, this model very well fits the experimental data for both the cytoplasm and nucleus. The obtained curves showed that both the cytoplasm and nucleus are more elastic at short frequencies, where \( G' \) is greater than the \( G'' \), and more viscous at longer frequencies. The cutoff in between these two regimes corresponds to the crossover frequency, where the two curves intersect. Also, both the \( G' \) and \( G'' \) curves were not lines, but visibly showed a two-slope behavior that is correctly captured by the two-power laws of the applied model, supporting that a model with one element would not be sufficient.

![Figure 10.5: Active microrheology](image)

**Figure 10.5: Active microrheology** (A) Sketch exemplifying the active microrheology routine based on oscillating a trapped bead (bead: grey circle, trap: red shaded area) in the cytoplasm, parallel to the nucleus, or at the nuclear envelope, perpendicularly to it. The bead is periodically oscillated along a path of 200nm at doubling frequencies in between 0.5 and 256Hz. (B) The force response to a sinusoidal displacement (grey line) for either elastic (teal line) or viscous (magenta line) materials. The elastic response is in phase with the displacement (as the force scales linearly with it) while the viscous response has a 90° phase shift (as it scales with the derivative of the displacement). (C) Fractional Kelvin-Voigt model with two fractional elements in parallel used for modeling the data. (D-E) \( G' \) and \( G'' \) data (dots) and fitted Kelvin-Voigt model (line) for rheological measurements in the cell cytoplasm (D) or nucleus (E).
We next performed active microrheology in the cytosol and at the nuclear envelope of various cells in suspension and modeled the data as just described. From the fractional Kelvin-Voigt model, the four parameters that can be obtained are the two exponents $\alpha$ and $\beta$, the pre-factors $c_\alpha$ and $c_\beta$ along with the crossover frequency that can be quantified as the intersection in between the $G'$ and $G''$ curves.

**Figure 10.6: The nucleus is stiffer than the cytoplasm** Results from the fit of the Kelvin-Voigt model on the active microrheology data performed in the cytoplasm or at the nuclear envelope of progenitor stem cells in suspension: (A) exponents $\alpha$ and $\beta$, (B) crossover frequency ($\omega_o$), (C) pre-factors $c_\alpha$ and (D) prefactor $c_\beta$. The exact values in mean ± standard deviation are: $\alpha_{Cyto} = 0.10 \pm 0.12$, $\alpha_{Nucl} = 0.15 \pm 0.12$; $\beta_{Cyto} = 0.81 \pm 0.05$, $\beta_{Nucl} = 0.77 \pm 0.07$; $\omega_o_{Cyto} = 3.2 \pm 2.5$Hz, $\omega_o_{Nucl} = 9.2 \pm 8.1$Hz; $c_\alpha_{Cyto} = 0.89 \pm 0.58Pa$, $c_\alpha_{Nucl} = 2.0 \pm 0.9Pa$; $c_\beta_{Cyto} = 0.16 \pm 0.1Pa$, $c_\beta_{Nucl} = 0.30 \pm 0.26Pa$; N=21. ** ** $P < 0.0001$, ** $P < 0.001$, n.s. not significant.

The data obtained by the active microrheology routine displayed similar values of the two exponents for the nucleus and cytoplasm. In both cases, $\alpha << \beta$, with $\alpha \approx 0$ and $\beta \approx 1$ (Figure 10.6 A). These values suggest that the $\alpha$-element is more similar to an elastic spring while $\beta$ mimics a viscous dashpot. This supports that both the cytoplasm and the nucleus are "more elastic" at low frequencies, where $G'$ dominates and thus they behave like a viscoelastic solid, and "more viscous" at higher frequencies, where $G''$ dominates, and thus they behave like viscoelastic fluids [205]. At the same time, both the crossover frequency and the prefactor $c_\alpha$ differ and are higher for the nucleus compared to cytoplasm (Figure 10.6 B-C), while the prefactor $c_\beta$ did not show a statistical difference (Figure 10.6 D).

The data show a high dispersion, partially explained by the low number of cells, but suggest that the low frequency stiffness is higher for the nucleus than the cytosol (because of the higher $c_\alpha$, Figure 10.6 C). The solid-like material property of the nucleus dominates its viscous property and the nucleus behave like a solid for a broader range of frequencies when compared to the cytosol (because of the higher crossover
frequency, Figure 10.6 B). In addition, the nuclear crossover frequency defines a characteristic time $\tau_{\text{Nucl}} \sim 0.1s$. For deformations that occurred on timescales longer than $\tau_{\text{Nucl}}$, the nucleus behaves like a viscoelastic solid, otherwise it behaves like a viscoelastic fluid. In the experiments presented throughout this thesis, mechanical confinement was gradually applied by lowering the pressure of the controller that moves the central piston of the suction cup (see Figure 7.1). This happens on the timescale of minutes. The results obtained from studying the mechanoresponse of progenitor stem cells in confinement suggested that the nucleus behaved like an elastic mechanosensing element (Chapter 8.1, 8.3). Indeed, this is consistent with the timescales in which the nucleus behaves like a viscoelastic solid ($t > \tau_{\text{Nucl}} = 0.1s$). The cytosol has a similar value, $\tau_{\text{Cyto}} = 0.3s$, but because of the lower $c_{\alpha}$ value the viscous component dominates. The fact that both cytoplasm and nucleus show this behavior is consistent with what was also suggested for tissues [184]. Moreover, the fluid-like components for nucleus and cytosol were found to be similar (Figure 10.6 D), resulting in a more effective behavior of the cytosol as a viscoelastic fluid. The fact that both exponents $\alpha$ and $\beta$ are comparable for the nucleus and cytosol (Figure 10.6 A) does not imply that the materials are identical, but rather that they have a similar composition (as discussed in [186] for the cytoplasm of cells during mitosis).

### 10.2 Conclusions

This Chapter described a novel protocol, developed to measure nuclear mechanics in suspension and confined cells [209]. Different approaches were tested: active micro-rheology was used to unveil the differences in between mechanical characteristics of the cytoplasm and the nucleus and stress relaxation routines were applied to address if nuclear mechanics are altered in deformed nuclei for cells in confinement. Further studies need to be performed to increase the sample size, given the high variability of the data, and to better characterize nuclear mechanics in control cells and under mechanical stress. In particular, active micro-rheology in confined cells will allow for a better characterization and comparison of both nucleus and cytosol material properties in suspension and under confinement. Our data put forward the role of the nucleus as an elastic mechanosensor, in agreement with its role in tailoring the cellular response to shape changes.

Specifically, performing stress-relaxation routines on the nucleus showed that both the relaxation time and the stored energy did not change when nuclei were deformed under confinement, suggesting that the viscoelastic properties of the nucleus were not altered when the nucleus was deformed.

Active micro-rheology in the cytoplasm and at the nuclear envelope together with modeling of the data by a fractional Kelvin-Voigt model, resulted in a higher $c_\alpha$ (where $\alpha \sim 0$ represents the elastic fractional element) and a higher crossover frequency for the nucleus with respect to the cytoplasm. Therefore, the nucleus appears to be stiffer than the cytoplasm and behave like a viscoelastic solid at long timescales. More specifically, the crossover frequency defines two timescales with different material regimes. For $t > \tau_{\text{Nucl}} = 0.1s$ the nucleus behaves like a viscoelastic solid, with higher stiffness
than the cytoplasm, while below this timescale it behaves like a viscoelastic fluid. In
the mechanical confinement experiments, the deformation was gradually applied (see
Figure 7.1) over a couple of minutes. Rheological measurements can be combined
with time-resolved studies of the cellular mechanoresponse under variable deformation
rates in next experiments to address time-dependent mechano-sensitive changes of
intracellular structures and adaptive cell responses.
Chapter 11

Single cell proprioception

In Chapter 9 we described how the nucleus can act as a mechano-gauge that enables cells to measure shape changes and regulate cell contractility levels and behavior. This is achieved because nuclear deformation upon confinement induces an unfolding of the INM, sensed by cPLA$_2$, that further activates myosin II via the release of AA and the Rho/ROCK pathway.

Single cells in living tissues are subjected to different kinds of forces as they get stretched or compressed during tissue development [4] or tissue damage [149, 168]. As a consequence different types of cell and nucleus deformations can occur. Whether progenitor stem cells use the same pathway, mediated by INM unfolding and cPLA$_2$ lipase activity, to sense various shape changes and regulate mechanosensitive cell behavior remained unclear.

11.1 Hypotonic cell swelling

11.1.1 Hypotonicity induces INM unfolding and myosin II activation

To study how cells respond to different shape changes, we induced hypotonic cell swelling to inflate cells. This cell deformation is associated with an increase in cell and nucleus volume, leading to an unfolding of the nuclear envelope as previously identified [168]. Furthermore, hypotonic stress was shown to activate the cPLA$_2$ mechanotransduction pathway upon tissue damage as a consequence of swelling in the zebrafish tail [149].

Applying hypotonic shock to progenitor cells in suspension, rapidly inflated cells and their nuclei, leading to an increase in their cross-sectional area (Figure 11.1 A-B-C) and induced INM unfolding (Figure 11.1 C-D), as visible from the higher nucleus area-to-perimeter ratio and invagination ratio. This change in nuclear envelope morphology was visualized by expressing the INM marker Lap2β--eGFP and the nuclear invagination ratio was quantified as shown in Figure 9.2. Notably, we did not observe a longer-time cell adaptation associated with a regulatory volume decrease as reported in previous
11.1. Hypotonic cell swelling

Figure 11.1: Hypotonic swelling inflates the cells, nuclei and unfolds INM (A) Cell area over time of progenitor stem cells expressing myosin II-eGFP in isotonic media ($t < 0$) and hypotonic media ($t > 0$) cultured in suspension. The time $t = 0$ has been defined as the moment of the addition of milli-Q water to induce the hypotonic shock. Only isolated cells were considered for this analysis. Iso: $N = 5$, Hypo: $N = 6$. (B) Exemplary confocal images of progenitor stem cells expressing myosin II-eGFP in isotonic media (Iso, top) or hypotonic (Hypo 0.5x, bottom) at indicated times. (C) Boxplot of nuclear area (top left), cell area (bottom left), nuclear area-to-perimeter ratio (top right) and invagination ratio (bottom right) for cells cultured in isotonic and hypotonic media. (D) Exemplary confocal images of progenitor stem cells expressing Lap2β-eGFP cultured in isotonic (top) and hypotonic (bottom) conditions. Scale bars 5µm. *** $P < 0.0001$.

studies for adherent HeLa cells [243]. Instead, progenitor stem cells maintained a larger cross-sectional area over the experimental time window (typically $\leq 1h$).

Notably, hypotonic stress was also associated with an increase in cortical myosin II levels as well as an increase in the relative bleb sizes when lowering medium osmolarity (Figure 11.2 A–B–C). However, hypotonic conditions led to significantly lower cortical myosin II accumulation compared to cell deformation in confinement (Figure 11.2 A), which further resulted in almost no polarized cells in hypotonic media (Figure 11.2 D), differently from the confinement case. However, INM unfolding in hypotonic conditions can reach similar levels as cells deformed in confinement. We found that the 0.5x hypotonic condition (median invagination ratio $IR_{hypo} = 0.082$) resulted in a comparable nuclear envelope unfolding as in cells deformed in 7µm confinement (median $IR_{conf} = 0.087$, statistical test between these data n.s.). Therefore, while hypotonic shock led to a similar INM unfolding for cell deformation in confinement and hypotonic swelling, cell swelling under hypotonic stress induced lower cortical myosin II levels and led to a significant reduction in the percentage of polarized cells.

Differences in this cellular response behaviour could be explained by various mecha-
A different mechanotransduction pathway might mediate myosin II recruitment to the cell cortex under hypotonic swelling, or other unknown factors could reduce the mechanosensitive activity of cPLA$_2$. To test if cPLA$_2$ was activated upon cell swelling in hypotonic conditions, we first performed Raman spectroscopy to quantify AA release. Indeed, we found that AA was released in the cytoplasm of swollen cells and this release occurred in a cPLA$_2$-dependent manner, as validated by treating cells in hypotonic conditions with Pyrrophenone (Figure 11.2 E-F), even if this reduction was not complete. These results suggested that there is another level of regulation of cPLA$_2$ activity that allows cells to distinguish comparable levels of INM unfolding in the conditions of cell squeezing versus cell swelling.
11.1.2 Calcium is differentially regulated upon swelling or confinement

cPLA$_2$ is a calcium-dependent lipase [169]. Previous studies and our results (discussed in Chapter 9.2, Figure 9.7) reported that its lipase activity can be tuned by regulating intracellular calcium levels [149]. We thus performed quantitative imaging of calcium dynamics in cells under hypotonic swelling and mechanical confinement. Interestingly, we found that calcium was differentially regulated in the two conditions. By labeling cells with the calcium reporter Calbryte520 and quantifying reporter intensities, we found that mechanical confinement induced higher calcium levels compared to hypotonic swelling, both in the cytoplasm and, even more significantly, in the nucleoplasm (Figure 11.3 A-B). Similar results were obtained by expressing a genetic calcium reporter that encodes gCamp-NLS (nuclear localization signal)-dTomato. The NLS-dTomato signal can be used as a fluorescence reference reporting on the gCamp levels in each cell (for normalization of gCamp intensities due to cell-to-cell variability in protein expression by mRNA injection). We therefore quantified intracellular calcium levels as the ratio in between the gCamp intensity in the entire cell normalised with the NLS-dTomato intensity (Figure 11.3 C). This analysis showed that, consistently with the Calbryte520 data, mechanical confinement induced higher intracellular calcium levels than hypotonic shock (Figure 11.3 C-D). We also quantified the nuclear-to-cytosolic gCamp intensity (which is independent of the expression level of the construct) and observed that this value decreased in inflated cells, while an increase in confinement was observed (Figure 11.3 C-D).

These experiments suggested that calcium levels are increased both in stretched cells in confinement and hypotonic conditions compared to the control case (Figure 11.3). However, calcium levels appear significantly higher in confinement versus hypotonic conditions (Figure 11.3 C-D). This difference is even more pronounced when analysing at nuclear calcium levels (Figure 11.3 A-B) or the ratio of nuclear-to-cytosolic calcium levels (Figure 11.3 C-D). These data, which were validated by using two different calcium reporters, support that calcium is differentially regulated depending on the applied cell deformation. Considering that cPLA$_2$ requires calcium in the nucleus for the binding of its C2-domain to the INM [169] that triggers lipase activity, we hypothesized that variable calcium levels would be sufficient to tune the activation of the cPLA$_2$/myosin II pathway.

11.1.3 cPLA$_2$ mechanotransduction requires INM unfolding and high calcium levels

To directly test if variable calcium levels are sufficient to tune cPLA$_2$ activity and to validate that the cPLA$_2$ mechanotransduction pathway is functional in hypotonic conditions, we thus monitored cell responses in hypotonic conditions under variable intracellular calcium levels by treating cells with ionomycin. The addition of ionomycin in hypotonic stress conditions rapidly induced a cPLA$_2$-dependent cortical myosin II accumulation and was associated with an increased rate of stable-bleb transformation (Figure 11.4 A-C). This condition was further accompanied by an increase of AA
Figure 11.3: Calcium is differentially regulated upon cell confinement or cell swelling (A) Normalized cellular (left) and nuclear (right) Ca^{2+} levels (Calbryte520) for isotonic-suspension (Susp./Iso.) and hypotonic (0.5×) conditions and mechanical confinement (13 and 7 μm). (B) Representative confocal images of cells stained with Calbryte520 in isotonic condition (Susp./Iso.), under 0.5x hypotonic shock or confinement at 13/7 μm height. Dashed lines outline the cell and nucleus perimeter. (C) Normalised gCamp/NLS-dTomato mean fluorescent intensity (left) and nuclear/cytosolic gCamp intensity (right) for cells co-expressing gCamp+NLS-dTomato in isotonic-suspension (Susp./Iso.) and hypotonic (0.5×) conditions and mechanical confinement (13, 10 and 7 μm). (D) Representative confocal images of cells expressing gCamp+NLS-dTomato in isotonic-suspension (Susp./Iso.) and hypotonic (0.5×) conditions and 7 μm confinement. Scale bars 10 μm. ∗ ∗ ∗ P < 0.0001, ∗∗ P < 0.001, ∗ P < 0.01, n.s. not significant.

production in the cytoplasm, as quantified by Raman spectroscopy (Figure 11.4 D). Importantly, while ionomycin treatment in hypotonic media led to a rapid increase of AA production and cortical myosin II levels, ionomycin addition to cells in isotonic conditions was not sufficient to evoke an increase in myosin II levels or stable-bleb transformation (Figure 9.7 D-E and Figure 11.4 B), supporting that calcium has a permissive role for cPLA2 activity. We next validated that stable-bleb cells in the hypotonic condition with ionomycin were motile. Similarly to other amoeboid cells [54,66,67,85], stable-bleb cells were only motile when cultured in 3D environments, such as a gentle confinement, to efficiently transmit forces to the environment for cell movement. When culturing cells at 16 μm confinement in the presence of hypotonic conditions and the addition of ionomycin we could observe their fast migration, as visible by the measured cell tracks and mean cell velocities of polarized versus non-polarized cells in
Hypotonic cell swelling \textit{vivo} (Figure 11.4 D-E-F). A similar hypotonic shock could also be induced \textit{in vivo}, by injecting hypotonic media with ionomycin in the live blastula embryos, leading to the appearance of stable-bleb polarized and motile cells (Figure 11.4 G).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure11_4.png}
\caption{Nuclear swelling and high calcium levels are required for cPLA$_2$ mechanotransduction (A) Relative cortical myosin II intensity for progenitor cells cultured in isotonic, hypotonic and hypotonic + ionomycin conditions with/without the supplement of cPLA$_2$ inhibitor Pyrrophenone alone or in combination with Bapta-AM. (B) Percentage of polarized stable-bleb cells under isotonic conditions, hypotonic conditions supplemented with $1\mu M$ ionomycin in control conditions (magenta), in the absence of extracellular calcium (orange), cPLA$_2$ inhibition (green) and cPLA$_2$ + Bapta-AM (blue). Statistical tests performed with respect to the hypotonic+ionomycin condition. N>300 for all conditions. (C) Exemplary confocal images of non-polarized (top) and stable-bleb polarized (bottom) expressing myosin II–eGFP in hypotonic conditions supplemented with ionomycin. (D) Scores of Raman component associated with intracellular AA levels in hypotonic condition (Hypo, N=27) and in hypotonic condition supplemented with ionomycin (Hypo + Iono, N=25). Red lines represent mean and sem. \textit{(Caption continuous on next page.)}}
\end{figure}


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Figure 11.4: Nuclear swelling and high calcium levels are required for cPLA$_2$ mechanotransduction (Figure on previous page.) (E) Cell tracks (left) and mean velocities (right) for stable-bleb polarized (blue, motile) and blebbing non-polarized (orange, non-motile) progenitor stem cells cultured in hypotonic media supplemented with 1$\mu$M ionomycin under 16$\mu$m confinement. Red lines represent mean and standard error of the mean (sem). (F) Mean square displacement (MSD) analysis of cell tracks related to (E) and Figure 8.3 E-F. A persistent random walk model is fit to the data (Fürth formula) for 7$\mu$m confined cells (blue points, velocity and tracks shown in Figure 8.3 E-F) and hypo+ionomycin treated cells under 16$\mu$m confinement (magenta points), with persistence time of $t = 1.9\min$ (2.8$\min$), respectively. (G) Representative in vivo image of a motile stable-bleb cell (dashed red line) in a zebrafish embryo at blastula stage (4$\text{hpf}$) injected with hypotonic media supplemented with ionomycin (10$\mu$M). Asterisk denotes cell front. (H) Sketch of cell polarization and motile cell behavior in 2D (top) versus 3D confined environments (bottom), for control conditions (isotonic media; first column) versus cPLA$_2$ interference (second column) and hypotonic condition alone (third column) or in the presence of ionomycin (fourth column). High/low T, high/low tension. Part of the control data of panel A-B have been acquired by F. Pezzano (CRG). Injections and image acquisition for panel G done by H. Häkkinen (CRG). Scale bars 10$\mu$m. ***$P < 0.0001$, n.s. not significant.

Taken together, as summarized in the sketch of Figure 11.4 H, we showed that:

- Mechanical confinement is sufficient to induce stable-bleb motility in a cPLA$_2$-dependent manner as it leads to both INM unfolding and high intracellular and nuclear calcium levels. These two factors, together, trigger a high cPLA$_2$ lipase activity associated with high cortical myosin II levels that are necessary for cell polarization and the migratory transformation into the stable-bleb phenotype.

- Hypotonic shock is not sufficient to induce stable-bleb motility as low intracellular and nuclear calcium levels are not sufficient to induce sufficient myosin II activation, despite the presence of INM unfolding.

- Hypotonic shock together with ionomycin treatment (to increase calcium levels) recapitulates the phenotype observed in confinement. In this condition, nucleus stretching and high calcium levels are combined and induce high cell contractility and stable-bleb transformation similar to cell compression in confinement.

These data further support that single progenitor stem cells can both sense and distinguish uniaxial deformation in confinement versus isotropic swelling in hypotonic media as the cPLA$_2$ mechanotransduction pathway is differentially activated in both scenarios. While we showed that INM unfolding is comparable in the case of cell deformation in confinement and hypotonic stress, intracellular and nuclear calcium levels vary significantly between both conditions. This further controls the different cell behavior in the two conditions and induces migrating cells in confinement, while cells remain non-motile and blebbing in hypotonic media. Overall, we validated that the cPLA$_2$ pathway is functional in hypotonic stress conditions, but cPLA$_2$ activity is limited by the low calcium levels and artificially increasing calcium (by adding ionomycin) was able to recapitulate the phenotype observed in upon cell deformation in confinement.
11.1.4 cPLA$_2$: a calcium-dependent membrane tension reporter

Given the role of cPLA$_2$ in sensing hypotonic swelling and its increased activity with ionomycin, we next thought of imaging the localization of cPLA$_2$ in all these conditions. Therefore, we expressed a fluorescently-tagged cPLA$_2$ construct via mRNA injection and imaged cells in isotonic/hypotonic conditions and under 7 μm confinement with and without the addition of ionomycin (Figure 11.5 A-B). In control (isotonic, suspension) cells cPLA$_2$ localized to the nucleus (Figure 11.5, as in Figure 9.2). Upon hypotonic swelling, cPLA$_2$ did not translocate to the INM, while a re-localization was observed in deformed nuclei only in 2% of confined cells, as discussed in Chapter 9.5 and Figure 9.5. Addition of ionomycin to cells under hypotonic stress or confinement significantly increased the percentage of cPLA$_2$ translocation events to the INM and its relative signal intensity at the INM (defined as the ratio of the cPLA$_2$-GFP fluorescence intensity at the INM and in the nucleoplasm). Moreover, this translocation was stable over time (Figure 11.5 A-B). In contrast, the addition of ionomycin to isotonic media led to a transient translocation of cPLA$_2$ to the INM, and neither a cortical myosin II enrichment nor AA production was detected in this case (Figure 9.7 D-E). These observations are in agreement with the observations of Enyedi et al. [149], showing that cPLA$_2$ translocation only occurred at high levels of intracellular calcium.

Concluding, in our model system, the INM translocation of the fluorescently-tagged cPLA$_2$ reporter does not appear to be representative of its activity, which should be assessed via complementary methods that directly measure AA production such as Raman spectroscopy. In fact, our measurements on AA levels using Raman spectroscopy show a high correlation with the relative cortical myosin II accumulation in all of the conditions tested (Figure 11.5 C). This observation further supports a direct connection in between AA production and myosin II activity.
Figure 11.5: cPLA₂ is a tension reporter at high calcium levels (A) cPLA₂ translocation from the nucleoplasm to the INM for cells cultured in isotonic/hypotonic conditions or under 7μm confinement with or without the addition of ionomycin. The grey-scaled bar indicates the percentage of cells in which a cPLA₂ translocation was observed; colormap indicates the intensity and dynamics of the cPLA₂ translocation ratio, defined as the ratio in between the fluorescence intensity of cPLA₂-GFP at the INM and in the nucleoplasm. (B) Representative confocal images of non-polarized progenitor cells expressing cPLA₂-GFP cultured in isotonic/hypotonic conditions or for 7μm confinement with or without the addition of ionomycin. Representative nuclei with or without cPLA₂-INM translocation are shown in columns as indicated. Scale bars 10μm. (C) Cortical myosin II accumulation as a function of relative AA Raman score for all tested conditions. Both values are normalized to suspension (isotonic, control). The line indicates a linear regression, $R^2 = 0.68$ (considering only the last three points: $R^2 = 0.99$). Data points and bars indicate the mean and standard error of the mean (sem).

11.2 Nuclear stretch and nucleus positioning enable cellular proprioception

11.2.1 Confinement leads to ER immobilization

Our results showed that calcium levels are differentially regulated upon cell swelling versus cell confinement (Figure 11.3). A key morphological difference in between these two conditions is that under hypotonic shock, cells swell due to an influx of water and the cytosol gets diluted, so that different organelles or compartments are also positioned further away from each other. Upon cell squeezing, in contrast, both the cell and large intracellular organelles like the nucleus substantially deform along the axis of cell confinement, bringing the nucleus and surrounding organelles closer to the cell cortex-PM interface. As shown in Figure 11.6 A (and previously in Figure 8.8 B), under mechanical confinement the nucleus gets compressed to a height that is even smaller than the confinement height imposed on cells. This increase in nucleus compression is caused by cytoplasmic structures and organelles, such as the endoplasmic reticulum (ER) that surround the nucleus and which get physically compressed in between the
PM and the nucleus upon cell confinement. TIRF imaging of the ER in confined cells at the PM interface showed that ER structures indeed get immobilized in between the PM and the central region of the cell where the nucleus is located (Figure 11.6 B). In these immobilized regions the sheet-like structures of the ER appeared static compared to the dynamic arrangements of the ER outside the confined region of the nucleus and in non-compressed cells (zoomed view of Figure 11.6 B).

The ER is the main calcium storage in a cell and plays a major role in regulating calcium dynamics, together with ion channels in the PM and other organelles such as mitochondria and lysosomes [244]. Immobilization of the ER in between the nucleus and PM/cell cortex implies that the nucleus is pushing the ER towards the PM and can lead to a build-up of friction in between the ER and nuclear envelope. In agreement with this hypothesis, we could observe small residual INM folds in the lower and upper contact areas of the nucleus (Figure 11.6 C), while the INM appeared completely unfolded at the nuclear cross-section, as shown in Figure 9.2. These small folds did not disappear by de-polymerizing the actin cortex via addition of LatrunculinA, suggesting that remaining INM folds in the nucleus-ER/PM contact area were independent of the presence of the actin cortex, but are rather generated by the buildup of friction between the nuclear envelope and ER/PM interface (Figure 11.6 D). A quantification of the nucleus contact area in confined cells (with a value of 0 for cells in suspension or hypotonic conditions) further showed that intracellular calcium levels strongly correlated with nucleus compression and an associated increase in the nucleus-PM contact area (Figure 11.6 E).

### 11.2.2 Nucleus-PM proximity controls STIM-ORAI complex activity

Increasing cell confinement promotes the contact between the nucleus and the plasma membrane along with an immobilization of the ER underneath the nucleus (Figure 11.6 D). Given the central role of the ER in regulating calcium dynamics [244], we next addressed how the nucleus-PM proximity could control intracellular calcium under external force loads. We showed that stretch-activated ion channels and extracellular calcium did not play a major role in controlling cortical contractility under confinement (Figure 8.7), while intracellular calcium was essential for the activation of the cPLA₂ pathway (Figure 9.7). Furthermore, treatment with 2-APB completely inhibited cortical myosin II enrichment in confined cells (Figure 9.7 A). 2-APB is an inhibitor of the IP₃ receptor and of the STIM-ORAI complex. The IP₃ receptor, when active, triggers calcium release from the ER [111,245] and was also found to localized in nuclear envelope folds where it regulates nuclear calcium [151,152]. STIM and ORAI are two ion channels, with STIM proteins localized in the ER and ORAI in the PM. When the ER is depleted of calcium, the cytosolic part of STIM undergoes a conformational change that allows binding with the C-terminal (cytosolic domain) of ORAI [246]. The two proteins have cytoplasmic domains with a size of approx. 6nm (STIM) and 10–14nm (ORAI) and they need to physically interact with each other in order to bind and form a functional junction complex [247]. Hence the ER and the PM must be closer than 20nm for the two proteins to interact. Upon binding, ORAI gets immobilized and
the STIM-ORAI complex is formed, creating a channel that connects the extracellular space with the ER lumen [246], as exemplified in Figure 11.7 A.

We thus monitored labelled STIM and ORAI proteins in confined cells. As expected, STIM-CFP localized to the ER and ORAI-YFP to the PM of progenitor stem cells (Figure 11.7 B-C). At 13μm confinement, ORAI showed a homogeneous distribution at the basal PM and no ER immobilization was observed (Figure 11.7 B-D). Interestingly, at 7μm confinement, ORAI accumulated in the region where the ER was immobilized between the nucleus-PM contact area, as indicated by the magenta arrows in Figure 11.7 B-C and quantified by the normalised fluorescence intensity that shows an enrichment in the same area (Figure 11.7 D).

To directly measure ORAI immobilization, we performed Fluorescence Recovery After Photo-bleaching (FRAP) on the basal side of 7μm confined cells expressing ORAI-YFP. In particular, we distinguished different regions of interests (ROIs), including the area
11.2. Nuclear stretch and nucleus positioning enable cellular proprioception

Figure 11.7: Nucleus to plasma membrane proximity promotes the formation of the STIM-ORAI complex (A) Sketch of the active STIM-ORAI complex. (B) Representative confocal images of cells expressing STIM-CFP and ORAI-YFP at 13 – 7µm confinement. Cross-sections (top) and basal membrane sections (bottom) are shown. (C) Transversal views of cells expressing STIM-CFP and ORAI-YFP for 7µm confinement. In (A-B) magenta asterisks indicate the nucleus and the arrow points at the region of the basal membrane for 7µm confinement where ORAI-YFP accumulates (ER-PM contacts). (D) ORAI-YFP intensity at the basal membrane for cells at 13 – 7µm confinement. Each line corresponds to one cell (13µm: N=6, 7µm: N=14). Black lines represent mean values. (E) Exemplary confocal snapshots of a FRAP routine (pre-bleach, bleach and recovery images) performed at the basal membrane of a cell expressing ORAI-YFP under 7µm confinement at the indicated times. In the two recovery images the magenta ROIs corresponds to the region below the nucleus, where ORAI accumulates, and cellular blebs, as shown in Figure 11.7 E. Comparing normalised FRAP curves for these regions, we observed that the nuclear region showed a significantly lower fluorescence recovery over time than the areas in cell blebs (Figure 11.7 F), indicative of a higher fraction of immobile ORAI channel in regions underneath the confined nucleus. Fitting of the FRAP curves obtained in

below the nucleus, where ORAI accumulates, and cellular blebs, as shown in Figure 11.7 E. Comparing normalised FRAP curves for these regions, we observed that the nuclear region showed a significantly lower fluorescence recovery over time than the areas in cell blebs (Figure 11.7 F), indicative of a higher fraction of immobile ORAI channel in regions underneath the confined nucleus. Fitting of the FRAP curves obtained in
different cells with a mono-exponential function confirmed that the immobile fraction is higher below the nucleus (∼ 25%) than in the bleb (∼ 2%, Figure 11.7 F, top). However, the recovery times $\tau$ of the two exponential curves were comparable (Figure 11.7 G), suggesting similar diffusion rates of the mobile receptor fraction in the PM of the bleb and underneath the nucleus. This suggested that ORAI did not accumulate underneath the nucleus because of a lower diffusion coefficient, but rather by the physical immobilization due to its interaction with STIM.

To understand the role of nucleus to PM proximity and, in particular, of the STIM-ORAI complex, in regulating intracellular calcium levels we monitored intracellular calcium levels by the expression of the gCamp+NLS-dTomato reporter in progenitor cells. Imaging was performed on cells in suspension and under mechanical deformation in confinement, in control conditions and upon treatments with either the cPLA$_2$ inhibitor or 2-APB. Inhibition of cPLA$_2$ did not affect calcium dynamics, while 2-APB specifically reduced the nuclear-to-cytoplasmic calcium ratio (Figure 11.8 A-B). These results support that 1) cPLA$_2$ mechanotransduction and the regulation of intracellular calcium levels are independent processes and 2) that the STIM-ORAI complex, activated upon nucleus-to-plasma membrane proximity, has a specific role in regulating nuclear calcium levels. The first conclusion specifically proves that there is no feedback process through which cellular contractility (and the activation of the cPLA$_2$ mechanotransduction pathway) controls intracellular and nuclear calcium levels. These data further support that the formation of the STIM-ORAI complex directly controls nuclear calcium levels relevant for the mechanosensitive activation of cPLA$_2$ upon nucleus stretch in deformed cells.

Figure 11.8: The STIM-ORAI complex controls nuclear calcium levels (A) Normalised gCamp/NLS-dTomato mean fluorescence intensity (left) and nuclear/cytosolic gCamp intensity ratio (right) for cells co-expressing gCamp+NLS-dTomato in suspension or under 7µm confinement in control conditions or upon treatment with the cPLA$_2$ inhibitor Pyrrophenone or 2-APB (inhibitor of the STIM-ORAI complex). (B) Representative confocal images of cells expressing gCamp+NLS-dTomato for control condition in suspension and under 7µm confinement and upon treatment with Pyrrophenone or 2-APB under 7µm confinement. The dashed magenta lines delimit the nucleus. Scale bars 10µm. ** $P < 0.001$, n.s. not significant.
11.3 Conclusions

In order to understand if cells use the same mechanosensitive pathway to sense different types of shape changes, we applied confinement and hypotonic shock conditions that induce an anisotropic cell compression or isotropic cell swelling of progenitor stem cells. As expected, cells in hypotonic shock conditions rapidly increased their cellular and nuclear volume and as a consequence, an unfolding of the INM was observed (Figure 11.1). INM unfolding correlated with a rapid increase of cortical myosin II levels and AA production (Figure 11.2). However, even if inflated and confined cells had a comparable INM unfolding, cell contractility was markedly different. Inflated cells had a much lower myosin II accumulation at the cortex with respect to confined cells, resulting in almost no cell polarization events and associated absence of a stable-bleb migration switch compared to confinement conditions (Figure 11.2). We identified that this difference was explained by the lower intracellular and nuclear calcium levels observed under hypotonic shock in comparison to mechanical cell confinement (Figure 11.3). In fact, calcium was shown to be required for the binding of cPLA$_2$ to the INM [100] and can tune its activity. Ectopically increasing calcium levels in hypotonic media by treating cells with ionomycin rapidly boosted cortical myosin II levels and the fraction of stable-bleb polarized cells (Figure 11.4).

These data can be illustrated by summarizing all tested conditions in a graphical phase diagram: INM folding and intracellular calcium levels are indicated in the y- and x-axis, respectively, and the colour-coding of the cells relates to their contractility level, as shown in Figure 11.9. Applying gradual mechanical confinement corresponds to moving along the diagonal axis of this diagram (arrow 1) were both INM unfolding, calcium levels and myosin II activity increase by cell deformation in confinement. In contrast, applying hypotonic shock conditions corresponds to a vertical transformation (arrow 2) characterized by INM unfolding, while calcium levels remain similar to suspension cells. Increasing intracellular calcium levels by the addition of ionomycin, thus moving parallel to the x axis, has no effect on cells in isotonic media, while it allows inflated cells in hypotonic conditions to increase contractility levels and acquire migratory competence (arrow 3), leading to a motile transition in the top-right corner of the plot similar to deformed cells in confinement. The cells in this corner have an unfolded INM, high calcium levels and therefore high cell contractility and can transform to a stable-bleb migratory state, while the remaining cells in the phase diagram are blebbing and non-motile.

Our data demonstrate that intracellular calcium levels are regulated by nuclear positioning. Under strong confinement, the nucleus is deformed and the ER gets immobilized in the contact region between the nucleus and the PM (Figure 11.6). This nucleus-PM proximity fosters the formation of the STIM-ORAI channel complex (Figure 11.7) that controls intracellular and nuclear calcium levels (Figure 11.8) and, as a consequence, the activation of the cPLA$_2$ mechanotransduction pathway and cellular contractility. In hypotonic conditions, the nucleus is further away from the PM, hence the STIM-ORAI complex cannot be formed and cells have lower calcium levels that do not permit high cPLA$_2$/myosin II activation. In this way, nuclear positioning translates the information on the type of cell deformation into the regulation of intracellular calcium levels.
Figure 11.9: INM unfolding and intracellular calcium levels define a unique readout of cell shape deformation

Phase diagram depicting the various stress conditions tested, with respect to INM folding (y axis, blue gradient), intracellular calcium levels (x axis, magenta gradient), cellular contractility levels (yellow-blue color-scale) and cell behavior (blebbing versus motile).

The starting point of the diagram is a control suspension cell showing a folded INM, low calcium level and low contractility (bottom-left corner, close to axis origin). Applying mechanical confinement corresponds to a transformation that follows the diagonal axis (along arrow 1), with cell squeezing leading to INM unfolding, an increase in intracellular calcium levels and as a result an up-regulation of myosin II activity and its binding to the cell cortex.

With increasing cell deformation strength, cells can reach a high INM unfolding (darker cyan) and a high intracellular calcium level (brighter magenta), a regime in which cells can undergo spontaneous cell polarization and become motile with a stable-bleb amoeboid migration phenotype (top-right region marked by the red dashed line). Hypotonic swelling (arrow 2) induces INM unfolding (dark cyan) but at lower calcium levels (light pink), corresponding to a transformation where cells are only slightly more contractile and remain in a non-motile blebbing state (top-left region of the phase-diagram). Boosting calcium levels by treating cells with ionomycin (horizontal transformation, bottom part of the diagram) has no effect on cells in an isotonic condition (folded INM), but increases contractility upon cell swelling in hypotonic conditions (arrow 3) and induces rapid stable-bleb transformation, thus bringing the cell into the polarized-migration regime. The top-right corner of this diagram (dark purple shading) corresponds to the region where cells undergo a motile stable-bleb cell transformation, while cells are blebbing and non-motile in the rest of the diagram.

This additional level of cellular shape sensing enables cells to distinguish different types of cell deformations, even if they lead to a similar degree of INM unfolding, as in confinement and hypotonic swelling conditions.

Concluding, the combination of INM unfolding and nuclear positioning provides a unique readout of the degree and type of cell shape deformation, here exemplified by anisotropic confinement and isotropic swelling. This allows cells to feel their 3D shape with respect to their environment and, accordingly, respond to changes in their environment.
environment, by tuning myosin II activity and migratory cell dynamics. This process recalls the sense of proprioception in animals that corresponds to the awareness of body posture and movements with respect to the surrounding space. Similarly, single cells can be aware of different types of shape changes, by using their nucleus as force sensor, and adapt their dynamic behavior to their local microenvironment.
Part IV

Conclusions and discussions
Chapter 12

Conclusions and discussions

In this work we show that the nucleus, being the largest and stiffest organelle in a cell, not only functions as a physical container for the genomic DNA, but can act as an elastic mechanosensor that allows cells to sense their physical environment and shape changes. Furthermore, by being a hub for mechano-transduction signalling cascades, the nucleus further enables to control cell dynamics and adapt cortical contractility levels to mechanical changes in the local microenvironment. This, together with complementary studies [85, 94, 115, 149, 181] represents a paradigm-shift from a classical molecular biology point of view, in which the nucleus controls cellular processes by the activation of transcription and translation programs.

Through this thesis, we understood and described how single cells can sense 3D deformations of their shape imposed by physical constraints or mechanical challenges in their environment. This process was shown to occur similarly in other model systems and cell lines and therefore appears to represent a widely conserved mechanosensitive mechanism [181].

12.1 Cell deformation controls cellular contractility and amoeboid motility

A systematic and quantitative study allowed us to describe and understand how cells respond to different degrees of physical shape deformations. Progenitor stem cells derived from zebrafish embryos were cultured in different controlled microenvironments and their dynamic behavior and cortical contractility was quantified.

First of all, we observed that cellular contractility increased for large cell deformations, exerted by lowering the height in planar micro-confinement assays (Figure 8.1). Defined confinements were applied at discrete steps and we found that myosin II motor proteins progressively accumulated at the cortex when increasing cell deformation (Figure 8.1). This was not accompanied by a change in cortical actin density, supporting that myosin II was specifically activated and recruited to the cell cortex. Cells showed bigger blebs and a higher rate of stable-bleb transformation (Figure 8.1, 8.3), supporting an
increase in cortical contractility upon cell deformation in confinement. Notably, cortical myosin II accumulation upon confinement was stable over time thus the degree of cell deformation determined the set-point of cellular contractility (Figure 8.2). When the confinement was released, myosin II motor proteins rapidly re-localized to the cytosol of progenitor stem cells and contractility levels returned to baseline values (Figure 8.2). This mechanosensitive response at the cell cortex further controls cell dynamics and migration. In fact, it was previously shown that highly contractile cells spontaneously transform to stable-bleb cells, thus acquiring a polarized shape [54] (Figure 8.3). When myosin II is activated, local fluctuations in the cortex can rapidly induce cell polarization [54]. As a result, cells can transform to a stable-bleb phenotype and become migratory in confinement. Consistently with this, we observed higher fractions of stable-bleb migrating cells when confinement heights were lowered (Figure 8.3). Interestingly, the mechanoresponse of myosin II activation and stable-bleb transformation under confinement did not depend on cell fate specification or pre-existing migration programs (Figure 8.5). Confining mesoderm, endoderm or ectoderm cells in non-adhesive substrates showed that all cell types responded to mechanical confinement in a similar way. Additionally, confining mesoderm cells cultured on fibronectin, rapidly induced a switch from slow and collective mesenchymal migration to single cell amoeboid motility. All together, these data support that:

1. single cells can measure different degrees of shape deformation and thus physical constraints of their environment;
2. shape deformation in confinement defines the set-point of cortical contractility, which is stable over time while cells remain deformed in confinement;
3. cellular sensing and adaption to shape deformations occurs continuously, rapidly and reversibly;
4. increasing cell contractility upon cell shape deformation controls the migratory behavior of cells;
5. myosin II activation and stable-bleb transformation upon confinement do not depend on fate specification or pre-existing migration programs.

A direct physiological consequence of these conclusions is that cells can sense tissue crowding and tight physical space constraints and that they appear to be equipped with an "evasion reflex mechanism" that allows cells to move away from confined or crowded regions (Figure 8.4).

At the same time, this set of experiments puts forward the idea that cell deformation must be sensed by a non-dissipative element, conserved during early zebrafish development and among different species [104,105,181]. An elastic mechanosensor would explain all of the observed characteristics of the cellular mechanoresponse under confinement as it would enable a rapid, continuous and reversible cell response to shape deformations. The easiest example of an elastic behavior is a spring, for which the force scales with the deformation and no dissipation occurs. Therefore, the mechanosensitive element that mediates myosin II activation under confinement should "behave like" a spring. This is particularly intriguing because biological tissues
and cells are typically described as viscoelastic elements and therefore have dissipative components [77, 184, 186, 202, 205, 207].

**Looking for a mechanosensor**

To identify the possible mechanosensitive pathway that mediates myosin II recruitment at the cortex upon cell shape deformation, we performed an image-based screening approach. We probed and interfered with various known mechanosensors and signalling pathways and quantified cortical myosin II levels in suspension and confined cells. By doing so, we concluded that stretch-activated ion channels or adhesion complexes (cell-cell and cell-substrate) were not involved in the process (Figure 8.6, 8.7). Also, myosin II activation upon confinement was not transcriptionally regulated or dependent on the pro-apoptotic activity of caspases (Figure 8.6).

Moreover, by confining cells at different heights we observed cells increased their contractility only below 13 µm confinement (Figure 8.1). By quantifying cell and nucleus deformation for variable confinement heights, we observed that this threshold correlated specifically with nucleus deformation. For the larger confinement heights tested (18 – 16 µm), cells got deformed, as observed by the increase in cellular diameter, while no increase in cortical contractility was observed. Instead, myosin II motor proteins were recruited at the cortex only below 13 µm, a height at which the cell nucleus started to be deformed as observed by the increase in its diameter (Figure 8.8). To further assess the relationship in between myosin II activation upon confinement and nucleus/cell deformation, we confined cells of variable sizes dissociated from different developmental stages. Notably, an increased cell deformation for larger cells at a similar confinement height did not correlate with higher cortical myosin II enrichment (Figure 8.9). Instead we identified a correlation with the deformation of the nucleus, measured as the relative increase in nuclear diameter in suspension versus confinement conditions (Figure 8.9).

Indeed, nucleus integrity appeared to be essential to recruit myosin II motor proteins to the cell cortex. Mitotic cells did not accumulate myosin II at the cortex when confined (Figure 8.10). Moreover, when a cell entered mitosis, myosin II cortical levels instantaneously dropped in association with nuclear envelope breakdown. In turn, when a cell completed division and the two daughter nuclei formed, high myosin II levels rapidly re-appeared at the cell cortex. However, cell division in confinement often led to the appearance of bi-nucleated (polarized or non-polarized) cells as the hyper-activation of myosin II compromised the formation of the mitotic cleavage furrow, as previously documented [248]. This dynamical control of myosin II activity further supported that cells were continuously sensing shape changes and were able to adapt their contractility level to the sensed deformation. To do so, the integrity of the nuclear envelope was required. To increase the fraction of mitotic cells with a disassembled nuclear envelope, we depolymerized microtubules by incubating cells with Nocodazole (Figure 8.10). Mitotic cells treated with Nocodazole were also not capable of recruiting myosin II at the cortex when confined, in contrast to Nocodazole-treatment interphase cells. However, Nocodazole-treated mitotic cells were able to activate myosin II and increase cortical contractility when treated with LPA, supporting that myosin II motor proteins could bind to the cell cortex upon microtubules depolymerization in mitotic
12.2 A nuclear ruler pathway allows cells to sense physical shape changes

To investigate how nuclear deformation controls myosin II activity, we imaged the nuclear envelope of suspension and confined cells by looking at the INM and ONM of progenitor stem cells. This, led to the observation that embryonic progenitor cells have several type II invaginations, containing both INM and ONM folds that are visible in the cross-section of cell nuclei (Figure 9.1). Sometimes, tubular structures that cross the entire nucleus in its transversal direction were also visible (Figure 9.1). Interestingly, upon mechanical confinement the nuclear envelope appeared smoother with an unfolded INM (Figure 9.1, 9.2).

This observation opened up various questions about the structure of the nuclear envelope and its connection with the ER. It is well known that INM and ONM are continuous and merge along nuclear pore complexes (that pinch the NE membranes) and that the ONM is continuous with the ER [120, 154]. If all three membranes are continuous, the INM should be connected to an infinite pool of membrane reservoir. When the nucleus is deformed, lipids could flow from the ER, to the ONM and then to the INM.
to prevent or equilibrate its stretching [154]. However, this was not happening in our system or similar studies [181], nor in previously shown studies reporting INM unfolding [149, 168], or related to nuclear pore opening upon nuclear deformation [93]. These studies along with our data suggested that the nuclear envelope can get under tension, suggesting that there must be a diffusion barrier in between these three membrane compartments that prevent lipid flow upon stress. At the same time, lipids are known to be able to diffuse from the ER, to the ONM and then to the INM as during lipid synthesis [154]. How this diffusion is controlled in standard conditions or under stress and, as a consequence, how the lipid composition of the nuclear envelope is regulated are still poorly understood [154, 155, 250, 251].

The fact that confinement induces an unfolding of the INM is what allows cells to sense shape deformation. The unfolding appears to be continuous by confining cells at heights lower than $13\mu m$, a threshold below which myosin II accumulation scales with nucleus deformation, and stable in time under confinement (Figure 9.2). INM unfolding was shown to trigger the activation of cPLA$_2$, as previously studied in the context of inflammatory responses [149, 168]. In our system, cPLA$_2$ allows cells to sense large shape deformation, controlling myosin II activity and amoeboid motility in a cell autonomous way (Figure 9.3). Chemical inhibition of cPLA$_2$ using Pyrrophenone completely inhibited the mechanoresponse upon confinement for different confinement heights at various developmental stages, independently of fate specification (Figure 9.3).

Furthermore, we showed that cPLA$_2$ activates a mechano-transduction pathway that mediates myosin II accumulation at the cortex. We directly validated its function by using a dominant negative cPLA$_2$ construct and performing a morpholino interference and a rescue experiment (Figure 9.4). We confirmed that the protein is encoded by the $pla2g4aa$ gene, consistent with [168]. However, the genetic interference approaches did not lead to a complete inhibition of myosin II accumulation in confined cells. The dominant negative construct competes with the endogenous protein and therefore residual myosin II activation can be observed. On the other hand, morpholino interference prevents mRNA splicing and transcription, thus it does not target proteins that are already present in the embryo before zygotic transcription starts. This represents the maternal protein contribution, that appears to be comparable with the protein levels present in the embryo during early blastula stages, as validated by PCR experiments (see Appendix A.1). However, the expression levels of cPLA$_2$ were generally very low in early zebrafish development, complicating its direct detection by protein based methods as western blots.

Our data showed that cPLA$_2$ localizes to the nucleus of progenitor stem cells, independently of the various constructs that we generated (different fluorophore tags and linker sequences) or of intracellular calcium levels (Figure 9.5), consistent with [149]. Upon cell shape deformation in confinement, cPLA$_2$-eGFP translocates to the INM only in the 2% of the cells, while in the rest its localization remained nucleoplasmic. This observation is not in agreement with various studies according to which the protein’s localization is representative of its activity [149, 169, 170, 228]. The much lower (few orders of magnitude) expression levels at the early stages of development with respect
to the stages studied in Enyedi et al. [149], as reported for mRNA and protein activity quantification [172], could explain this inconsistency. In fact, expressing a fluorescent construct might saturate the endogenous levels at early stages and does not allow to observe the endogenous protein dynamics. This problem was overcome in HeLa cells by performing fluorescence-activated cell sorting (FACS) to select cells with an optimal expression level of the fluorescent construct [181]. In zebrafish this problem could have been solved by expressing the cPLA\(_{2}\)-GFP in the morphant background, to avoid the over-expression of the protein. However, probably because of the partial effect of the morpholino and of the very low protein levels at blastula stages, this approach did not change the outcome. At the same time, various trials of rescue experiments with the fluorescently-tagged reporter failed, suggesting that the catalytic activity of these proteins might be partially or fully affected. However, as the experiments presented through this thesis showed, label-free approaches such as Raman spectroscopy have a higher specificity and sensitivity in quantifying cPLA\(_{2}\) activity compared to monitoring the protein localization (Figure 9.5).

The specific localization of cPLA\(_{2}\) in suspension cells as well as in live embryos also opens multiple questions. As its name indicates, the protein was initially described to be cytosolic and to translocate to the perinuclear space and ER membranes upon activation [169,170,228]. Various studies now showed that cPLA\(_{2}\) localizes to the nucleus in different types of non-stimulated cells, as in adult zebrafish tissues [149], in non-confined HeLa cells [181] and in our study. How cells control the nucleus-to-cytosolic localization of cPLA\(_{2}\) and why different cell lines differentially regulate protein localization is still open questions. In particular, it would be interesting to understand if protein localization is controlled genetically or mechanically (e.g. by nuclear pore opening and active import upon nuclear envelope stretch, as shown for YAP [94]) and whether protein localization can tune mechanosensitive cell responses.

Given the key role of cPLA\(_{2}\) in mediating myosin II activation, we then investigated in more detail the cPLA\(_{2}\) mechanotransduction pathway and how myosin II is activated. From these experiments we concluded the following:

1. cPLA\(_{2}\) is required in the nucleus of progenitor stem cells, validated by a cPLA\(_{2}\)-NES construct that was injected on a cPLA\(_{2}\) morphant background and combined with Leptomycin B, an inhibitor of nuclear export, to tune its localization. We observed a full rescue of cortical myosin II levels in confined cells only when the cPLA\(_{2}\)-NES construct localized in the nucleus upon Leptomycin B treatment (Figure 9.5).

2. When active, cPLA\(_{2}\) produces AA that is released into the cytosol (Figure 9.5). We specifically detected a cPLA\(_{2}\)-dependent increase of AA in the cytosol of confined cells using Raman spectroscopy.

3. AA oxidation or the activity of COX and LOX pathways, involved in the downstream production of pro-inflammatory cytokines and chemo-attractants as eicosanoids, are not involved in mediating myosin II activation downstream of AA (Figure 9.6). Myosin II accumulation upon confinement was a cell-autonomous process, not mediated by an autocrine or paracrine pathway (Figure 9.6).
4. The Rho/ROCK pathway mediates myosin II activation downstream of cPLA$_2$ (Figure 9.6). Interference with RhoA using a dominant-negative construct or with ROCK using Y-27632, prevented myosin II accumulation at the cortex of confined cells. The MLCK and MEK1b/ERK do not appear to be involved. Also, using a RhoA-FRET sensor showed that RhoA is activated in confined cells and that the activation depends on cPLA$_2$ activity.

5. As cPLA$_2$ is a calcium-dependent lipase, calcium is needed for its activity, intracellular calcium (but not extracellular calcium) is necessary to mediate myosin II activation (Figure 9.7). However, myosin II is functional upon calcium depletion or cPLA$_2$ inhibition and increasing intracellular calcium is not sufficient to activate myosin II (Figure 9.7).

6. The inhibition of cPLA$_2$ activity or increase in contractility upon confinement does not affect INM unfolding (Figure 9.8). This excludes the presence of feedback loops or cross-talk between nuclear envelope architecture and the cPLA$_2$ mechanotransduction pathway. Moreover, when confined cells divide, we observed that daughter cells have an unfolded INM (Figure 9.8). This suggests that the folding state of the INM is imposed by the confinement and that cells are not capable of adapting their nuclear envelope state over the time scales assessed in our experiments (approx. 1 hour).

This last observation opened up relevant questions regarding the physical structure and the regulation of the nuclear envelope and specifically the INM. It is not yet fully understood how the nuclear envelope assembled under mechanical stress, what limits the amount of INM in confined cells and how is lipid homeostasis maintained. Given the fundamental importance of the INM as a signalling platform within the cell, future studies will be required to address the structural and dynamic regulation of the nuclear envelope under mechanical stress conditions.

In addition, knocking out the pla2g4aa gene that encodes for cPLA$_2$ using CRISPR-Cas9 induced a compensatory mechanism in KO embryos. Unexpectedly, cells derived from KO embryos were capable of transforming to the stable-bleb phenotype when confined and this process was inhibited by treatment with the cPLA$_2$ inhibitor Pyrrophenone (Figure 9.10). As suggested by previous studies [241, 242], we observed that the KO embryos had higher expression levels of gene paralogs and we specifically identified four genes encoding for lipases capable of releasing AA acid from membrane phospholipids that are calcium-independent (Figure 9.10). Consistently, stable-bleb transformation in KO-cells was not affected by chelating intracellular calcium with BAPTA-AM (Figure 9.10). Still, it is not clear why the genetic compensation occurs for specific genes and how the process is regulated. It is intriguing to speculate that cPLA$_2$ is essential for early zebrafish embryogenesis and this is why the genetic compensation mechanism is triggered. Moreover, some of the genes that are over-expressed in KO embryos, such as pla2g6 and pla2g15, are also expressed in WT. Therefore, the calcium-independent proteins encoded by these genes could contribute to AA production and myosin II activation in WT cells as well and also explain the partial reduction of the mechanosensitive cell response via the morpholino approach. Their possible roles and/or differential regulation in WT embryos still remains to be investigated.
Concluding, we showed that cPLA$_2$ is the mechanosensitive element in the nucleus that allows cells to measure shape changes. cPLA$_2$ senses INM unfolding upon nuclear deformation and translates this information in a calcium-dependent manner into AA production, which regulates myosin II activity via the Rho/ROCK pathway. This mechanotransduction pathway further controls cell behavior and amoeboid motility under confinement. These data highlight that the nucleus is not only a static and large organelle, that represents a physical obstacle or hindrance for cells during migration [72,86], but that the nucleus also has an active role in regulating cell dynamics. It was recently shown that the nucleus acts as a ruler that allows immune cells to chose the migration path of least resistance preventing large nucleus deformations [85], or that it can function as a piston to help cell protrusion growth in dense 3D environments [84]. Our study extends these findings and propose a new role of nucleus as an active cellular force sensor that actively controls cell behaviour and migration plasticity. Importantly, the same nuclear rule pathway is conserved in other cell lines such as HeLa or immune cells [181] or human melanoma cells [182]. Cancer cells, in particular, often show high levels of cPLA$_2$ expression that correlate with elevated migration and invasion capacity [170,178]. High levels of Rho/ROCK activity are also observed at the invasive front of cancer [53] and nuclear mechanics control nuclear plasticity to enable migration in confined spaces [89]. If cPLA$_2$ plays a role in cancer cell migration and metastasis in vivo is therefore a direct relevant question to address. Moreover, recent studies suggested that INM stretch induces cPLA$_2$ activation also upon nuclear swelling during mitotic entry in RPE-1 cells [183]. In this work, the authors showed a correlation in between nuclear envelope swelling, cPLA$_2$ recruitment at the INM and translocation of specific factors (in this case cyclin B1) from the cytosol to the nucleus [183]. It would be indeed fascinating to understand how NE tension controls both the cPLA$_2$ mechanotransduction pathway and nuclear pore opening [94,159] and their potential cross-talk as a regulator of nuclear import/export of specific proteins and of cPLA$_2$ itself.

It is also noteworthy that the cPLA$_2$ pathway is conserved across very different cell lines, that have diverse NE composition and thus distinct nuclear mechanics. In fact, NE composition is crucial for a cell to respond to mechanical forces. Changing Lamin A/B levels in HeLa cells [181] and down-regulating or over-expressing emerin in melanoma cells [182] alters the cPLA$_2$ pathway. How nuclear mechanics and mechanosensitivity are coupled among various tissues and cell lines is still not known.

### 12.3 Nuclear mechanics

In order to characterize nuclear mechanics in our model system, we developed a new method based on optical tweezers [209], described in Chapter 10. By injecting 1$\mu$m beads into 1-cell stage embryos and dissociating progenitor stem cells at blastula stage, we could perform intracellular force measurements in live cells by manipulating single beads within cells and recording the associated trap forces. In this way, we can specifically deform the nucleus in both suspension and confinement (Figure 10.1). Multiple indentations showed that the nucleus is stiffer than the cytosol, as a higher force was necessary to deform it. It still needs to be investigated further if energy dissipated
observed during each indentation cycle changed over time, indicative of viscoelastic changes of the nucleus upon mechanical stimulation. The main limitation of our measurement approach was nuclear movement observed during the indentation routines. This could be overcome by using a second trap on the distal side opposite of the indenting trap, to hold the nucleus in place. At the same time, this second trap would provide the information on the force transmitted through the nucleus while indenting it.

By performing stress-relaxation routines in both suspension and confinement conditions we observed that the viscoelastic properties of the nucleus did not change upon cell deformation in confinement (Figure 10.4). Moreover, combining this experimental approach with active microrheology (Figure 10.5) showed that the nucleus behaves like a viscoelastic solid, which is stiffer than the cytosol at time scales larger than $\tau_{\text{Nucl}} \sim 0.1s$ (Figure 10.6). This time scale is an important parameter to be considered when applying shape deformations to single cells and studying their mechanical response. It would be certainly intriguing to be able to confine cells at different velocities and to understand the interconnection in between nuclear mechanics and the activation of mechano-transduction pathways. It is also important to consider that these rheological routines were performed in the cytoplasm, away from the nucleus (via parallel oscillations) and directly at the nuclear envelope that was gently indented (via perpendicular oscillations). Ideally it would further be interesting to perform active micro-rheology in the cytoplasm and directly inside the nucleus, but the required experimental implementation is not straightforward as bead injections into the nucleus are difficult to achieve. Also, the small oscillation amplitudes and low laser powers required by this approach prevent a large indentation of the nucleus and only a probing of the outermost surface was possible. This might also lead to a contribution of cytoplasmic mechanics (mainly ER and perinuclear structures) to the measured mechanical response and a more detailed modelling of these measurements would be required. However, overall this method represents a promising approach to quantitatively characterize the cytoplasm and nucleus of both suspended and confined cells in a frequency-dependent manner to fully understand their mechanics. Future applications to study the mechanics of diverse intracellular structures in live cells can also be envisioned. It would therefore be interesting to extend this method in order to unveil nuclear mechanics in different cell lines or model organism, both under normal and mechanical stress conditions. By doing so, we could understand better why different cell types have diverse nuclear shapes and mechanics and how intracellular mechanics, in general, impact on (or control) cellular mechanosensation and -transduction and force-dependent adaptions in cells.

12.4 Sensing different forces

Single cells in their 3D microenvironment can be subjected to different kinds of shape changes as various physical forces act on them. Confinement mimics anisotropic cell deformations that can occur because of crowding or as cells migrate in tight tissue spaces. Isotropic stretching, in contrast, can happen during cell swelling as a consequence of a tissue damage when cells are exposed to hypotonic conditions [149,168]. This was also the biological context in which cPLA$_2$ was found to act as a mechano-transducer of
inflammation. In order to understand if progenitor stem cells could sense different types of shape deformations through the same mechanotransduction pathway, we decided to induce cell swelling by hypotonic shock. Inflated cells rapidly increased their cell and nuclear area after the hypotonic shock was applied (Figure 11.1). As a consequence, their INM unfolded (Figure 11.1) and myosin II motor proteins rapidly activated and were recruited at the cortex, leading to an increase in cell contractility (Figure 11.2). However, cell contractility did not reach the same levels as those measured in confined cells, along with a low fraction of cell polarization that induced a stable-bleb phenotype (Figure 11.2). The process appeared to be mediated by cPLA\(_2\) since AA was produced in hypotonic conditions in a cPLA\(_2\) dependent manner (Figure 11.2). This discrepancy was explained by the fact that intracellular calcium levels were differentially regulated upon cell swelling versus confinement, with significantly lower intracellular calcium levels in hypotonic conditions compared to cells in confinement (Figure 11.3). Consistently, increasing calcium levels by adding ionomycin in hypotonic conditions, but not in isotonic media, induced a rapid increase of cortical myosin II and led to a high fraction of polarized stable-bleb cells (Figure 11.4). These cells were migratory if cultured in confined environments or \textit{in vivo}, as stable-bleb cells are non-motile in suspension but require a confined microenvironment to transmit traction forces for movement [54,66].

These data support that calcium has a permissive role on the cPLA\(_2\) pathway and varying calcium levels can tune cellular dynamics even at comparable INM unfolding. The exact role of calcium for cPLA\(_2\) activation is not fully understood in previous studies. It has been reported that calcium is sufficient to induce cPLA\(_2\) translocation to the nuclear envelope and protein translocation was used as a readout of its activity [170, 176, 228, 229]. In our model system, calcium can induce a temporary translocation of cPLA\(_2\) to the INM but this transient re-localization did not correlate with AA production or a change in myosin II activity (Figure 11.5). In fact, we found that INM stretch is required to generate a stable protein translocation over time (Figure 11.5). The fraction of cells showing cPLA\(_2\)-INM translocation increased by adding ionomycin, but in any case the re-localization of our probe did not correlate with AA production or myosin II activity. Therefore we suggest that a direct measurement of AA production will yield a more robust readout of lipase activity. More studies are demanded to understand the mechanisms of cPLA\(_2\) activity and its mechanosensitive localization in different cell types. Notably, our data suggest that cPLA\(_2\)-GFP can be used as a INM tension reporter at high calcium levels.

Another open question concerns the specific mechanism how myosin II is recruited at the cortex upon hypotonic cell swelling. Inhibition of cPLA\(_2\) reduced the increase of cortical myosin II levels by hypotonic shock and ionomycin. When cells were cultured in hypotonic media, treatment with Pyrrophenone reduced AA production but did not completely block the increase in contractility upon cell swelling (Figure 11.4). Given the mechanosensitive role of myosin II binding on actin [16,29], we could speculate that mechanical stretching of the actomyosin cortex leads to a cooperative binding process of myosin II motor proteins at the cortex. Still, how this cortical enrichment is maintained over time while the cortex undergoes rapid turnover is still not clear.
Nucleus positioning controls calcium levels

The differential regulation of intracellular calcium levels explains why inflated cells in hypotonic conditions have lower contractility than confined cells. But how are calcium levels differentially regulated in these two conditions? An obvious difference in between cell swelling and cell compression in confinement is that cells in hypotonic media get larger and their cytoplasmic content is diluted, leading to a higher distance between the nucleus and plasma membrane. In confinement, however, the nucleus appears squeezed and the ER gets squeezed in between the nucleus and the PM (Figure 11.6). Our data showed that the PM/ER membranes and nuclear envelope are in close proximity and friction originates underneath the nucleus, leading to residual INM folds in the basal membrane and to ER immobilization (Figure 11.6). In this central region, we observed the recruitment of the STIM-ORAI complex, with ORAI in the PM and STIM in the ER (Figure 11.7). The STIM-ORAI complex forms a double ion channel that connects the extracellular space with the ER lumen when they are in close proximity. Indeed, ORAI gets immobilized in the central region of the cell, as shown by the increased fraction of immobile receptors measured by FRAP (Figure 11.7). Inhibition of the STIM-ORAI complex by using 2-APB inhibited intracellular and nuclear calcium and myosin II activity (Figure 11.8, 9.7).

The region of ORAI immobilization was larger than the cross-section of nucleus, as visible by the side view images of Figure 11.7. We observed that the nucleus moved during cell blebbing and we speculate that the nucleus behaves like a piston that can squeeze the ER closer to the PM. This might induce the formation of the STIM-ORAI complex even when not directly located underneath the nucleus. Also, the formation of the STIM-ORAI complex requires the activation of STIM upon ER calcium depletion [246,247]. Indeed, in confined cells, the ER appeared darker than the cytoplasm or the nucleus when monitoring the signal of different calcium reporters in confined cells (Figure 11.3). Also, 2-APB that was used to interfere with the function of the STIM-ORAI complex, can also inhibit IP3 receptors that might play a role in controlling nuclear calcium levels and nuclear envelope folding [147,151–153]. Hence, it would be interesting to quantify intracellular calcium levels in an organelle-specific way to understand how calcium is regulated within various cellular compartments and identify the main players involved in this process.

Concluding, each cell can feel its own 3D shape and dynamic shape deformations by combining information on INM unfolding and nuclear positioning. These two parameters control cellular contractility and cell behavior via the cPLA₂ mechanotransduction pathway. In this way, cells can dynamically and reversibly adapt their behavior to changes in their local microenvironment and, for example, become motile as a result of anisotropic deformation in confined environments, but not upon hypotonic swelling during tissue damage. This process recalls the sense of proprioception in animals that corresponds to the awareness of body posture and movements with respect to the surrounding space, achieved thanks to proprioceptive neurons along the entire body. Similarly, single cells can be aware of different types of shape changes, by using their nucleus as a deformation sensor that integrates information on nucleus stretch and positioning to adaptively regulate dynamic cell behavior. This process, summarized in
Figure 12.1, establishes a new role for the nucleus as a module for cellular proprioception and controller of migration plasticity.

**Figure 12.1:** The nucleus measures shape changes for cellular proprioception to control dynamic cell behavior. The nucleus allows cells to sense and distinguish anisotropic deformation in confinement or isotropic swelling in hypotonic conditions. This is achieved through the mechanosensitive activity of cPLA\(_2\) that, in the presence of high calcium, senses INM unfolding. When active, cPLA\(_2\) releases AA from the INM into the cytosol. This further controls myosin II activity and cell behavior as highly contractile cells spontaneously become motile and acquire and amoeboid stable-bleb phenotype. Moreover, nuclear positioning within the cell controls intracellular and nuclear calcium levels via nucleus-PM proximity, which tunes cPLA\(_2\) activity. This further leads to a different cell response regarding myosin II activity and dynamic cell behavior in confinement versus cell swelling in hypotonic conditions. All together, these data establish the nucleus as a module for cellular proprioception.
Appendix

A.1 cPLA$_2$ sequence, expression, constructs and KO line

All the molecular biology experiments presented here have been performed by S. Jiménez-Delgado and F. Pezzano (CRG).

As a validation of the results discussed in this thesis, we decided to study in more detail the sequence and functioning of cPLA$_2$ and to characterize its expression levels and localization during early zebrafish development. The protein cPLA$_2$ refers to the 85kDa cytosolic phospholipase A2 group IVA or cPLA$_2$$\alpha$. In zebrafish, the protein is encoded by the genes pla2g4aa and pla2g4ab (because of a genome duplication). The first gene was also identified to mediate leukocyte recruitment at wounding sites in zebrafish [168]. The two genes show very low expression levels in general (in comparison with proteins like myosin II, VastDB database). However, the pla2g4aa gene appears to be more expressed than pla2g4ab. Also, the pla2g4aa gene is expressed at different developmental stages, in almost all the tissues in the adult and its expression increases during development [172]. Instead, pla2g4ab is specifically expressed in the heart and testicles only (VastDB). Using specific morpholinos to interfere with both genes, as presented in Chapter 9.2, supported that the identified mechanosensitive protein is encoded by pla2g4aa. The protein sequence contains a C2-domain, the phospholipid binding region, and a catalytic domain (Figure A.1).

We used semi-quantitative PCR and quantitative-PCR (qPCR) to measure the relative expression levels of the cPLA$_2$ DNA during zebrafish development, as shown in Figure A.2 A-B. For the qPCR we used the following primers: cPLA2-qF2: CATCAACCGCTACGTCAAA; cPLA2-qR2 TGTCCATTCTCCCTGGGAATA. Indeed, we found low expression levels at early stages and comparable levels of maternal contribution. At the same time, we designed a probe for in situ hybridization that recognizes the 3'UTR region of the RNA (Figure A.1) to observe its endogenous localization. At 64 cell stage, it appeared to be homogeneously localized in the cell cap, while at sphere we found a higher intensity in the inner cell mass (deep cells) and low expression in the outer surface (EVL) of the embryo (Figure A.2 C).
cPLA₂ localization in the cell  Considering its name, cytosolic cPLA₂ was described to localize to the cytosol of different cell types and to translocate to the nucleus or ER and Golgi membranes upon calcium stimulation [228]. In other cell types and model systems, however, such as zebrafish and HeLa cells, cPLA₂ was also found in the nucleus. Its localization and the mechanisms regulating cytoplasmic versus nucleoplasmic localization are not yet fully understood [170]. Calcium is a main factor known to trigger cPLA₂ translocation from the cytosol to perinuclear regions, however, the protein also has a high affinity for PIP₂ (lipid second messenger phosphatidylinositol-4,5- bisphosphate)-rich membranes even at low calcium levels [170]. An increase of intracellular PIP₂ was shown to induce AA release via cPLA₂ in a calcium-independent way [169]. Recent studies supported a role for a kinase-dependent phosphorylation (in specific Serines) of cPLA₂ by MAPK/ERK and PKC in regulating its localization and activity [169,170,229]. More specific analysis of the human cPLA₂ amino-acid sequence led to the identification of two putative nuclear localization signals (NLS) and a putative nuclear export signal (NES) [229]. The same regions could be identified in zebrafish by aligning the two amino-acids sequences. The two potential NLSs are one in the C2-domain (position 54 – 60, PDSRKRT) and the other in the PLA2-domain (position 269 – 283, PQKV KRYVESLWKKK). The putative NES sequence is in the PLA2-domain (position 552 – 562, LTF NLPY PLIL). Also, a cPLA₂ activating protein (PLAA) was found to be highly expressed during blastula zebrafish stages (VastDB database) and responsible to tune the activity of cPLA₂ and other lipases in macrophages [252]. These results show a complex framework in which cPLA₂ localization can be dynamically regulated in development or in a tissue-dependent manner by various factors and proteins.
Figure A.2: cPLA$_2$ expression and localization during early zebrafish development (A) cPLA$_2$ expression by semi-quantitative PCR. cDNA concentration was 50ng/$\mu$l per tube and 40 PCR cycles were used. (B) Relative cPLA$_2$ expression by q-PCR, normalised by eef$_{1\alpha}$. Results expressed in percentage. cDNA concentration was 8ng/$\mu$l per well. (C) Whole mount in situ hybridization at 64 cells stage (2 hpf) and sphere stage (4 hpf).

Dominant-negative cPLA$_2$ Two different mutations that affect cPLA$_2$ activity have previously been reported for the human protein [227]. Exchanging a Serine (S) in position 111 with a Proline (P) led to a mutant (named S111P) that could not bind calcium or translocate to the INM, while its catalytic activity was maintained. Exchanging an Arginine (R) in position 478 with a Histidine (H) generated an inactive enzyme (mutant R478H) with normal membrane binding capabilities but no catalytic activity. In order to design a zebrafish-specific dominant negative construct, we aligned the two sequences and found that the Serine (S) of position 111 in human was not present in zebrafish, but both sequences had a Serine in position 110 (corresponding to position 104 of the zebrafish gene) and the Arginine (R) in position 485 (corresponding to position 478 of the zebrafish gene) was aligned with another R. Thus, we designed a double-mutated mRNA encoding the $pla2g4aa$ zebrafish gene with S104P and a R478H mutations. To do so, we defined six primers and generated the construct using a Gibson cloning system to merge the three fragments together.
A.2. Constructs

### Primers

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**cPLA$_2$-KO line** To generate a stable cPLA$_2$ knock-out line using CRISPR-Cas9, 1-cell stage (zygote) embryos were injected with the guide RNA (gRNA) specific for the $pla2g4aa$ gene together with the Cas9 protein (TrueCut Cas9 Protein v2, Thermo Scientific, A36497). To synthesize the gRNA, we first annealed the specific primer with the T7-universal primer (which contains the T7 RNA polymerase promoter) and then we filled in with T4 DNA polymerase. RNA synthesis was then performed with the KIT MAXI-script (Invitrogen). cPLA$_2$ guide: GAAATTAATACGACTCATTATAG

AGTGGTGTCGCCCATGATGG

GTTTTAGAGCTAGAAATAGCAAG (the bold part represents the specific sequence recognising the $pla2g4aa$ gene); T7-universal primer: AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCT-TATTTTAACTTGCTATTTTCTAGCTCTAAAAC.

### A.2 Constructs

Construct cloning and mRNA preparation was done by S. Jiménez-Delgado and F. Pezzano (CRG).

The following constructs were subcloned in a pCS2+ or pCS2+-eGFP vector, linearized with BamH1-EcoR1 restriction enzymes, using the Gibson cloning system: LAP2$_\beta$-eGFP was amplified from a pME 185-FL3 vector (clone 2643665, Dharmacon); cPLA$_2$$_\alpha$ ($pla2g4aa$) plasmid was amplified from a PCR4-TOPO vector (clone 9037889, Dharmacon). NES-cPLA$_2$$_\alpha$-eGFP was amplified from the pCS2+-cPLA$_2$$_\alpha$-eGFP vector using primers encoding for an N-terminal NES sequence (LPPLERLTL). cPLA$_2$-linker-eGFP was amplified from the pCS2+-cPLA2-coGFP vector using primers encoding a linker sequence (GHGTGSTGSGSS) between the cPLA2 and eGFP coding sequences. Human STIM and Orai were subcloned from mCFP-N1 and mYFP-N1 plasmids (provided from the lab of M. A. Valverde, UPF), respectively, into a pCS2+ vector and linearized with EcoRI-XbaI restriction enzymes. All cDNAs were amplified using Phusion HF DNA Polymerase (Thermofisher F530S), see primers below. The following constructs were provided from the following labs: pCS2-DNRhoA N19 (courtesy S. Schneider-Maunoury); pCS2+cyclops [253]; pCS2+-lefty, Casanova and H2A-mCherry (courtesy C. P. Heisenberg); pCS2+ Lyn-TdTomato (courtesy B. Alsina); pCS2+ gCamp-NLS dTomato (courtesy A. Pauli); pTriEx-RhoA FLARE.sc Biosensor WT was a gift from Klaus Hahn (Addgene plasmid 12150; RRID:Addgene-12150). The oligonucleotides used for cloning are shown in the following table:
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Glossary

NM-II  non-muscle myosin II
RLC  regulatory light chains
ROCK  Rho-associated protein kinase
MLCK  Myosin light chain kinase
MYPT1  myosin light chain phosphatase
BTFs  bipolar thick filaments
GEFs  guanine nucleotide exchange factors
GAPs  GTPase-activating proteins
GDIs  GDP dissociation inhibitors
Rho  Ras homology
PM  plasma membrane
LPA  Lysophosphatidic Acid
ECM  extracellular matrix
PDMS  polydimethylsiloxane
NE  nuclear envelope
MAT  mesenchyma-to-amoeboid transition
EVL  enveloping cell layer
FAs  focal adhesions
YAP  Yes-associated protein
ONM  outer nuclear membrane
ER  endoplasmic reticulum
INM  inner nuclear membrane
NPCs  nuclear pore complexes
NR  nucleoplasmic reticulum
IP3  inositol trisphosphate
LINC  linker of nucleus and cytoskeleton
cPLA2  cytosolic phospholipase A2
AA  arachidonic acid
PC  phosphatidylcholine
COX  cyclooxygenase
LOX  lipoxygenase
AFM  Atomic Force Microscopy
OT  Optical Tweezers
ε  strain
σ  stress
G(t)  relaxation modulus
J(t)  creep compliance
G'(ω)  storage modulus
G''(ω)  loss modulus
hpf  hours post fertilization
DMEM  Dulbecco’s minimum essential medium–nutrient mixture F-12
BF  bright field
TIRF  total internal reflection
FRET  Förster resonance energy transfer
FRAP  Fluorescence recovery after photobleaching
NES  nuclear export signal
NLS  nuclear localization signal
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