Doctoral Thesis

The Mechanism of Mitotic Rounding
Role of the Actomyosin Cortex

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Publication Date:
2014

Permanent Link:
https://doi.org/10.3929/ethz-a-010348082

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THE MECHANISM OF MITOTIC Rounding: ROLE OF THE ACTOMYOSIN CORTEX

A thesis submitted to attain the degree of

DOCTOR OF SCIENCES of ETH ZURICH

(Dr. sc. ETH Zurich)

presented by

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2014
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SUMMARY

Actomyosin-dependent mitotic rounding occurs both in cell culture and tissue, where it is involved in cell positioning and epithelial organization. While the importance of F-actin for mitotic rounding is established, due to conflicting data, the role of cortical myosin in mitotic rounding is not well understood. Therefore, in the first part of our work, we investigated how actomyosin dynamics shape mitotic cells against surrounding extracellular confinement (Chapter 2). Using atomic force microscopy to characterize the mechanics of single mitotic cells and confocal microscopy to image the cell cortex, we related the intracellular pressure and cortical tension of a rounded cell with the dynamics of the actomyosin cortex throughout mitosis. We found that at mitotic onset, the assembly of a uniform F-actin cortex coincides with initial rounding. Thereafter, cortical enrichment of F-actin remained stable, while myosin II progressively accumulated at the cortex and directly correlated with increasing intracellular pressure. Next, we used Cdk1 inhibitors to force cells arrested in metaphase to exit mitosis and followed the reversal of what happens to the actomyosin cortex during mitotic entry. Cdk1 inhibition reduced the cortical enrichment of myosin II, but not of F-actin, and also decreased the intracellular pressure and cortical tension of mitotic cells. These experiments reveal the dynamics of how cortical myosin II enrichment throughout mitosis correlates closely with intracellular pressure and cortex tension.

Since the progressive enrichment of cortical myosin II is a salient feature of mitotic rounding, in the second part, we wanted to understand the role of myosin II activity in mitotic cell mechanics (Chapter 3). We found that within 10 minutes of myosin II inhibition, the cortex tension and intracellular pressure reduced five-fold. Although myosin activity is known to influence F-actin dynamics in the leading edge of crawling cells, cleavage furrow as well as neuronal growth cones, we found cortical F-actin localization and turnover to be largely unperturbed by myosin II inhibition during mitosis. Furthermore, time-dependent mechanical response to confinement showed that while F-actin devoid of myosin II activity can provide short-term (<10 s) resistance to deformation, myosin II is required to sustain intracellular pressure for longer duration (>60 s). Thus, during mitosis in confined tissue-like
environments, where cells have to maintain round morphology for over 30 minutes, cortical myosin II plays a critical role in resisting deformation.

Given the importance of Cdk1 activity for enrichment of cortical myosin, in the third part, we sought to understand the mechanistic details of how Cdk1 signaling is transduced to bring about changes to the actomyosin cortex (Chapter 4). As expected, inhibition of Ect2, a Cdk1 substrate, resulted in a reduction in not only cortex tension and intracellular pressure but cortical F-actin and myosin II as well. Next, we tested the role of three most prominent Rho GTPases downstream of Cdk1 — RhoA, Rac1 and Cdc42 — in mitotic rounding and found only RhoA to be crucial for the maintenance of cortical F-actin and myosin II. Interestingly, although Ect2 and RhoA regulated both cortical F-actin and myosin II, the RhoA effector — Rho kinase — was essential only for maintaining cortical myosin II enrichment. For maintaining cortical F-actin, mitotic cells required the formin DIAPH1, but not Arp2/3. Finally, we identify p21-activated kinases as negative regulators of cortical myosin II and propose that Cdk1 controls the progressive accumulation of cortical myosin II by linking the opposed activities of Rho kinase and p21-activated kinases.

Overall, this thesis provides insights into how recruitment of F-actin and myosin II are coordinated at the cortex to generate cortex tension and intracellular pressure to facilitate mitotic rounding in confined tissue-like environments.
ZUSAMMENFASSUNG


Da die fortlaufende Anreicherung von kortikalem Myosin II eine auffallende Besonderheit der mitotischen Zellrundung ist, wollten wir im zweiten Teil unserer Arbeit die Rolle von Myosin II in der Mechanik mitotischer Zellen besser verstehen (Kapitel 3). Wir konnten beobachten, dass 10 Minuten nach Inhibition von Myosin II
sowohl die Kortexspannung als auch der intrazelluläre Druck auf ein fünftel reduziert wird. Obwohl die Aktivität von Myosin in anderen Prozessen dafür bekannt ist, die Dynamik von F-Aktin zu beeinflussen – so zum Beispiel im Leitsaum von migrierenden Zellen, in der Teilungsfurche sowie in neuronalen Wachstumskegeln - zeigte sich in unseren Experimenten sowohl die Lokalisierung von F-Aktin als auch dessen Umsatz mehrheitlich unbeeinflusst von der Inhibierung von Myosin II. Des Weiteren zeigte die mechanische Reaktion auf räumliche Einengung einzelner mitotischer Zellen, das F-Aktin ohne aktives Myosin II diesen kurzzeitig (<10 s) Stabilität verleihen kann, dass aber Myosin II notwendig ist, um den intrazellulären Druck über längere Zeit (>60 s) aufrecht zu erhalten. Daraus folgt, dass in gewebeähnlichen räumlich einschränkenden Umgebungen, in welchen mitotische Zellen ihre runde Form während mehr als 30 Minuten aufrecht erhalten müssen, kortikales Myosin II eine entscheidende Rolle spielt bei der Vermeidung von Zelldeformationen.

durch die Verbindung der gegensätzlichen Aktivitäten von Rho-Kinase und p21-aktivierten Kinasen kontrolliert.

Zusammenfassend erlaubt die vorliegende Dissertation einen Einblick in die Koordination der Anreicherung von F-Aktin und Myosin II am Kortex zum Aufbau von Kortexspannung und intrazellulärem Druck, welche die Abrundung mitotischer Zellen in einer räumlich einschränkenden gewebeähnlichen Umgebung ermöglichen.
1. INTRODUCTION TO MITOTIC ROUNding

The maintenance of cell shape is central to many aspects of biology such as organism growth and form. One ubiquitous example of cell shape change occurs during mitosis, where animal cells round up to divide\textsuperscript{1–6} (\textbf{Fig. 1.1}). Mitotic rounding has been demonstrated to play a role in maintaining tissue organization. For example, it is involved in mouse epidermis differentiation\textsuperscript{7}, tracheal invagination in \textit{Drosophila}\textsuperscript{8} and the maintenance of pseudostratified epithelia in \textit{Drosophila} wing discs\textsuperscript{9}. Cell culture studies show that flattening round mitotic cells by confinement results in prolonged or defective mitosis due to failure of the mitotic spindle to properly capture and organize chromosomes\textsuperscript{10,11}. Retraction fibers are strands of actin-rich filamentous protrusions that anchor mitotic cells to the substrate\textsuperscript{12}. Spindle orientation, another key aspect of mitotic geometry, is controlled by the arrangement of retraction fibers\textsuperscript{13,14}. The cortical tension generated during mitotic rounding translates the adhesion pattern into cortical guidance cues for spindle orientation in an actin-dependent manner\textsuperscript{15}. Thus, it is clear that rounding is important for multiple aspects of mitosis.

\textbf{Figure 1.1. Cell shape change during mitotic rounding in \textit{Drosophila} wing disc.} (A) Image of the apical plane of \textit{Drosophila} wing disc, fixed and stained with antiphosphohistone (PH3) and discs large (DLG). The stages of mitosis are indicated below the images. (B) Image of wing disc cross section, fixed and stained with phallodin. The yellow arrows indicate rounded mitotic cells. Images in \textbf{A} and \textbf{B} are taken from Meyer et al., 2011\textsuperscript{5}. 

During mitosis, regulatory pathways trigger cells to de-adhere from their substrate and remodel their actin cytoskeleton into cortical F-actin\(^{16-18}\). A build-up of intracellular pressure acting against contractile forces of the actin cortex drives mitotic rounding\(^{19,20}\). In the next four sections of the introduction, we will examine the contribution of each of these processes — de-adhesion, cytoskeleton remodeling, intracellular pressure change and the role of regulatory pathways — in mitotic rounding.

**De-adhesion and mitotic rounding**

Mitotic entry is accompanied by a drastic remodelling of cell-substrate adhesion\(^{12,16}\). The cells disassemble focal adhesion complexes, the cellular machinery for substrate adhesion, by inhibiting focal adhesion kinase (FAK), paxillin and p130\(^{\text{CAS}}\) during mitosis\(^{21}\). The activity of Rap1, a small GTPase involved in the regulation of focal adhesions, has been shown to decrease at mitotic entry and recover at the end of mitosis when the daughter cells start to spread\(^{18}\). Furthermore, when expressing a constitutively active mutant version of Rap1, which inhibits the disassembly of focal adhesions, mitotic cells fail to round up\(^{18,10}\). Despite the disassembly of focal adhesions, cells still retain retraction fibers throughout mitosis\(^{12}\). The arrangement of the mitotic retraction fibers thus provides spatial clues to, the otherwise symmetric, round mitotic cells and influence spindle orientation\(^{13,14}\). While integrin mediated focal adhesions are disassembled during mitosis, fluorescence as well as electron microscopy of epithelial tissue show the presence of intact adherens junctions as well as desmosomes and hemidesmosomes throughout mitosis\(^{22}\).

**Role of cell cytoskeleton in mitotic cell shape**

Loss of substrate adhesion due to trypsin treatment is sufficient for the rounding of isolated interphase cells. These trypsin rounded interphase cells, unlike round mitotic cells, do not generate increased intracellular pressures and cannot round up against confining structures like tissue\(^{20,10}\). Hence, mitotic rounding, especially in confining tissue-like environment, requires other mechanisms in addition to simple de-adhesion from the cell substrate. In cell culture, filamentous actin (F-actin) is predominantly basally organized into bundled fibers called stress fibers during interphase\(^{23,24}\). Stress
fibers serve to anchor interphase cells to their substrate through focal adhesions\textsuperscript{25–27}. At the onset of mitosis, the nuclear envelope breaks down and the cell starts to change its shape; the cell margin retracts in a manner dependent on actin, but not on myosin\textsuperscript{12,28}. Soon, the actin cytoskeleton gets organized at the periphery of the round cell to form an actin cortex\textsuperscript{29}. This formation of an actin cortex is essential for the stiffness of round mitotic cells\textsuperscript{20,30}. While the role of cortical F-actin in mitotic rounding is clear, the role of myosin II is nebulous (Fig. 1.2). Imaging actin and myosin localizations throughout mitosis has not reflected an obvious role in mitotic rounding, but emphasized their well-established role in anaphase and cytokinesis\textsuperscript{31}. Some images of cells fixed prior to anaphase show diffuse localizations of myosin\textsuperscript{32–35} while others report unreliable degrees of cortical myosin enrichment\textsuperscript{5,7,17,36,37} (Table 1).

Figure 1.2. Hypothetical scenarios of actomyosin cortex reorganization during cell rounding. Three scenarios show how the actomyosin cytoskeleton could be reorganized from the transition from an adherent interphase state, where F-actin (red) and myosin-II (green) are predominantly in the stress fibers, to a round mitotic cell. In scenario 1, F-actin is enriched at the cell cortex devoid of myosin II. In scenario 2 and 3, both F-actin and myosin II are enriched at the cortex although cortical myosin II is inactive (blue) in the former case.
Apart from cortical F-actin, mitotic rounding is also dependent on RhoA and Rho kinase, the regulators of the actin cytoskeleton, as well as actin modulators such as ezrin, radixin and moesin (ERMs), WDR1, and other accessory proteins.

Unlike the actin cytoskeleton, microtubules are not essential for mitotic rounding; when microtubules are depolymerized mitotic rounding is not detered. Intermediate filaments, the metazoan specific cytoskeletal system, play an important role in maintaining cell and tissue architecture. Although intermediate filaments undergo extensive mitosis-specific reorganization, their role in mitotic rounding has not been investigated.

**Role of Intracellular hydrostatic pressure in mitotic rounding**

During mitosis, the inward acting contractile force provided by the actin cortex is balanced by outward acting intracellular hydrostatic pressure. This is evidenced by the presence of membrane protrusions called blebs in mitotic cells upon treatment with actin inhibitors. Partial perturbation of cortical F-actin results in the inability of the cell to contain the intracellular pressure and causes blebbing as a result of membrane detaching from the actin cortex. Activity of the Na\(^+\)/H\(^+\) exchanger NHE1 increases the osmolarity of the cell due to the influx of Na\(^+\) and is critical for recovery of cell volume after hypertonic shock. The perturbation of osmotic balance during mitosis with either α-toxin, which breaks down monovalent cation gradients of the cell, or by inhibiting NHE1 reduces the intracellular pressure and cell volume. Furthermore, the elevated activity of NHE1 at the start of mitosis and the associated increase in intracellular pH due to influx of Na\(^+\) suggests that NHE1 plays an important regulatory role in mitosis.

**Cdk1 and Rho GTPases in mitotic rounding**

Cdk1 is a highly conserved kinase that phosphorylates a wide range of substrates and plays a vital role in coordinating the cell cycle. Cdk1 is tightly regulated and its activity is altered by its binding to CyclinB in mitosis. Cdk1 activity is essential for mitotic entry and a drop in activity of Cdk1 correlates with anaphase and mitotic
Injecting active Cdk1 into interphase rat fibroblasts produces mitosis-like phenotype including chromosome condensation, cell rounding and disassembly of stress fibers. In mitosis, Cdk1/CyclinB interacts with a range of cytoskeletal proteins, including Rho kinase, which regulate the actin cytoskeleton. Furthermore, Ect2, a Cdk1 substrate, has been shown to be critical for mitotic F-actin cortex and mitotic cell stiffness. On the other hand, in vitro and in vivo studies indicate that Cdk1 directly phosphorylates myosin in an inhibitory fashion. Hence, as with most mechanisms in mitosis, mitotic rounding is also likely to be regulated by Cdk1. However, the details of how Cdk1 regulates the mitotic actin cytoskeleton are still unclear.

Rho GTPases, a family of over 25 proteins, are regulators of actin cytoskeleton. Rho GTPases toggle between active and inactive states when bound to GTP or GDP respectively. GTPase activating proteins (GAPs) retard Rho GTPase activity by catalyzing GTP hydrolysis while Rho GTPases are activated by guanine nucleotide exchange factors (GEFs) that promote GTP exchange. Guanosine nucleotide dissociation inhibitors (GDIs) maintain Rho GTPases in the inactive state by preventing the exchange of GDP for GTP.

The most prominent and well-studied Rho GTPases are RhoA, Rac1 and Cdc42. In motile interphase cells, Rac1 and Cdc42 are important for the formation of lamellipodia, filopodia and for the maintenance of the actin scaffold structure at the leading edge. In contrast, RhoA activity promotes actin contractility and promotes retraction of the cell membrane at the rear. The RhoA pathway is critical for mitotic rounding; upon perturbation of RhoA, mitotic cells are unable to round and cortical F-actin localization is punctate. Furthermore, inhibition of the upstream Rho GEF, Ect2, or the downstream RhoA effector, Rho kinase, also deters mitotic rounding. While RhoA activity is essential for mitotic rounding, the roles of other Rho GTPases, especially Cdc42 and Rac1, have not been investigated.
Altogether, previous results establish the central role of the actomyosin cortex in mitotic rounding. However, the quantitative details of how cortical actin and myosin is enriched during mitosis are nebulous (Table 1). Therefore, in the next chapter we develop an assay to quantify the enrichment of cortical F-actin and myosin. By correlating the enrichment of cortical F-actin and myosin with cell mechanics, we want to understand the details of how the mitotic actomyosin cortex governs cell rounding.
2. CORRELATING ENRICHMENT OF CORTICAL F-ACTIN AND MYOSIN WITH CELL MECHANICS

Confocal microscopy of the actomyosin cytoskeleton

We set out to study the roles of F-actin and myosin in mitotic rounding. The reports on actin and myosin localization from early mitosis to metaphase, the timeframe corresponding with mitotic rounding, are inconclusive (Table 1). Therefore, we imaged HeLa cells that stably express myosin heavy chain 9 (MYH9)-GFP, a GFP-labeled version of the heavy chain subunit of myosin IIA, and Lifeact-mCherry, a red fluorescent protein-based marker for F-actin, through mitosis (Fig. 2.1). With the start of nuclear envelope breakdown (NEBD), we observed that basal actomyosin structures disassembled and cells changed shape from flat to round.

![Image of confocal microscopy](image)

**Figure 2.1.** F-actin and myosin-II are enriched at the cortex during mitosis. Trans-mitotic confocal images of MYH9-GFP Lifeact-mCherry expressing HeLa cells with DIC (grey), mCherry (red) and GFP (green). Images were acquired every 8 minutes at three focal planes 4 µm apart where zero was at the base of the cell. Between 8 and 16 minutes, NEBD is evidenced by ingression of MYH9-GFP signal into the nuclear space ($n = 7$). Scale bar 20 µm, applies to all images.
Subsequently, F-actin and myosin were redistributed into a cortex. The initial reorganization of actin to the cortex is presumably driven by mitosis-specific disassembly of focal adhesions$^{64-66}$, which would otherwise recruit actin to basal adhesion clusters and stress fibers. Thereafter, actin and myosin were enriched at the cortex until the expected accumulation at the midbody during anaphase. Thus, we conclude that F-actin and myosin II is enriched at the cell cortex during mitosis.

**Quantifying the enrichment of cortical F-actin and myosin II**

During mitosis, although F-actin and myosin II is enriched at the cell cortex compared to the cytosol, we were unable to gain insight into the dynamics of the enrichment process. Hence, we quantified the enrichment of actomyosin cortex during mitosis. Confocal images of HeLa cell expressing MYH9-GFP and Lifeact-mCherry were used to determine the cortex/cytoplasm ratio and full width at half maximum (FWHM) of F-actin and myosin (Fig. 2.2, see methods section for a full description).
Figure 2.2. Quantification of cortex/cytoplasm ratio and cortical full width at half maximum (FWHM) of labeled F-actin and myosin II in live cells. (A) Confocal image of a MYH9-GFP Lifeact-mCherry expressing HeLa cell. The purple circle denotes the center of the cell. 60 points (yellow markers) are 6° apart at the cell periphery. (B) Fluorescence intensity profile (cyan) taken from the average signal along a 0.6 µm thick radial line (yellow) through a representative peripheral point (yellow marker). (C) The 60 fluorescence intensity profiles (thin traces) through the peripheral yellow markers were aligned at the cell periphery (0 µm) and superimposed. The thick green trace represents the average of all 60 fluorescent intensity profiles taken from one cell. (D) Determination of cortex/cytoplasm ratio ($R$) of the whole cell from the average fluorescence intensity profile. Within each average profile, the cortex/cytoplasm ratio ($R$) of the whole cell from the average fluorescence intensity profile. Within each average profile, the
(Figure 2.2 continued)
peak intensity (p) between -2 and 0.5 µm from the cell edge and the mean intensity (m) between -2 and -1 µm from the cell edge were extracted. The cortex/cytoplasm ratio (R) for the fluorescence image was determined as the ratio of p to m. (E) Determining the cortical FWHM of the average fluorescence intensity profile shown in D. The half peak height (l) is the intensity halfway between p and m. Cortical FWHM is the width (in µm) of the signal at l. (F) The yellow markers from the MYH9-GFP image were copied into the Lifeact-mCherry fluorescence image. (G-J) Utilizing the same method as in the MYH9-GFP image, the cortex/cytoplasm ratio (R) and FWHM were determined from the average fluorescence intensity profile of Lifeact-mCherry image.

Next, we wanted to test our method to quantify enrichment of F-actin and myosin at the cortex by comparing cells with and without prominent F-actin cortex. The average radial intensity profile and the distribution of cortex/cytoplasm ratio of F-actin and myosin II were compared between untreated cells and cells treated with 20 µg ml⁻¹ C3-toxin, an inhibitor of cortical F-actin accumulation in mitosis. In the C3-toxin treated cells, the localization of cortical F-actin and myosin II were diminished when compared with untreated cells (Fig. 2.3A). Average intensity profiles quantifying the radial distribution of the actomyosin cortex show F-actin and myosin II to be enriched at the cell periphery. While the enrichment of actin and myosin was concurrent at the inner regions of the cortex, the enrichment of myosin II was diminished at the outer regions of the cortex when compared to the enrichment of F-actin in the same region (Fig. 2.3B). This could be due to the presence of retraction fibers — actin rich, myosin poor membrane tethers between mitotic cells and the substrate — at the cell periphery.

In C3-toxin treated cells, the enrichment of peripheral F-actin and myosin II was diminished and comparable to the intracellular regions of the cell (Fig. 2.3A,C). The distributions of the cortex/cytoplasm ratios further confirm that the enrichment of cortical F-actin and myosin II is diminished in C3-toxin treated cells (Fig. 2.3D,E). These results show that the assay to determine the enrichment of cortical F-actin and myosin II can indeed distinguish the difference between the actomyosin cortex of untreated and C3-toxin treated cells.
Figure 2.3. Testing the method to quantify the cortex/cytoplasm ratio of F-actin and myosin. (A) Confocal images of STC-arrested mitotic MYH9-GFP Lifeact-mCherry expressing HeLa cells confined to a height of 10 µm. Cells were untreated or pre-incubated for at least 30 minutes with C3-toxin. Scale bars, 10 µm. (B) Average intensity profile quantifying the radial distribution of F-actin (red) and myosin II (green) in the untreated mitotic cell from A. (C) Average intensity profile quantifying the radial distribution of F-actin (red) and myosin II (green) in a mitotic cell treated with 20 µg ml⁻¹ C3-toxin from A. (D,E) Histogram showing the cortex/cytoplasm ratio of F-actin, in D, and myosin II, in E, from the 60 segmented fluorescence intensity profiles from each of the single cells that were untreated or incubated with 20 µg ml⁻¹ C3-toxin (n = 5).
Comparing actomyosin cortices in interphase and mitosis

In the absence of substrate adhesion, and whether or not in mitosis, actomyosin typically exists in the form of a peripheral cortex underlying the plasma membrane. We reasoned that examining the actomyosin cortex of cells in a detached state might provide insight into mitosis-specific changes otherwise not evident during the transition from flat to round (Fig. 2.4A). To investigate this, the enrichment of cortical F-actin and myosin II in rounded interphase and arrested mitotic cells were quantified by determining cortex/cytoplasm ratios (Fig. 2.2). Using this method, we found a ~25% rise in cortical F-actin localization in mitosis (Fig. 2.4B,C). This change occurred without a noticeable increase in homogeneity, which was assessed by comparing the ratios of each of the 60 segments around the cell periphery (Fig. 2.4D). Myosin II localization differences were more striking, as the cortical enrichment more than doubled from interphase to mitosis (Fig. 2.4B,C,E). These results demonstrate that both cortical F-actin and myosin II are significantly enriched in mitosis and the timing of these changes are related to cell cycle progression.

Figure 2.4. F-actin and myosin II enrich at the mitotic cell cortex. (A) Procedure for imaging the actomyosin cortex in round interphase and mitotic cells. STC treated cells (Figure 2.4 continued in the next page)
were trypsinized, seeded on glass and imaged, after four hours, while still round. Interphase and arrested mitotic cells were identified by the presence or absence of an intact nucleus, respectively. (B) Representative confocal microscopy images of rounded interphase and STC-arrested mitotic MYH9-GFP Lifeact-mCherry expressing HeLa cells. Scale bars, 10 µm. (C) F-actin (red) and myosin II (green) cortex/cytoplasm ratios in MYH9-GFP Lifeact-mCherry expressing HeLa cells. Cells were in rounded interphase (n=10) or STC-arrested mitotic states (n=13). Each circle represents one cell characterized. The diamond-box contains 25-75% percentiles of the data and the bar denotes the median. Mann-Whitney significance tests of cells with respect to data plotted in the interphase column are indicated: (*) 0.05≥U>0.005, (**) U≤0.005. (D,E) Histograms showing the cortex/cytoplasm ratio of F-actin, in D, and myosin II, in E, from all the segmented fluorescence intensity profiles from cells that were rounded in interphase (orange) or STC-arrested in mitosis (purple).

**AFM based confinement assay to study cell mechanics**

Previously we used AFM (atomic force microscope) as a tool to measure the force exerted by round cells against the cantilever\(^{20,67}\). In simple terms, a typical AFM setup consists of a micro-cantilever overhanging from a fixed chip (Fig. 2.5A). Incoming light from a laser source is reflected from the top of the cantilever. When an upward force is exerted on the cantilever, the resulting cantilever deflection causes change in the angle of the reflected light from the cantilever. This change in the angle of reflected light is detected by a photodiode. Thus, the deflection of the cantilever due to a force exerted on the cantilever can be accurately measured. The spring constant of the cantilever is determined using the thermal noise method\(^{68}\). Using Hooke’s law the force exerted on the AFM cantilever can be calculated as the product of cantilever deflection and its spring constant.

To determine the intracellular hydrostatic pressure (\(\Delta P\)) and the cortical tension (\(T\)) of a round mitotic cell, we applied an AFM based assay (Fig. 2.5B, see methods section for a full description). For accurate intracellular pressure and cortical tension measurements, we incorporated advancements to the earlier assay. As opposed to the use of traditional AFM cantilevers with an inherent 10° tilt, the use of wedged AFM cantilevers provided true parallel plate uniaxial confinement\(^{69}\). Thus, cell sliding, a frequently observed complication in the case of non-wedged cantilevers with a ten-
degree contact angle, was prevented. This improvement allowed precise
determination of cell shape to improve the accuracy of intracellular pressure
measurements and calculate cortical tension\textsuperscript{70}.

**Figure 2.5. Schematic of the AFM assay to measure intracellular pressure and cortical tension.** (A) Schematic of an AFM, wherein the cantilever deflection ($\Delta D$) upon application of a force ($F$) is shown. Cantilever deflection changes the path of reflected laser, which is quantified by the detector as voltage change ($\Delta V$). (B) Illustration of a cell uniaxially confined to a height ($H$) with an AFM cantilever. $F$ is the force exerted by the cell against the cantilever. $A_C$ is the cell-cantilever contact area. $P_c$ and $P_o$ are intracellular and extracellular pressures, respectively. $T$ is the cortical tension of the cell. $R_1$ and $R_2$ are the radii of principle curvature at point $X$. Equations use the Laplace’s law to calculate hydrostatic pressure difference across the cortex ($\Delta P$) and cortical tension ($T$).
In order to assess the mechanics of cells going through mitosis, MYH9-GFP Lifeact-mCherry labeled HeLa cells that were rounding in early prometaphase were identified and engaged for experiments. Cells were first confined with a wedged cantilever to a height of 10 µm, which represents ~50% of the height they would attain if unconfined (Fig. 2.6A). In our previous experiments, we confined mitotic cells to a height of approximately 10 µm (~50% confinement) and found that the time taken by the confined cells to complete mitosis was not different from unconfined cells20. On the other hand, confining mitotic cells to less than 35% of their normal height retards spindle formation and hinders mitosis10. Based on these two results, a cell confinement height of 10 µm was deemed appropriate for our experiments.

Once confined, cells were allowed to proceed through mitosis and the force exerted by the mitotic cell against the cantilever was measured. The force exerted by the mitotic cell followed the same trend as observed in our earlier study20. From prometaphase until the start of metaphase, there was a continuous build-up of force. Subsequently, the force plateaued off until the start of anaphase. Throughout the force measurement, the cell was imaged with confocal microscopy to monitor the enrichment of cortical F-actin and myosin II. Kymographs of F-actin and myosin constructed from the fluorescent images indicate that from prometaphase to metaphase there was a steady increase in the cortical enrichment of myosin II, which was not accompanied by an increase in cortical F-actin enrichment (Fig. 2.6B). These results indicate that the accumulation of cortical myosin II, rather than of F-actin, confers the mitotic cell the ability to push against confinement.
Figure 2.6. Trans-mitotic localization of cortical myosin II coincides with mitotic rounding force against confinement. (A) Trans-mitotic confinement of a single cell to determine its intracellular pressure, cortical tension and actomyosin dynamics. Representative plot showing the cell radius (teal markers), height of uniaxial confinement with wedged cantilever (orange line) and measured force (purple trace) exerted by a MYH9-GFP Lifeact-mCherry expressing HeLa cell. A confocal optical section was acquired through the middle of the cell every 2 minutes to determine cell dimensions and quantify cortex/cytoplasm ratios and cortical full-width-at-half-maximum (FWHM) for F-actin and myosin II. The measurement started at early prometaphase and ended at cytokinesis. (B) F-actin and myosin II intensity kymographs from a cell, extending from the extracellular region (ECR) through the cortex and into the cytosol. During the 36 minutes indicated, the cell proceeds from early prometaphase to anaphase. In the image, gold represents the maximum fluorescence intensity and black the minimum.

Trans-mitotic correlation of mechanics and cortex composition

We sought to probe the observed changes in cortical F-actin and myosin II to cell mechanics throughout mitosis. Therefore, we quantified the cortical F-actin and myosin II enrichment and thickness with fluorescent imaging by measuring their cortex/cytoplasm ratio and FWHM throughout mitosis and correlated that to intracellular hydrostatic pressure and cortex tension. During mitosis, the cortex/cytoplasm ratio of myosin II increased from 1.86±0.14 to 3.63±0.27 (mean±s.e.m, n=11) with a commensurate rise in intracellular pressure, from 135.60±21.04 Pa to 546.66±44.03 Pa and cortex tension, from 0.42±0.07 mN m⁻¹ to 1.69±0.15 mN m⁻¹ (Fig. 2.7A,C,E). The higher pressures than those previously measured²⁰ arise from a more accurate analysis of cell contact area. In contrast to
increases in pressure, tension and myosin II enrichment, the cortical F-actin enrichment remained almost unchanged throughout mitosis (Fig. 2.7A,C,E). The mean F-actin cortex/cytoplasm ratio of mitotic cells was 2.42±0.15 (n=11). FWHM is a commonly used parameter to quantify the width of an object without sharp edges. In order to verify the reduction of cortical F-actin thickness observed in kymographs (Fig. 2.6B), we measured cortical F-actin FWHM. Indeed, with increasing cortical tension and pressure, the cortical F-actin FWHM decreased from 0.72±0.04 µm to 0.54±0.02 µm (n=11) indicating a reduction in F-actin cortex thickness. In the same timeframe, cortical myosin II FWHM changed from 0.38±0.02 µm to 0.43±0.02 µm (n=11) (Fig. 2.7B,D,E). Overall, these data show that the enrichment of myosin II at the cell cortex correlates with cortical tension and intracellular pressure throughout mitosis. Previous results show that with mitotic rounding the increase in the outward acting osmotic pressure is contained by the cortical F-actin cortex\(^{20}\). This could in-turn raise the cortex tension and intracellular pressure of mitotic cells without any additional contractility provided by the activity of myosin (Fig. 1.2). Our results, showing a lack of a positive correlation between cortical F-actin enrichment and cortical tension indicates that, in round mitotic cells, F-actin itself does not generate tension but rather provides a scaffold for myosin II.
Figure 2.7. Trans-mitotic localization of cortical myosin II coincides with buildup of intracellular hydrostatic pressure under confinement. (A,B) Intracellular hydrostatic pressure plotted with cortex/cytoplasm ratio, in A or cortical FWHM of F-actin and myosin II, in B, of a single cell. (C,D) Scatter plots of the intracellular hydrostatic pressure of MYH9-GFP Lifeact-mCherry expressing HeLa cells \( (n=5) \) starting from early prometaphase and ending in anaphase. Plotted are cortex/cytoplasm ratios, in C, or cortical FWHM, in D, of F-actin and myosin II. (E) Pressure, tension, cortical FWHM and cortex/cytoplasm ratio of F-actin or cortical FWHM and cortex/cytoplasm ratio of myosin II during mitosis \( (n=11) \). The data plotted are for prometaphase (Prometa) and metaphase when the force exerted by the cell on the cantilever reaches a maximum (Meta-max). The diamond-box contains 25-75% percentiles of the data and the bar denotes the median. Mann-Whitney significance tests of mitotic cells with respect to data taken in prometaphase are indicated: (NS) \( U>0.05 \), (*) \( 0.05 \geq U>0.005 \), (**) \( U \leq 0.005 \).
Cortical Myosin II Enrichment Depends on Cdk1 activity

In order to explore the regulatory process behind mitotic rounding, we proceeded to investigate Cdk1, the master regulator of mitosis. The role of Cdk1 activity in regulating myosin II activity is unclear. Previous experiments have indicated that Cdk1 could directly inhibit myosin II in mitosis and the increased activity of myosin in the cleavage furrow is a result of inactivation of Cdk1 at the start of anaphase. On the other hand, more recent experiments linking the role of Ect2, a Cdk1 substrate, to the enrichment of mitotic F-actin cortex has hinted the contrary. We used inhibitors of Cdk1 to investigate the role of Cdk1 activity in regulating the enrichment of cortical myosin II in mitosis. To examine the role of Cdk1 activity in mitotic cell shape and spindle morphology, we introduced the Cdk1 inhibitor, Cdk1/2 III, to mitotic HeLa cells expressing H2B-mCherry and tubulin-GFP. For this experiment, we used cells arrested in mitosis using drugs to inhibit the proteasome (MG132), kinesin Eg5 (STC) or microtubule (nocodazole). By imaging the mitotic spindle with H2B-mCherry and tubulin-GFP, we find that the cells arrested with MG132 had bipolar spindle morphology indistinguishable from non-arrested control metaphase cells. In contrast, STC and nocodazole treated cells had monopolar and no spindle, respectively.

Figure 2.8. Morphology of the spindle when cell are arrested in mitosis using various inhibitors
Confocal images of H2B-mCherry (red) and tubulin-GFP (green) expressing mitotic HeLa cells. The cells were either untreated or treated with the indicated inhibitor to induce mitotic arrest. Protease inhibitor, MG132, treated cells exhibited bipolar morphology similar to untreated cells. STC, an Eg5 inhibitor, treated cells had mono-polar spindle morphology. Microtubule depolymerizer, nocodazole (Noc), treated cells were devoid of mitotic spindle. Scale bar of 10 μm applies to all images.
Evidenced by the reformation of nucleus and cell spreading, all cells, irrespective of their spindle morphology, exited mitosis upon Cdk1 inhibition. The morphology of the contractile actomyosin cleavage furrow, on the other hand, depended on the morphology and orientation of the mitotic spindle (Fig 2.9).

Figure 2.9. Cdk1 inhibition in the presence of mitotic spindle triggers anisotropic localization of cortical F-actin and myosin II. Confocal images of MYH9-GFP (green) and Lifeact-mCherry (red) expressing mitotic HeLa cells. Images were acquired every 4 minutes. Cells were untreated (n=5), or preincubated for 30 min with MG132 (n=5), STC (n=5) or nocodazole (Noc, n=5) at concentrations indicated. Nine minutes after starting the experiment 1.5 µM Cdk1/2 inhibitor (Cdk1/2 III) was introduced. Scale bar of 10 μm, applies to all images.
After ~10 minutes upon Cdk1 inhibition, mitotic cells with bipolar spindle orientation (control and MG132 treatment) started to form a normal, symmetric actomyosin cleavage furrow. When Cdk1 was inhibited in STC treated cells, which have a monopolar spindle, actin and myosin was enriched in a cup like region opposite to the spindle pole. By contrast, in Cdk1 inhibited cells with no mitotic spindle, as a result of nocodazole treatment, there was no heterogeneous accumulation of F-actin and myosin II.

At the onset of anaphase, when Cdk1 is inactive, the centralspindlin complex recruits Ect2 which in turn recruits RhoA to form the actomyosin rich cleavage furrow. Thus, as seen in our results, when Cdk1 is inhibited, the complex cleavage furrow-like structure that forms is dependent on the morphology of the mitotic spindle (Fig 2.10). Hence, these results also indicate that when Cdk1 is inhibited, the formation of anaphase actomyosin cleavage furrow due to centralspindlin complex activity can be avoided by depolymerizing the microtubules with nocodazole treatment.

Figure 2.10. Scheme of the mitotic spindle organizing the cortical enrichment of actomyosin when Cdk1 is inhibited. In Cdk1 inhibited cells with a bi- (untreated or MG132 arrest) or mono- (STC arrest) polar spindle, cortical actomyosin is enriched in regions furthest from the spindle poles. Mitotic cells with no spindle (nocodazole arrest) are devoid of anisotropic enrichment of cortical F-actin or myosin after Cdk1 inhibition.
In order to investigate the role of Cdk1 in regulating the mitotic actomyosin cortex, we used nocodazole arrested mitotic HeLa cells expressing MYH9-GFP and Lifeact-mCherry. We used confinement with cantilever and live imaging to correlate intracellular pressure and cortex tension with enrichment of cortical F-actin and myosin II before and after introduction of the Cdk1 inhibitor, Cdk1/2 III (Fig. 2.11A,B). Ten minutes after introducing Cdk1/2 III, we observed that the cortex/cytoplasm ratio of myosin II decreased from 4.34±0.27 to 2.28±0.26 (mean±s.e.m, n=14) with a commensurate drop in intracellular pressure from 493.15±27.21 Pa to 199.50±35.17 Pa and cortex tension from 0.63±0.11 mN m⁻¹ to 0.42±0.07 mN m⁻¹ (Fig. 2.11B-E). Introduction of Ro3306, another Cdk1 inhibitor, in lieu of Cdk1/2 III reproduced the same results. In contrast to decreases in pressure, tension and myosin II enrichment, the F-actin cortex/cytoplasm ratio remained mostly unchanged at around 2.17±0.35 (n=10) upon introduction of either Cdk1/2 III or Ro3306 (Fig. 2.11B,C,F). The cells started to slowly respread on the substrate 15 minutes after the introduction of either Cdk1/2 III or Ro3306. Previous experiments, both in vitro and in vivo, looking at phosphorylation states of myosin II indicate that Cdk1 inhibits myosin⁵³,⁵⁴,⁸². On the other hand, Ect2 is essential for mitotic rounding suggesting that Cdk1 should activate myosin¹⁷. Our results show that activity of Cdk1 is essential for maintaining the enrichment of myosin II at the cell cortex, which is in turn tightly correlated with cortical tension and intracellular pressure during mitotic rounding.
Figure 2.11. Cdk1 activity is essential for the maintenance of cortical myosin II enrichment and elevated intracellular pressure. (A) Cell confinement experiment to interrogate the role of Cdk1 in mitotic cell mechanics. A wedged cantilever is used to confine a nocodazole-arrested mitotic HeLa cell expressing Lifeact-mCherry and MYH9-GFP to correlate intracellular pressure with enrichment of cortical F-actin and myosin II. After at least 15 minutes, the cell was treated with Cdk1 inhibitor. (B) F-actin and myosin II intensity kymograph of a cell, extending from the extracellular region (ECR) through the cortex and into the cytosol. After ~15 minutes (indicated by orange dotted line), 3 µM Cdk1/2 III was introduced to previously untreated (UT) medium. In the images, gold represents the maximum fluorescence intensity and black the minimum. (C) Intracellular hydrostatic pressure plotted with cortex/cytoplasm ratio of F-actin and myosin II of a single cell. Orange region indicates when 3 µM Cdk1/2 III was introduced to UT medium. Blue and yellow regions highlight measurements before and after the introduction of Cdk1/2 III, respectively. (Figure 2.11 continued in the next page)
Cortical F-actin is essential for mitotic rounding, but the role of myosin in mitotic rounding is unclear (Table 1). In this chapter, we quantified the enrichment of myosin II and F-actin at the cell cortex during mitosis, and correlated it to cell mechanics. From our experiments, we make the following conclusions:

1. F-actin is already enriched at the cell cortex at the early stages of mitosis and the enrichment does not increase thereafter.
2. The accumulation of myosin at the cell cortex increases steadily during mitosis.
3. The rise in intracellular pressure and cortex tension of mitotic cells correlates directly with the enrichment of cortical myosin.
4. The enrichment of myosin and the associated cortex tension is dependent on Cdk1 activity.

Thus far, we studied the dynamics of cortical myosin accumulation and the buildup of cortex tension in mitosis. The enrichment of cortical myosin in mitosis could influence cell mechanics directly, by increasing the contractility of the cell cortex, or indirectly, by the changing cortical F-actin enrichment. In the next chapter, we explore this theme and examine how enrichment of cortical myosin governs mitotic cell mechanics.
3. ROLE OF CORTICAL ACTIN AND MYOSIN IN RESISTING CONFINEMENT

Role of myosin activity in maintaining cortical F-actin

We were intrigued by the observed decrease in the thickness of cortical F-actin during mitosis (Fig. 2.6B, 2.7B,D,E). According to the active gel theory of the cell cortex, cortical tension is the product of active stresses exerted by myosin motors and the F-actin cortex thickness. Experimental evidence also suggests F-actin cortical thickening correlates with higher cell cortex tensions in interphase cells. One explanation for the decline in thickness during mitosis is that cortical F-actin thickness and myosin II activity are inversely related. To check this possibility, we again used confinement with cantilever and live imaging of the cortex to analyze cortical tension and F-actin FWHM in mitotic cells before and after introduction of blebbistatin, a myosin II ATPase inhibitor (Fig. 3.1A). While tension was reduced around fivefold, after 12 minutes, neither F-actin cortex enrichment nor width was affected (Fig. 3.1B-D). These data indicate that, in mitosis, cortex tension and F-actin thickness are not related and suggest that increased tension can be ascribed primarily to the activity of non-muscle myosin II and not to changes in cortex thickness.

Next, we investigated whether myosin II affects F-actin cortex turnover dynamics, another key aspect of F-actin behavior. Indeed, actin dynamics can be influenced by the transient crosslinking and contractile activity of myosin II at the cleavage furrow, neuronal growth cones or leading edge of crawling cells. To examine F-actin dynamics in mitosis, HeLa cells that express β-actin-GFP were tested by photobleaching a ~2 µm region of the F-actin cortex and analyzing recovery and turnover (Fig. 3.2A). We found the turnover of cortical actin (half-time, 5.9±1.1 s) in mitotic HeLa cells was similar to the turnover in blebbing filamin-deficient M2 melanoma cells in interphase, investigated previously.
Figure 3.1. Maintenance of cortical F-actin enrichment is independent of myosin II activity. (A) Cell confinement experiment to test the relationship between cortical tension and distribution of F-actin. A wedged cantilever is used to confine an STC-arrested mitotic cell and determine cortex tension, F-actin cortex/cytoplasm ratio and cortical F-actin FWHM. HeLa cells expressing MYH9-GFP Lifeact-mCherry were imaged to visualize mCherry and treated with 20 µM blebbistatin to inhibit myosin II. (B) F-actin intensity kymograph from a cell, extending from the extracellular region (ECR) through the cortex and into the cytosol. After ~25 minutes (indicated by orange dotted line) blebbistatin was introduced to previously untreated (UT) medium. In the image, gold represents the maximum fluorescence intensity and black the minimum. (C) Cortex tension, cortical F-actin FWHM and F-actin cortex/cytoplasm ratio of an STC-arrested mitotic MYH9-GFP Lifeact-mCherry expressing HeLa cell. The orange region indicates when 20 µM blebbistatin was introduced to the UT medium. Blue and the yellow regions highlight measurements before and after the introduction of blebbistatin, respectively. (D) Cell radius, intracellular pressure, cortex tension, cortical F-actin FWHM and F-actin cortex/cytoplasm ratio of mitotic cells (n=15). Plotted are data taken four minutes prior and 12 minutes after introducing 20 µM blebbistatin to the medium. Mann-Whitney significance tests of mitotic cells with respect to data recorded without blebbistatin are indicated: (NS) U>0.05, (**) U≤0.005.
Since blebbistatin gets photoinactivated by blue light\textsuperscript{92}, we could not use blebbistatin to inhibit myosin II and track β-actin-GFP in photobleaching experiments. Hence, myosin II was depleted using RNAi instead. Since Myosin IIA is the exclusive non-muscle isoform expressed in HeLa cells, we were able to use MYH9 RNAi to deplete myosin II\textsuperscript{93}. This depletion had no detrimental affect on the recovery of cortical actin. Within 60 s, the cortical β-actin-GFP signal in photobleached regions of cells transfected with control or MYH9 siRNA recovered to ~72% or ~79% of the levels of the prebleached cortex, respectively (Fig. 3.2C). Together, these results indicate that the recruitment, enrichment and thickness of F-actin in the mitotic cortex are regulated neither by the binding of myosin II nor by the tension myosin II provides through contraction.

Figure 3.2. Enrichment and turnover of cortical actin during mitosis is not dependent on myosin II. (A) Images showing a cross sectional region of an STC-arrested mitotic β-actin-GFP expressing HeLa cell used in a photobleaching experiment. The region inside the red circle (diameter, 2 µm) was photobleached for three seconds before time zero. The actin cortex/cytoplasm ratio of the unbleached cell cortex is the ratio of mean pixel intensity in the yellow square (0.32 µm\textsuperscript{2}), outside the bleach zone, and the magenta rectangle (0.32×1.18 µm\textsuperscript{2}), which is 0.32 µm away from the unbleached region of the cortex in the cytosol. The actin cortex/cytoplasm ratio of the bleached cell cortex is the ratio of mean pixel intensity in the green square (0.32 µm\textsuperscript{2}), within the bleach zone, and the magenta rectangle. Scale bar, 1.25 µm, applies to all images. (B) The actin cortex/cytoplasm ratio for unbleached (black) and bleached (magenta) cell cortex. The blue trace is the exponential fit of the data, post-bleaching. The half-time and recovery for the cell are indicated within the plot. (C) Plot of the time taken after photobleaching to reach half the final actin cortex/cytoplasm ratio (Half-time) and the percentage recovery of actin cortex/cytoplasm ratio compared to pre-bleached cortex. Cells were transfected at least 48 hours prior to the experiment with control (n=9) or MYH9 (n=9) siRNA.
Imposed confinement of mitotic cells

Next, we wanted to probe mitotic cell mechanics and its relationship to cortex architecture under various perturbations to regulators downstream of Cdk1. However, because many inhibitors of the cytoskeleton can prevent or severely delay mitotic rounding\textsuperscript{12,16}, precise measurements of intracellular pressure and cortex tension in perturbed cells is difficult with a trans-mitotic approach (as in Fig. 2.6). Allowing cells to round up before introducing the perturbation of interest can circumvent this problem. But the intracellular pressure and cortex tension determined using this method had a wide-spread of data, presumably because the cells were not in identical stages of mitosis. Previous experiments suggest that round mitotic cells subject to an imposed uniaxial confinement exhibit similar cortex tension and intracellular pressure compared to those allowed to round up against constant confinement\textsuperscript{20}. Furthermore, S-trityl-L-cystine (STC), a kinesin eg-5 inhibitor that arrests cells in mitosis by inducing mono-polar spindles\textsuperscript{74} (Fig 2.8), did not significantly alter the intracellular pressure\textsuperscript{20}, indicating that mitotic arrest with STC could be used to enrich candidate cells for testing. To assess intracellular pressure and cortical tension at higher throughput and to decrease the spread of data, we sought to validate an approach using mitotic arrest with STC followed by imposed confinement with a wedged cantilever (Fig. 3.3A).

In rheological terms, the imposed confinement is a constant strain experiment, and viscoelastic materials, such as cells or polymer gels, exhibit a characteristic force (or stress) response profile that decays to a steady value\textsuperscript{94}. Upon imposed mechanical confinement, STC-arrested mitotic cells show such a viscoelastic response (Fig. 3.3A,B). We refer to the initial peak as transient force and the steady value as persistent force, and pressures and tensions are termed accordingly. In the case of mitotic HeLa cells confined to 10 \( \mu \text{m} \) (~50% uniaxial deformation), the transient forces were \( \sim 40\% \) greater than the persistent values, and the decay time was approximately five seconds (Fig. 3.3B). This force decay time is comparable to the time taken for actin turnover at the cell cortex (half-time, 5.9±1.1 s, Fig. 3.2).
Figure 3.3. Imposed confinement of rounded cells yields a characteristic time-dependent mechanical response. (A) Scheme depicting the force response behavior of an STC arrested mitotic cell via imposed confinement with a wedged AFM cantilever. Representative plot showing the height of uniaxial confinement with wedged cantilever (orange line) and the force (purple trace) exerted by STC-arrested MYH9-GFP Lifeact-mCherry expressing HeLa cells. Upon confinement to a height of 10 µm at time zero, the cell exerts a transient force peak (red box), which decays to a persistent value (blue box). (B) Force response behavior of 24 cells to imposed confinement. In purple are individual force traces, aligned at their force peak. The thick grey trace (grey) is the average force trace of all cells.
Comparing the imposed confinement and trans-mitotic assays

We wanted to test if the mechanics and the enrichment of cortical F-actin and myosin II measured through imposed confinement and the trans-mitotic assays were similar. The enrichment of cortical F-actin and myosin II and the cell mechanics of confined STC-arrested mitotic cells were compared to non-arrested cells in metaphase. The cortex/cytoplasm ratio of F-actin and myosin II as well as the persistent pressure and tension of arrested mitotic cells were not significantly different from non-arrested mitotic cells measured when the force exerted by the cell on the cantilever reaches a maximum during mitotic progression (Fig. 3.4). This result shows that the mechanics and the enrichment of cortical actin and myosin II measured through imposed confinement and the trans-mitotic assays are similar.

Figure 3.4: Imposed confinement and trans-mitotic assays are comparable.
Quantification of cortex/cytoplasm ratio of F-actin (red), myosin II (green), intracellular hydrostatic pressure (grey) and cortical tension (blue) in mitotic MYH9-GFP Lifeact-mCherry expressing HeLa cells confined to 10 µm. The cells were either STC pretreated (persistent, n=16) or untreated (meta-max, n=5). STC pretreated cells were subjected to imposed confinement and measured 60 seconds after confinement was established. Meta-max cells were subjected to trans-mitotic confinement assay in prometaphase and measured when the force exerted by the cell on the cantilever reaches a maximum, prior to anaphase, during mitotic progression. Mann-Whitney significance tests of mitotic cells with respect to data plotted in the first column are indicated: (NS) U>0.05.
The role of confinement on cell mechanics and actomyosin cortex

Next, we wanted to understand the influence of the degree of confinement of mitotic cells on the actomyosin cortex and cell mechanics. We exposed STC arrested mitotic cells to increasing degrees of confinement (16, 14, 12, 10 and 8 µm) and correlated cell mechanics with enrichment of cortical F-actin and myosin. As dictated by cell geometry, with increasing confinement the maximum cell radius increased while there was no significant change in the cell volume (Fig 3.5A). The enrichment of cortical F-actin and myosin II was independent of the extent of confinement throughout the range of 16 µm to 8 µm (Fig 3.5B). Although the force exerted by the cell on the cantilever increased with increasing confinement, there was a reduction in the intracellular pressure and cortex tension (Fig 3.5C). This decrease in intracellular pressure and cortex tension could be due to the increase in the surface area of the cell cortex upon confinement. The increase in cortex surface area would reduce the number of myosin motors per cortical area, which in turn could reduce cortex tension and intracellular pressure. Alternatively, cellular blebbing at confinements of 10 µm and 8 µm could also release intracellular pressure and reduce cortex tension. These results show that with increased confinement, the enrichment of cortical F-actin and myosin does not change. Furthermore, cell confinement does not induce a rise in intracellular pressure and cortical tension. Hence, the height of confinement is not critical for the intracellular pressure and cortex tension measurements. These results also indicate that the influence of intracellular organelles in resisting confinement is not substantial.
Figure 3.5 Increasing cell confinement does not increase cortex tension and intracellular pressure in mitotic cells. (A) Plot of cell radius and volume of STC arrested mitotic MYH9-GFP Lifeact mCherry expressing HeLa cells subjected to imposed confinement. The cells were confined to heights indicated (16 µm, n=15; 14 µm, n=11; 12 µm, n=14; 10 µm, n=13; 8 µm, n=11) and measured at least for 60 seconds after confinement was established. (B) Quantification of cortex/cytoplasm ratio and cortical FWHM of F-actin (red) or myosin II (green). The cells were confined to heights indicated and measured 60 seconds after confinement was established. (C) Quantification of transient and persistent force (purple), pressure (grey) or tension (blue) of cells. The diamond-box contains 25-75% percentiles of the data and the bar denotes the median. Mann-Whitney significance tests of mitotic cells with respect to data plotted in the first column are indicated: (NS) U>0.05, (*) 0.05≥U>0.005, (**) U≤0.005.
Comparing the mechanics of interphase and mitotic cells

To verify the applicability of imposed confinement, we applied the assay on rounded interphase or arrested mitotic cells (Fig. 3.6A). The transient and persistent pressures as well as tensions of mitotic cells were over seven-fold higher than that of interphase cells (Fig. 3.6B). This suggests that the enrichment of cortical F-actin and myosin II during mitosis (Fig. 2.4, 2.7) plays an essential role in resisting deformation upon confinement both transiently (<5 s) and for longer durations (>60 s). The imposed confinement assay has several advantages: it is comparable to the already established trans-mitotic assay, can replicate results from earlier studies, improve the throughput and reduce the spread of intracellular pressure as well as cortex tension measurements of rounded cells (Fig. 2.7, 3.4, 3.6). Hence, we will use the imposed confinement with STC arrest for characterization of rounded mitotic cells in the rest of the study.

Figure 3.6. Interphase cells cannot sustain hydrostatic pressure at any time scale. (A) Average force traces of MYH9-GFP Lifeact-mCherry expressing HeLa cells confined to a height of 10 µm. Time zero represents arrival at the height of 10 µm after the AFM-cantilever descended at a speed of 1 µm s\(^{-1}\). Cells were either round interphase (red trace) or STC-arrested in mitosis (orange trace). (B) Transient and persistent pressure (grey) or tension (blue) of cells under confinement. Cells were either round interphase (n=7) or STC-arrested in mitosis (n=7).
The differential roles of cortical F-actin and myosin II in mitotic rounding

Having established the imposed confinement of STC-arrested cells as an appropriate assay, we employed inhibitors to test the roles of F-actin and myosin II in sustaining pressure and tension. Myosin II and F-actin were perturbed with blebbistatin and latrunculin A, respectively (3.7A,B). Latrunculin A (200 nM) treatment abolished cortical enrichment of F-actin and myosin, leaving only punctate aggregates while, interestingly, blebbistatin (20 µM) did not perturb the enrichment of cortical F-actin or myosin (Fig. 3.1, 3.7A,B). These blebbistatin results confirm that, unlike in cases such as migrating endothelial cells\(^9^5\), tension provided by myosin II is not required for F-actin recruitment and maintenance of cytoskeletal architecture in mitotic cells.

As for the mechanical response, confined cells treated with latrunculin A lost the ability to produce substantial pressure or tension both transiently (<5 s) and for longer durations (>60 s). In contrast, cells treated with blebbistatin showed only slight reductions in transient pressure and tension, while the persistent pressure and tension decayed to less than half over ~60 s (Fig. 3.7C-E).

In order to verify these results, we used RNAi to perturb the enrichment of cortical F-actin, with Ect2 siRNA\(^1^7\), and myosin II, with MYH9 siRNA\(^9^3\) (Fig. 3.8A,B). The mechanics of mitotic cells from siRNA experiments showed the same trends as the latrunculin A and blebbistatin experiments. Perturbation with Ect2 siRNA affected both the transient and persistent response while MYH9 siRNA affected only persistent pressures and tensions (Fig. 3.8C,D). Therefore, in response to imposed confinement with the AFM cantilever descending at a speed of 1 µm/s, our results demonstrate that the short term response (<10 s) is dominated by resistance of the F-actin cortical meshwork to cortex surface area expansion. This is in the same time scale as the turnover of cortical actin (half-time, 5.9±1.1 s) measured by photobleaching (Fig. 3.2). Hence, after several tens of seconds, the stretched elements of cortical F-actin can turnover and relax. Subsequently, the persistent tension and pressure are set by baseline myosin II contractility. These results complement the trans-mitotic data (Fig. 2.7) and provide insight into how cortical myosin II sustains persistent pressure during mitosis.
Figure 3.7. Myosin II inhibited mitotic cells are unable to sustain persistent pressure. (A) Representative confocal images of STC-arrested mitotic MYH9-GFP Lifeact-mCherry expressing HeLa cells confined to a height of 10 µm. Cells were untreated or pre-incubated for at least 30 minutes with latrunculin A (LatA) or blebbistatin (Blebb). Scale bars, 10 µm. (B) Quantification of cortex/cytoplasm ratio of F-actin (red) and myosin II (green) in STC-arrested mitotic MYH9-GFP Lifeact-mCherry expressing HeLa cells confined to a height of 10 µm. Cells were untreated (n=16) or pre-incubated for at least 30 minutes with latrunculin A (LatA, n=7) or blebbistatin (Blebb, n=10). (C) The illustration shows the wedged cantilever assay used to determine transient and persistent pressure and tension of an (Figure 3.7 is continued in the next page)
(Figure 3.7 continued)

STC-arrested mitotic cell. (D) Average force traces of STC-arrested mitotic MYH9-GFP Lifeact-mCherry expressing HeLa cells confined to a height of 10 µm. Time zero represents arrival at the height of 10 µm after the AFM-cantilever descended at a speed of 1 µm s⁻¹. Cells were untreated, treated with latrunculin A or blebbistatin. (E) Transient and persistent pressure (grey) or tension (blue) of cells under confinement. Cells were untreated (n=16) or pre-incubated for at least 30 minutes with latrunculin A (n=7) or blebbistatin (n=10). The diamond-box contains 25-75% percentiles of the data and the bar denotes the median. Mann-Whitney significance tests of mitotic cells with respect to data plotted in the first column are indicated: (*) 0.05≥U>0.005, (**) U≤0.005.
Figure 3.8. Mitotic cells transfected with Ect2 siRNA are compromised in sustaining both transient and persistent pressure. (A) Representative confocal images of STC-arrested mitotic MYH9-GFP/Lifeact-mCherry expressing HeLa cells confined to a height of 10 µm. Cells were transfected at least 48 hours prior to experiments with control (n=9), Ect2 (n=13) or MYH9 (n=10) siRNA. Scale bars, 10 µm. (B) Cortex/cytoplasm ratios of F-actin (red) and myosin II (green). Cells were transfected at least 48 hours prior to experiments with control (n=9), Ect2 (n=13) or MYH9 (n=10) siRNA. (C) Average force traces of STC-arrested mitotic MYH9-GFP/Lifeact-mCherry expressing HeLa cells confined to a height of 10 µm. Time zero represents arrival at the height of 10 µm after the AFM-cantilever descended at a speed of 1 µm s⁻¹. Cells were transfected with control (orange, n=9), Ect2 (red, n=13) or MYH9 (purple, n=10) RNAi. (D) Transient or persistent pressure (grey) and tension (blue) of mitotic cells under confinement. Cells were transfected at least 48 hours prior to experiments with control (n=9), Ect2 (n=13) or MYH9 (n=10) siRNA.
Mitotic rounding is characterized by a progressive increase in cortical myosin enrichment accompanied by an augmentation of intracellular pressure (Fig 2.7). In this chapter, we explore how mitotic cell mechanics is influenced by the enrichment of cortical myosin. We find that the activity of myosin does not influence the enrichment or turnover of cortical F-actin in mitosis. Next, we develop the imposed confinement assay to track the mechanical response of STC treated cells arrested in mitosis in short (<10 s) as well as long (>60 s) time scales. The imposed confinement assay revealed the importance of cortical myosin in resisting confinement. While the F-actin cortex devoid of myosin can resist confinement only for short timescales, myosin activity was essential to resist confinement over timescales relevant to mitosis.

The imposed confinement assay developed here presents the following advantages over the trans-mitotic assay used in the previous chapter:

1. The cortex tension and intracellular pressure of round cells can be measured more quickly.
2. Data thus acquired is of higher quality because the arrested cells are in the same mitotic state.
3. The assay can distinguish between the mechanical contributions of cortical F-actin and myosin.
4. The round mitotic cells are arrested with STC before the introduction of perturbants. Hence, the assay allows us to study the effect inhibitors that can prevent mitotic rounding.

Because of these advancements, we are now ideally placed to systematically interrogate the role of actomyosin regulators in mitotic rounding using the imposed confinement assay.
4. REGULATION OF MITOTIC ROUNding

Role of Rho GTPases: RhoA, Rac1 and Cdc42

We wanted to investigate the role of signaling pathways known to regulate the actomyosin cytoskeleton in determining mitotic cell mechanics. While the role of Ect2 in regulating the actomyosin contractile ring in cytokinesis is established, Matthews et al., 2012 report that Ect2 is activated at the start of mitosis and its activity is essential for the stiffness of mitotic cells. In agreement with this result, we observe that Ect2 is involved in cortical enrichment of F-actin and myosin in mitosis and is essential for intracellular pressure as well as cortex tension (Fig. 3.8). Therefore, we sought to address effectors downstream of Ect2, which can activate several major Rho GTPases that regulate actomyosin dynamics, including RhoA, Rac1 and Cdc42. We analyzed the mechanics of mitotic cells treated with inhibitors against each of these Rho GTPases. The RhoA inhibitor, C3-toxin, almost totally abolished cortical tension while neither inhibitors of Rac1, NSC23766 and EHT1864, nor of Cdc42, Pirl1 and ML141, consistently reduced cortical tension by more than 20% (Fig. 4.1).

Figure 4.1. Unlike RhoA; perturbation of Rac1 or Cdc42 does not affect mitotic cortex tension or intracellular pressure. Plotted are persistent pressure (grey) and tension (blue) of STC-arrested mitotic HeLa cells confined to a height of 10 µm. The cells were untreated (n=84) or incubated with C3-toxin (n=11, 8 and 18), NSC23766 (NSC, n=47 and 12), EHT1864 (EHT, n=37 and 15), Pirl1 (Pirl, n=46 and 32) or ML141 (n=35 and 22) at concentrations given. The diamond-box contains 25-75% percentiles of the data and the bar denotes the median. Mann-Whitney significance tests of cells with respect to data plotted in the control column are indicated: (NS) $U>0.05$, (*) $0.05\leq U>0.005$, (**) $U\leq 0.005$. 

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To confirm these results, STC arrested mitotic HeLa cells transiently expressing GFP tagged wild type, dominant positive (DP) and dominant negative (DN) mutants of the three Rho GTPases RhoA, Rac1 and Cdc42 were tested. Except for the control condition where cells were transfected without plasmids, only cells expressing GFP were examined. Among the cells transfected with mutant RhoA plasmids, only DP RhoA transfected cells showed significantly higher cortical tension (3.23±0.17 mN m⁻¹, n=21 for DP RhoA versus 1.02±0.07 mN m⁻¹, n=47 for no plasmid control). None of the Rac1 and Cdc42 constructs changed cortical tensions significantly (Fig. 4.2). Previous results have shown that RhoA is required for mitotic cell stiffness and for maintaining cortical F-actin in mitosis. Our results are consistent with this finding and show that RhoA activity is essential for elevated intracellular pressure and cortex tension in mitotic cells. Furthermore, these results also suggest that neither Rac1 nor Cdc42 are required to maintain intracellular pressure and cortex tension in mitotic cells.

**Figure 4.2.** Constitutively active RhoA elevates intracellular pressure and cortex tension in mitosis. Plotted are persistent pressure (grey) and tension (blue) of STC-arrested mitotic HeLa cells confined to a height of 10 µm. The cells were transfected with plasmid expressing either wild type (WT RhoA, n=17; WT Rac1, n=11 and WT Cdc42, n=17), dominant positive (DP RhoA, n=21; DP Rac1, n=19 and DP Cdc42, n=19) or dominant negative (DN RhoA, n=23; DN Rac1, n=15 and DN Cdc42, n=15) mutants of RhoA, Rac1 or Cdc42. No-plasmid (n=47) and GFP (n=51) plasmid transfections are the controls. The diamond-box contains 25-75% percentiles of the data and the bar denotes the median. Mann-Whitney significance tests of cells with respect to data plotted in the control column are indicated: (NS) \( U > 0.05 \), (*) \( 0.05 \geq U > 0.005 \), (**) \( U \leq 0.005 \).
Mechanism of how RhoA sustains mitotic rounding

To further probe the action of RhoA on the actomyosin cortex, we treated mitotic cells with the RhoA activator, calpeptin\textsuperscript{103}, and the RhoA inhibitor, C3-toxin. Calpeptin treatment induced extensive blebbing and increased the cortical myosin II accumulation by 40% (Fig. 4.3, 4.4A,B). Compared to untreated cells, calpeptin treated cells produced substantially higher pressure or tension both transiently (<5 s) and for longer durations (>60 s) (Fig 4.4C,D). In contrast, C3-toxin abolished pressure and tension, and markedly decreased the enrichment of cortical F-actin and myosin II (Fig. 4.4A,B). Furthermore, cells treated with C3-toxin lost the ability to produce substantial pressure or tension both transiently (<5 s) and for longer durations (>60 s) (Fig 4.4C,D). These results show that RhoA activity is essential for the enrichment of F-actin and myosin at the cell cortex, and that RhoA controls mitotic cell mechanics by regulating the enrichment of cortical F-actin and myosin II.

Figure 4.3. Activation of RhoA or inhibition of Pak1 increases mitotic blebbing. Plots characterizing STC-arrested mitotic HeLa cells expressing Lifeact-mCherry and MYH9-GFP that were untreated (n=82) or pre-incubated for at least 30 minutes with latrunculin A (LatA, n=10 and 7), blebbistatin (Blebb, n=10 and 10), calpeptin (Calp, n=10 and 9), C3-toxin (C3, n=7 and 7), Y27632 (Y27, n=10 and 10), SMIFH2 (SMI, n=11 and 10), CK666 (CK, n=10 and 10) or IPA3 (n=9 and 10) at the concentrations given (method as in Fig. 3.3). Bars (grey) indicate the percentage of round mitotic cells that blebbed when confined to 10 µm. Number of cells tested is noted above each bar.
Figure 4.4. RhoA sustains enrichment of cortical F-actin and myosin II.
(A) Representative confocal images of STC-arrested mitotic MYH9-GFP Lifeact-mCherry expressing HeLa cells confined to a height of 10 µm. Cells were untreated or pre-incubated for at least 30 minutes with calpeptin (Calp) or C3-toxin (C3) at concentrations given. Scale bars, 10 µm. (B) Quantification of cortex/cytoplasm ratio of F-actin (red) and myosin II (green) in STC-arrested mitotic MYH9-GFP Lifeact-mCherry expressing HeLa cells confined to a height of 10 µm. Cells were untreated or pre-incubated for at least 30 minutes with calpeptin (n=10 and 9) or C3-toxin (n=7 and 7) at concentrations given. (C) Average force traces of STC-arrested mitotic MYH9-GFP Lifeact-mCherry expressing HeLa cells, confined to a height of 10 µm. Time zero represents arrival at the height of 10 µm after the AFM-cantilever descended at a speed of 1 µm s⁻¹. Cells were untreated (orange) or perturbed as indicated (purple). (D) Transient and persistent pressure (grey) or tension (blue) of cells under confinement.
**Role of kinases: Rho kinase and PAKs**

Rho kinase, a RhoA effector, regulates both F-actin and myosin in interphase cells. By phosphorylating LIM kinase, which down-regulates cofilin — an F-actin severing protein — Rho kinase stimulates F-actin assembly\(^{104}\). Rho kinase also stimulates myosin II activity directly through myosin phosphorylation and indirectly by inhibiting myosin phosphatase\(^{105,106}\). Indeed, Rho kinase has been shown to be essential for retraction of the cell cortex from the substrate during mitosis\(^{16}\). We asked whether Rho kinase activity influences the mitotic actomyosin cortex. In mitotic cells treated with Rho kinase inhibitor, Y27632\(^{107}\), the cortical myosin enrichment was reduced to less than than 50%. In contrast, there was no reduction in cortical F-actin in Y27632 treated mitotic cells ([Fig. 4.5A,B](#)). There was also no decrease in the transient tension and pressure of Y27632 treated mitotic cells, although the persistent tension and pressure was reduced to less than 50% ([Fig. 4.5C,D](#)). Ect2 and RhoA, both upstream regulators of Rho kinase, were essential for the enrichment of cortical F-actin and myosin II in mitosis ([Fig. 3.8, 4.4](#)). Interestingly, here we observe that Rho kinase activity is essential only for the enrichment of cortical myosin II but not cortical F-actin.

p21-activated kinases (PAKs) is a family of myosin kinases downstream of Rac1 and Cdc42\(^{108}\). PAKs are essential for mitotic progression and are phosphorylated by Cdk1\(^{109,110}\). To examine the role of PAKs in regulating the mitotic actomyosin cortex, we used PAK1-3 inhibitor IPA3\(^{111}\) ([Fig. 4.5A](#)). Upon PAK1-3 inhibition, mitotic cells blebbed extensively while cortical myosin II was enriched by up to 35% ([Fig. 4.3, Fig. 4.5B](#)). Despite this, PAK inhibited cells did not exhibit altered cortical tension, perhaps because blebbing released excess intracellular pressure ([Fig. 4.5C,D](#)). In interphase cells, PAK1 activity has been shown to phosphorylate myosin light chain kinase and thus inhibit myosin II activity\(^{112}\). Our results show that PAKs activity inhibits myosin II in mitosis as well.
Figure 4.5. Localization of myosin II depends on Rho kinase activity. (A) Representative confocal images of STC-arrested mitotic MYH9-GFP Lifeact-mCherry expressing HeLa cells confined to a height of 10 µm. Cells were untreated or pre-incubated for at least 30 minutes with Y27632 (Y27) or IPA3 at concentrations given. Scale bars, 10 µm. (B) Quantification of cortex/cytoplasm ratio of F-actin (red) and myosin II (green) in STC-arrested mitotic MYH9-GFP Lifeact-mCherry expressing HeLa cells confined to a height of 10 µm. Cells were untreated or pre-incubated for at least 30 minutes with Y27632 (n=10 and 10) or IPA3 (n=9 and 10) at concentrations given. (C) Average force traces of STC-arrested mitotic MYH9-GFP Lifeact-mCherry expressing HeLa cells, confined to a height of 10 µm. Time zero represents arrival at the height of 10 µm after the AFM-cantilever descended at a speed of 1 µm s⁻¹. Cells were untreated (orange) or perturbed as indicated (purple). (D) Transient and persistent pressure (grey) or tension (blue) of cells under confinement.
The activity of Cdk1 is increases sharply at the start of mitosis\textsuperscript{50}. Since PAK1 is phosphorylated by Cdk1\textsuperscript{110} and our results show PAKs to be inhibitors of myosin II (Fig. 4.5), we proceeded to investigate the role of PAKs in regulating myosin from prophase to metaphase. After NEBD, IPA3 treated mitotic cells accelerated the recruitment of myosin II to the cortex (Fig. 4.6A). This was accompanied by a four-fold increase in the rate of pressure generation (Fig. 4.6B, C). These results indicate that at the start of mitosis, the activity of PAKs inhibit the recruitment of cortical myosin II. Thus, in early mitosis, PAKs function as negative regulators of myosin activity.

Figure 4.6. Pak inactivated mitotic cells exhibit accelerated intracellular pressure buildup in early mitosis. (A) Fluorescence time-lapse images of two MYH9-GFP H2B-mCherry expressing HeLa cell confined to a height of 10 µm. The cells measured were untreated or pre-incubated for at least 3 hours with 20 µM IPA3. The measurements started at NEBD (defined as time zero). Scale bar, 20 µm, applies to all images. (B) Representative plots of the intracellular pressure of the cells confined to a height of 10 µm. (C) The maximum pressure attained during mitotic progression (max pressure) and the time taken from NEBD until a pressure of 300 Pa was reached (time to 300 Pa) by MYH9-GFP H2B-mCherry expressing HeLa cell. Cells were untreated (n=8) or pre-incubated for at least 3 hours with 20 µM IPA3 (n=5). The diamond-box contains 25-75% percentiles of the data and the bar denotes the median. Mann-Whitney significance tests of mitotic cells with respect to data plotted in the first column are indicated: (NS) $U>0.05$, (** $U \leq 0.005$).
Although Ect2 is already activated at the start of mitosis\textsuperscript{17}, the enrichment of cortical myosin is gradual and it closely coincides with the buildup of cortex tension (Fig 2.7). We questioned if this gradual buildup of cortical myosin could be simulated by linking the opposed activities of mitotic myosin activator — Rho kinase — and inhibitor — PAKs — by co-introducing 60 µM IPA3 and Y27632 (0, 0.1, 0.5, 1.25, or 2.5 µM) to mitotic cells (Fig 4.7A). IPA3 induced cortical enrichment of myosin was reversed by Y27632 treatment (Fig 4.7B). Introduction of another PAK1-3 inhibitor, FRAX486, to mitotic cells also increased the enrichment of cortical myosin (Fig. 4.7C column - 0 µM Y27). Furthermore, the results from Fig 4.7B could be reproduced by the introduction 10 µM FRAX486 instead of IPA3 (Fig 4.7C). These results suggest that the inhibitory action of PAKs in regulating the kinetics of myosin II recruitment to the mitotic cortex could be opposed by Rho kinase mediated myosin stimulation.

Figure 4.7. Rho kinase and PAKs play opposing roles in regulating cortical myosin II enrichment. (A) Scheme to probe the opposing roles of Rho kinase and PAKs in regulating cortical myosin II enrichment. STC-arrested mitotic MYH9-GFP and Lifeact-mCherry expressing HeLa cells were treated with Rho kinase and PAK1-3 inhibitors simultaneously (Figure 4.7 continued in the next page)
while the enrichment of cortical myosin II and F-actin were tracked. (B) Myosin II and F-actin cortex/cytoplasm ratio of mitotic cells. The data plotted are taken eight minutes prior to (control, $n=71$) or 20 minutes after the introduction of 60 µM IPA3 and Y27632 (0 µM, $n=12$; 0.1 µM, $n=8$; 0.5 µM, $n=15$; 1.25 µM, $n=11$; 2.5 µM, $n=15$) at the indicated concentration. (C) Myosin II and F-actin cortex/cytoplasm ratio of mitotic cells. The data plotted are eight minutes prior to (control, $n=50$) or 20 minutes after the introduction of 10 µM FRAX486 and Y27632 (0 µM, $n=8$; 0.1 µM, $n=7$; 0.5 µM, $n=10$; 1.25 µM, $n=8$; 2.5 µM, $n=10$) at the indicated concentration. The diamond-box contains 25-75% percentiles of the data and the bar denotes the median. Mann-Whitney significance tests of cells with respect to data plotted in the untreated column are indicated: (NS) $U>0.05$, (*) $0.05 \geq U>0.005$, (**) $U \leq 0.005$.

**Role of actin nucleators: formins and Arp2/3**

We have established that, in mitotic cells, Ect2 and RhoA regulate the mechanics and the cortical enrichment of both F-actin and myosin II (Fig. 3.8, 4.4). Interestingly, Rho kinase, which is downstream of Ect2 and RhoA, regulates enrichment of cortical myosin II without affecting F-actin (Fig. 4.5). While myosin II modulates the cortical tension and intracellular pressure in mitosis, a critical amount of F-actin is obviously required to host myosin II. Indeed, F-actin is more enriched at the mitotic cortex compared to interphase (Fig. 2.4). To understand how cortical F-actin is nucleated and maintained in mitosis, we investigated the role of the F-actin regulators, formins, in mitotic rounding. We treated cells with a broad spectrum formin inhibitor SMIFH2 and correlated mitotic enrichment of F-actin and myosin enrichment at the cortex with cell mechanics (Fig. 4.8A). Inhibition of formins nearly abolished cortical tension accompanied by a drastic decrease cortical F-actin and myosin II enrichment (Fig. 4.8C-D). An RNAi screen of select formins revealed DIAPH1 to be essential for enrichment of cortical F-actin (Fig. 4.9). These results reveal that formins, in particular DIAPH1, are essential for maintaining the enrichment of cortical F-actin during mitosis.
Next, we investigated the role of another major F-actin regulator, Arp2/3\textsuperscript{115}, in regulating the mitotic F-actin cortex. In contrast to inhibition of formins, the addition of Arp2/3 specific inhibitor, CK666\textsuperscript{116}, reduced neither the cortical tension nor cortical F-actin enrichment (Fig. 4.8).

**Figure 4.8.** Cortical F-actin scaffolding is sustained by formin-mediated nucleation. (A) Representative confocal images of STC-arrested mitotic MYH9-GFP Lifeact-mCherry expressing HeLa cells confined to a height of 10 \(\mu\)m. Cells were untreated or pre-incubated for at least 30 minutes with SMIFH2 (SMI) or CK666 (CK) at concentrations given. Scale bars, 10 \(\mu\)m. (B) Quantification of cortex/cytoplasm ratio of F-actin (red) and myosin II (green) in STC-arrested mitotic MYH9-GFP Lifeact-mCherry expressing HeLa cells confined (Figure 4.8 continued in the next page)
(Figure 4.8 continued)

to a height of 10 µm. Cells were untreated or pre-incubated for at least 30 minutes with SMIFH2 (n=11 and 10), CK666 (n=10 and 10) at concentrations given. (C) Average force traces of STC-arrested mitotic MYH9-GFP Lifeact-mCherry expressing HeLa cells, confined to a height of 10 µm. Time zero represents arrival at the height of 10 µm after the AFM-cantilever descended at a speed of 1 µm s\(^{-1}\). Cells were untreated (orange) or perturbed as indicated (purple). (D) Transient and persistent pressure (grey) or tension (blue) of cells under confinement. The diamond-box contains 25-75% percentiles of the data and the bar denotes the median. Mann-Whitney significance tests of cells with respect to data plotted in the control column are indicated: (NS) \(U>0.05\), (*) \(0.05\geq U>0.005\), (**) \(U\leq0.005\).
Figure 4.9. DIAPH1 siRNA transfected mitotic cells are compromised in cortical F-actin enrichment. (A) Representative confocal images of STC-arrested mitotic MYH9-GFP and Lifeact-mCherry expressing HeLa cells confined to a height of 10 µm. Cells were transfected at least 48 hours prior to experiments with control, MYH9, DIAPH1_1 (C1) or DIAPH1_2 (C2) siRNA. Both C1 and C2 target the same DIAPH1 gene. Scale bars, 10 µm. (B) Cortex/cytoplasm ratios of F-actin (red) and myosin II (green). Cells were transfected at least 48 hours prior to experiments with control (n=10), MYH9 (n=10), DIAPH1_1 (C1, n=10) or DIAPH1_2 (C2, n=8) siRNA. (C) Transient or persistent pressure (grey) and tension (blue) of mitotic cells under confinement. The diamond-box contains 25-75% percentiles of the data and the bar denotes the median. Mann-Whitney significance tests of cells with respect to data plotted in the control column are indicated: (NS) U>0.05, (*) 0.05≥U>0.005, (**) U≤0.005.
In mitotic cells, dynamic clusters of F-actin have been reported to revolve along the cortex in the cytosol with a constant angular speed. This phenomenon is Arp2/3 dependent\textsuperscript{117}. We wanted to confirm whether Arp2/3 was actually inhibited by CK666 in mitotic cells. Within 20 minutes after CK666 introduction to arrested mitotic cells, we found that the cytosolic F-actin clusters start to disappear (Fig. 4.10). This indicates that CK666 inhibits Arp2/3 activity in mitosis. These results show that, unlike formins, Arp2/3 does not contribute to maintaining cortical F-actin enrichment in mitosis. Indeed, formin-nucleated bundled F-actin networks tend to be more conducive to host myosin II contractility as opposed to Arp2/3-mediated branched F-actin\textsuperscript{118}, and this pattern holds true in the case of the mitotic cortex.

Figure 4.10. Monitoring Arp2/3 dependent revolving clusters in mitotic cells. Shown are confocal images of Lifeact-mCherry to visualize amorphous cytosolic F-actin structures in STC-arrested mitotic MYH9-GFP and Lifeact-mCherry expressing HeLa cells. Images were acquired every 2 minutes. The acquisition time is indicated in the top left corner. At time zero, 40 µM CK666 was introduced to previously untreated medium. An asterisk indicates the presence of amorphous cytosolic F-actin structures in the vicinity. Scale bar of 10 µm applies to all images.
Cdk1 and its downstream effector, Ect2, regulate the actomyosin cortex and determine cell mechanics in mitosis (Fig. 2.11, 3.8). In this chapter, we investigate the signaling pathway downstream of Cdk1 using the imposed confinement assay. We find RhoA activity to be indispensable for cortical F-actin and myosin enrichment. On the other hand Cdc42 and Rac1 are not essential for the mitotic actomyosin cortex. Although Rho kinase is downstream of RhoA, its activity is essential for the enrichment of only cortical myosin but not F-actin. Additionally, PAKs have emerged as negative regulators of cortical myosin enrichment. Our data indicates that the progressive accumulation of myosin to the mitotic cell cortex likely requires the activation of PAKs, which inhibit myosin recruitment, and of Rho kinase, which stimulates myosin recruitment to the cortex.

Since Rho kinase does not regulate cortical F-actin, we investigated other regulators of F-actin. Formins, which are downstream RhoA and nucleate F-actin by bundling, regulate F-actin in mitosis. Furthermore, Arp2/3, which nucleate F-actin by bundling, is not essential for cortical F-actin in mitosis. Our data indicate that, during mitosis, cortical F-actin is predominantly nucleated by actin-bundling mechanism as opposed to actin branching.

In the second chapter, we discovered that, during mitosis, the cortical enrichment of F-actin remains stable while myosin II progressively accumulates at the cortex. The results from this chapter reveal how the signaling pathway downstream of Cdk1 regulates the dynamics of cortical F-actin and myosin accumulation in mitosis.
5. DISCUSSION

The ability of mitotic cells to resist deformation during mitosis \textit{in vivo} is central to tissue morphogenesis and maintenance\textsuperscript{5-7}. Mitotic rounding defects in tissue leads to loss of spindle orientation, cell delamination and epithelial-to-mesenchymal transition (EMT) which can in turn trigger the formation of basally localized tumor-like masses\textsuperscript{9}. Our study simulates such confined environments and elucidates the roles of enrichment of cortical F-actin and myosin II in resisting confinement during mitotic rounding (\textbf{Fig. 5.1}).

\textbf{Figure 5.1.} 2D-confinement assay mimics the confined environment of mitotic cells in tissue. Schematic depicting the morphological changes from prometaphase to metaphase in different environments. (\textbf{A}) Mitosis in 2D cell culture. At early prometaphase, F-actin (red) reorganizes into a uniform cortex while myosin II (blue) is in the cytoplasm. At metaphase, cortical F-actin enrichment remains unchanged while myosin II (green) accumulates at the cell cortex. (\textbf{B}) Mitosis in 2D cell culture with confinement. At prometaphase, the mitotic cell does not produce force against the cantilever upon confinement. Enrichment of cortical myosin II, during mitosis, produces mitotic force against the confinement. (\textbf{C}) Mitosis in tissue environment. Mitotic cell is not round at the start of mitosis. With mitotic progression the cell rounds up and deforms the surrounding tissue.
In this thesis, we analyze the relationship between cortical actomyosin dynamics and mechanical properties in mitotic cells. To do this, we used microcantilever-based uniaxial confinement to measure the intracellular pressure and cortical tension of mitotic cells while simultaneously imaging the cell cortex with confocal fluorescence microscopy. As cells progressed through mitosis, we find that both F-actin (~2:1 cortex/cytoplasm ratio) and myosin II (~3:1 cortex/cytoplasm ratio) converge toward a stable enrichment at the mitotic cell cortex and were comparable to observations in fixed samples \(^7,17\). Our experiments revealed that progressive accumulation of myosin II determines the capacity of cells to produce persistent pressure by generating tension in the cortex, while F-actin enrichment remains unchanged and does not correlate with intracellular pressure and cortex tension (Fig 5.2A).

Furthermore, as this myosin II-based tension does not affect the enrichment, thickness, or turnover of cortical F-actin, we conclude that myosin II activity is essential for >90% of tension and pressure generated by mitotic cells.

Once cortical myosin II accumulation saturates in mitosis, pressure and tension reach a stable pre-anaphase plateau. By arresting cells in this state with STC, it was possible to systematically probe the mechanical behavior and biochemical regulation of the mitotic cortex. These experiments involved deforming a round cell by imposing uniaxial confinement and analyzing the response dynamics. We observed a characteristic viscoelastic response with pressure and tension reaching a transient peak upon initial deformation before decaying to persistent values, which were not significantly different from the stable pre-anaphase pressure measured in trans-mitotic experiments. This transient peak was dependent only on the enrichment of cortical F-actin and not myosin II. This indicates that the transient peak is a result of the increased tension due to cell surface area expansion upon confinement. Subsequently, cortical F-actin is remodeled to dissipate cortical tension. Indeed, the time required for the transient mechanical response to dissipate correlated closely with F-actin turnover times measured by fluorescence recovery after photobleaching (FRAP) (~5s).
Figure 5.2. Trans-mitotic characteristics of the actomyosin cortex and resultant intracellular pressure and cortical tension. (A) Schematic depicting transformation of the actomyosin cytoskeleton throughout mitosis. During interphase, F-actin (red) and myosin II (green) are predominantly associated with basal adhesions and stress fibers. At the start of mitosis (early prometaphase), F-actin reorganizes into a uniform cortex while myosin II (blue) is in the cytoplasm. As the cell proceeds through mitosis, cortical F-actin enrichment remains unchanged (red trace) while myosin II (green trace) accumulates at the cell cortex concomitant with a rise in cortical tension (blue trace) and intracellular pressure (black trace). When the cell enters anaphase, myosin II localizes to the cleavage furrow to constrict the contractile ring, thereby initiating cytokinesis. (B) Regulatory pathways, from the literature and this study, involved in generating persistent cortical tension and intracellular hydrostatic pressure during mitosis. Links with arrowheads, bars and dots denote stimulation, inhibition and no discernable effect, respectively. Relevant studies are denoted alongside the links. Blue and teal links denote mitotic rounding-specific and general relationships, respectively. Brown and grey links indicate mitosis-specific relationships confirmed in this study. The relative thickness of grey links indicates the strength of the relationship.
In contrast, the persistent cortical tensions and intracellular pressures are dependent on myosin II activity at the remodeled cortical F-actin. Hence, as in the trans-mitotic case, the persistent mitotic pressure was set by the cortical enrichment of myosin II. In a tissue scenario, we propose that the role of F-actin is to counter fast deformations and host myosin II, while myosin II generates active cellular forces that push against confinement over the timespan of mitosis, thereby setting the persistent pressure and tension. Others have used microneedles, AFM or optical cell stretchers and observed that inhibition of upstream actomyosin regulators or accessory proteins compromise the ability of mitotic cells to resist deformations imposed over several seconds\textsuperscript{16,30,17}. Our experiments place these results in the framework of whole-cell mechanics and dissect the contribution of F-actin and myosin II at different timescales, ranging from seconds to minutes.

We then turned our attention to another key question: which biochemical pathways are involved in regulating the mitotic mechanics and enrichment of cortical F-actin and myosin II? While much is known about biochemical regulation of actomyosin dynamics in cytokinesis, cell migration and epithelial morphogenesis, the regulation of the mitotic cortex is relatively poorly explored. As with most events in mitosis, the regulation of the mitotic cortex must be underpinned to some extent by Cdk1/Cyclin B activity\textsuperscript{119}. Indeed, threshold levels of Cdk1/CyclinB activation correlate with NEBD, cell rounding, and mass phosphorylation of actomyosin proteins\textsuperscript{50,50,52}. The Cdk1 substrate, Ect2, was recently shown to regulate mitotic rounding and localization of actin by activating RhoA\textsuperscript{17}. Our RNAi results confirm this and demonstrate that both F-actin and myosin II cortical localization require Ect2 and RhoA. However, we found that Rho kinase, a downstream effector of RhoA, only influenced myosin II and not F-actin localization. Inhibitors against other potential regulatory proteins that govern F-actin assembly indicated that formins, but not Arp2/3, are required for F-actin enrichment at the mitotic cortex. As opposed to Arp2/3-mediated branched F-actin, formin-nucleated bundled F-actin networks tend to be more conducive to host myosin II contractility\textsuperscript{118}. From our study, this is a pattern that holds true in the case of the mitotic cortex. As formins are downstream
of RhoA, our data sheds light on how RhoA, Rho kinase, and formins work together to regulate enrichment of F-actin and myosin II at the mitotic cortex (Fig 5.2B).

As part of our study, we tested another Cdk1 substrate\textsuperscript{109,110}, PAK family kinases. Inhibition of PAK1-3 with the inhibitor IPA3 accelerated accumulation of myosin II to the cortex in early mitosis, indicating that PAKs might be involved in the timing of myosin II accumulation to the cortex. The effect observed in IPA3 treated cells was similar to the consequences of over-activation of RhoA with calpeptin, which points to an antagonistic role of PAK1-3 to Rho kinase signaling. Indeed, PAK inhibition induced cortical myosin enrichment was reversed by simultaneous Rho kinase inhibition. If RhoA is a common regulator of F-actin and myosin II enrichment, the disparity in timing between their trans-mitotic accumulations is an enigma. Since PAK1-3 inhibition augmented myosin II enrichment, it is possible that PAKs act as a secondary regulator of myosin II activity in mitosis. Indeed, PAK1-3 has been reported to attenuate myosin II activity through inhibition of myosin light chain kinase\textsuperscript{112} and Cdk1 dependent activation of PAK1 is at a maximum in early mitosis before diminishing thereafter\textsuperscript{109,110}. Although both formins and Rho kinase are up-regulated by RhoA at prometaphase, the enrichment of cortical F-actin precedes that of myosin. PAKs could inhibit myosin activity at the start of mitosis, thereby allowing staggered accumulation of cortical myosin through mitotic progression. Thus, our work suggests that although F-actin and myosin are under control of RhoA, the cell differentiates between the two using PAKs.
6. OUTLOOK

An introduction to ERMs, the membrane-cortex linkers

Outward acting intracellular pressure opposed by inward acting contractile actomyosin cortex drives mitotic rounding. Previously, we investigated the buildup of intracellular pressure\(^{20}\) and in this thesis we focused on the role of the actomyosin cortex in mitosis. An outstanding question concerning the mechanics of mitotic rounding is the identity and the regulation of the mitotic linker proteins that transduce the contractile forces from the actomyosin cortex to the cell membrane (Fig. 6.1). Ezrin, radixin and moesins, known collectively as ERMs, are linkers of the actomyosin cortex and the membrane\(^{120}\). Although moesin, the sole member of ERM in *Drosophila*, is important for mitotic rounding in *Drosophila* S2 cells\(^{30}\), a corresponding role for ERMs has not been demonstrated in mammals\(^{30,38,121}\). In this chapter, we present preliminary results highlighting the importance of the ERMs in mitotic rounding. Furthermore, we explore new methods that could be developed in order to investigate the role of mitotic membrane-cortex linkers and discuss the possible insights that could be obtained through such a study.

**Figure 6.1. The role of the membrane-cortex linkers in mitotic rounding.** In this depiction of a mitotic cell, the black and red arrows depict the outward acting intracellular pressure and the inward acting contractile actomyosin cortex. The membrane-cortex linkers (blue) couple the forces generated by the actomyosin cortex (red) with the cell membrane (black).
**Ezrin is essential for mitotic rounding**

To investigate the mechanism of how ERM family members influence mitotic rounding in mammalian cells, we tested HeLa cells expressing dominant negative ezrin (TA-ezrin or FERM domain) and dominant positive ezrin (TD-ezrin). Trans-mitotic analysis of cells revealed that expression of TA-ezrin or TD-ezrin resulted in mitotic rounding defects. When compared to the untransfected control, cells expressing mutant ezrin remained adhered to the substrate during metaphase and were unable to completely retract their cell cortex from the substrate to round up (Fig. 6.2A).

Figure 6.2. Ezrin is essential for mitotic rounding. (A) Representative images of mitotic H2B-GFP expressing HeLa cells confined to a height of 10 µm. Cells were transfected with no-plasmid (control), dominant negative ezrin (TA-ezrin) or dominant positive ezrin (TD-ezrin) one day prior to measurements. Scale bars, 10 µm. (B) Representative force traces of mitotic cells, confined to a height of 10 µm. Time zero represent NEBD. The time and force exerted by cell on the cantilever at mitosis (M) and anaphase (A) are indicated within the plot. Cells were transfected with no-plasmid (control) or dominant negative ezrin (TA-ezrin). (C) Plots of the force exerted by the mitotic cell, against the cantilever, at metaphase and anaphase. The diamond-box contains 25-75% percentiles of the data and the bar denotes the median. Mann-Whitney significance tests of mitotic cells with respect to data plotted in the first column are indicated: (NS) $U>0.05$, (*) $0.05\geq U>0.005$, (**) $U\leq0.005$. 

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Interestingly, the force exerted against AFM based confinement by the mitotic cell expressing mutant ezrin was diminished in metaphase but not during anaphase (Fig. 6.3B,C). These results show that ezrin plays an important role in mitotic rounding. The mechanism behind how ezrin strengthens only the pre-anaphase cortex, but does not seem to play a role in anaphase remains to be investigated.

**Role of NHE1 in maintaining cortical F-actin enrichment**

NHE1, the primary Na+/H+ exchanger in most cells\(^{123}\), is crucial for maintaining intracellular pressure and it has been suspected that it could be involved in increasing osmotic pressure inside the mitotic cell\(^{19,20}\). Furthermore, NHE1 directly binds to ezrin and regulates the actin cytoskeleton in interphase fibroblasts cells\(^{123}\) (Fig. 6.3). In order to understand the role of NHE1 activity on the mitotic actomyosin cortex, we tested mitotic cells treated with the NHE1 inhibitor EIPA\(^{124}\). Both transient and persistent pressures as well as tensions were reduced by 50% in EIPA treated cells (Fig 6.4A). This was accompanied by reduction in cortical enrichment of both F-actin and myosin II (Fig 6.4B,C). These results suggest that the activity of NHE1 is essential for the maintenance of the actomyosin cortex in mitosis.

![Figure 6.3. Ezrin directly interacts with NHE1 and F-actin. The schematic shows ezrin (blue) in two conformations; the inactive hairpin and in elongated active form. The N-terminus of active ezrin interacts with the cell membrane (grey) and NHE1 (orange) while the C-terminus interacts with F-actin (red).](image-url)
The decrease in mitotic intracellular pressure upon EIPA treatment is likely to be because of the reduction in cortical F-actin enrichment and may not be due to intracellular osmotic shift as hypothesized in earlier studies\(^{20}\). Although these results do not rule out a role for NHE1 in increasing the osmolarity of the mitotic cell, NHE1 seems to be important for the maintenance of cortical F-actin enrichment.

**Figure 6.4. Cortical F-actin enrichment is sustained by NHE1 activity.** (A) STC-arrested mitotic MYH9-GFP Lifeact-mCherry expressing HeLa cells confined to a height of 10 \(\mu m\). Cells were untreated or pre-incubated for at least 30 minutes with EIPA at concentrations given. Plotted are the transient or persistent pressures (grey) and tensions (blue) of cells under confinement. (B) Representative confocal images of STC-arrested mitotic MYH9-GFP Lifeact-mCherry expressing HeLa cells confined to a height of 10 \(\mu m\). Cells were untreated or pre-incubated for at least 30 minutes with EIPA at concentrations given. Scale bars, 10 \(\mu m\). (C) Quantification of cortex/cytoplasm ratio of F-actin (red) and myosin II (green) in STC-arrested mitotic MYH9-GFP Lifeact-mCherry expressing HeLa cells confined to a height of 10 \(\mu m\). Cells were untreated or pre-incubated for at least 30 minutes with EIPA at concentrations given. The diamond-box contains 25-75\% percentiles of the data and the bar denotes the median. Mann-Whitney significance tests of mitotic cells with respect to data plotted in the first column are indicated: (*) \(0.05 \geq U > 0.005\), (**) \(U \leq 0.005\).
AFM based methods to study blebs

Blebs are local cell membrane extrusions from the cell body due to decoupling of the cell membrane from the F-actin cortex\(^{125}\). Blebbing plays an important role in embryonic development, cell migration and stabilizes the cleavage furrow during anaphase\(^{126-129}\). Bleb formation can occur due to either an increase in intracellular pressure (Fig. 4.2, 4.3), a perturbation of cortical F-actin or a perturbation of the membrane-cortex linkers\(^{84,125,130,131}\). Although perturbation of membrane-cortex linkers like the ERMs and NHE1 could weaken the membrane and lead to the formation of blebs, membrane-cortex linker perturbation is often accompanied by a reduction of intracellular pressure (Fig 6.2, 6.4) and cell stiffness\(^{30,38}\). Hence, the membrane-cortex linkers inhibited cells may not bleb. Actin regulators like RhoA are essential for assembly of cortical F-actin and, hence, could play a crucial role in bleb retraction. But these experiments face the same bottleneck; RhoA inhibited cells have lower intracellular pressures and hence do not bleb (Fig. 4.3)\(^{132}\). Thus, the propensity of these cells to generate and retract blebs cannot be studied by conventional methods like laser ablation of the cell cortex or using constitutively blebbing filimin-deficient M2 cells\(^{84,125}\).

To quantify the contribution of the ERMs and NHE1 to maintaining membrane-cortex linkage, we developed an AFM-based assay to generate blebs. An AFM cantilever was lowered on metaphase HeLa cell at a rate of 0.05 \(\mu m/s\) to progressively confine the cell while simultaneously imaging the cell with light microscopy (Fig 6.5A). The transient intracellular pressure, transient cortex tension and the time when the confined cell extruded nascent blebs were measured. Cells typically started to extrude the first bleb when confined to a height of \(\sim 10.6\pm0.8 \mu m\) when the cell generated a transient force of \(\sim 63.33\pm17\) nN against the cantilever, a transient cortex tension of \(\sim 0.09\pm0.01\) mN/m and a transient intracellular pressure of \(\sim 350\pm60\) Pa \((n = 3)\). Further confinement resulted in increase in the initial bleb size and generated more nascent blebs (Fig. 6.5B).

In order to only study membrane-cortex decoupling in the generation of blebs and exclude the contribution of myosin II in generating as well as retracting blebs, the
The aforementioned experiment was carried out in the presence of 10 µM blebbistatin. Blebbistatin treated cells started to extrude the first bleb when confined to a height of 6.18±0.2 µm when the cell generated a transient force of 215±15 nN against the cantilever, a transient cortex tension of 0.18±0.005 mN/m and a transient intracellular pressure of 490±20 Pa (n = 3) (Fig. 6.5C).

**Figure 6.5.** Blebs generation through increasing cell confinement. (A) Scheme depicting the method to generate blebs by reducing the height of the cell at 0.05 µm/s to raise the transient intracellular pressure. (B) The plot of the effective cantilever height that confined a mitotic cell, the resulting transient force exerted by the cell on the cantilever, transient cortex tension and transient intracellular pressure. Shown above the plot are DIC images of cell at the start of the experiment (time zero) and subsequently when the cells started to extrude new blebs. (C) The plot of the effective cantilever height that confined a mitotic cell treated with 10 µM blebbistatin, the resulting transient force exerted by the cell on the cantilever, transient cortex tension and transient intracellular pressure. Shown above the plot are DIC images of cell at the start of the experiment (time zero) and subsequently when the cells started to extrude new blebs. Scale bar, 10 µm, applies to all images.
These results indicate that increasing transient intracellular pressure with AFM is a viable method to generate blebs and investigate the membrane-cortex coupling in cells. This method to generate blebs is effective even in myosin II inhibited cells that normally do not bleb. Our results also suggest that there is a threshold cortex tension (~0.2 mN/m) above which the cells could bleb because of disruptions to cortex integrity, although this needs to be verified with more experiments.

Preliminary experiments also point towards another novel method to generate blebs. After HeLa cells were confined to a height of 10 µm with AFM, the cantilever was oscillated at a frequency above 1.3 Hz and amplitude of about 5.8 nm (Fig 6.6A). Since the cantilever height data was under-sampled at 6 Hz, the exact frequency and amplitude of oscillation could not be estimated. As expected, confinement of a cell with an oscillating cantilever also resulted in oscillations in the force exerted against the cantilever by the cell (Fig 6.6B-D). Oscillating the height of the cellular confinement resulted in the formation of blebs in both mitotic and interphase cells (Fig 6.6B), accompanied by increased localization of cortical F-actin and myosin II (Fig 6.6C,D). These experiments show that, by oscillating the AFM cantilever, we can study bleb generation and retraction while simultaneously tracking the cortical components and measuring cell mechanics. Opposed to previous methods, we could generate blebs in cells with low intracellular pressure, as is the case in round interphase cells.
**Figure 6.6. Blebs generation through cantilever oscillation.** (A) Scheme depicting the method to generate blebs by confining the cell to 10 µm and oscillating the cantilever. (B) Plot of cantilever height and the force exerted by a confined round interphase cell against a non-oscillating cantilever. The right side panel is a magnified region from the left side panel. Measured amplitude and frequency of the cantilever height oscillation are denoted within the plot. (C) Plot of cantilever height and the force exerted by a confined round interphase cell against an oscillating cantilever. The right side panel is a magnified region from the left side panel. Measured amplitude and frequency of the cantilever height oscillation are denoted within the plot. (D) Representative confocal images of MYH9-GFP Lifeact-mCherry expressing HeLa cells. Cells were either STC-arrested in mitosis or rounded in interphase. Additionally, the cells were unconfined, confined with a non-oscillating or confined with an oscillating cantilever, as indicated. Scale bar, 10 µm.
A discussion on the role of ERM in mitosis

While most approaches to studying mitotic rounding have focused on contractility of the actomyosin cortex, how the forces from the actomyosin cortex are transduced to the cell membrane is an open question. The ERM family has emerged as prime candidates to play the crucial role of linking the actin cytoskeleton to the cell membrane\textsuperscript{120}. Depleting moesin, the sole member of the ERM family in Drosophila, results in severe mitotic rounding defects accompanied by a reduction of cortical actin enrichment\textsuperscript{30,38}. This result has not been reproduced in mammalian systems, presumably due to functional redundancy provided by the ezrin and radixin in addition to moesin. We show that in HeLa cells, the expression of either the dominant negative form of ezrin or FERM domain, caused mitotic rounding defects accompanied by a reduction in the ability of the mitotic cell to resist confinement. ERM is hypothesized to exist in two conformations; the inactive hairpin, where the N- and C-terminus interact with each other, and in elongated active form. The FERM domain at the N-terminus of active ERM binds to the cell membrane while the C-terminus interacts with F-actin\textsuperscript{133}. The dominant negative — TA-ezrin — is hypothesized to be inactive, albeit in a stretched conformation allowing radixin and moesin to be sequestered by the free N- and C-termini of TA-ezrin. Thus, in cells expressing TA-ezrin or the FERM domain, the activity of ERM could potentially be hindered despite the functional redundancy of ERM. The expressing moesin siRNA in Drosophila reduces cortical actin enrichment during mitosis\textsuperscript{30,38}. Further experiments are necessary to ascertain whether TA-ezrin or the FERM domain expressing HeLa cells have defective cortical F-actin during mitosis.

Although expression of dominant negative ezrin reduced the ability of the mitotic cell to resist confinement until metaphase, the cell produced substantial force against the confinement during anaphase. The mechanism behind how ezrin strengthens only the pre-anaphase cortex, but does not seem to play a role in anaphase remains to be investigated. One possibility is that the role of ERM in linking membrane and actomyosin cortex is substituted by other membrane-cortex linkers during anaphase when the ERM are relocated from the cell cortex to the cleavage furrow\textsuperscript{134}. 
Our results show NHE1 activity to be required for the enrichment of cortical F-actin enrichment during mitosis. Indeed, apart from regulating cellular osmolarity and pH, NHE1 has been shown to bind to ERM proteins and interact with a range of cytoskeletal proteins and regulators. Furthermore, cellular pH rises sharply along with transient NHE1 activity at the start of mitosis. Numerous modulators of the actin cytoskeleton as well as the actin cytoskeleton itself have been shown to be sensitive, if not regulated, by pH change. Studies combining intracellular pH measurements, control of NHE1 activity and monitoring of actomyosin cytoskeleton components could be performed while tracking cell mechanics and osmolarity in order to further dissect the mechanism driving mitotic rounding.

Blebs are the hallmark for defective actomyosin cortex-membrane linkage, and indeed, the ERMs are one of the first proteins to localize to the membrane detached from the actomyosin cortex. In order to dissect the role of linker proteins, particularly ERMs, in mitotic cell mechanics, we present two AFM-based assays to investigate defective actomyosin cortex-cell membrane linkage, by analyzing bleb generation and retraction. While these two methods are in the preliminary developmental stage, they could serve as a platform to address the intricate interplay between NHE1, ERMs and Cdk1 in maintaining the actomyosin cortex during mitosis.

**Conclusion**

In this thesis, we addressed the role of the contractile actomyosin cortex in counterbalancing intracellular pressure during mitosis and investigated the key regulatory pathways involved in this processes. Still many questions regarding the details of how the actomyosin cortex is regulated, especially relating to the offset in the timing of how cortical F-actin and myosin are enriched, remain unanswered. Cdk1, shown in our study to be essential for the enrichment of myosin II in mitosis, phosphorylates over 75 proteins and directly interacts with six known regulators of actomyosin. One approach to gain insight into how mitotic rounding is regulated could be to systematically interrogate the role of these Cdk1 targets with the approaches used in this thesis.
With Stewart et al., 2011, we showed that the contractility of the actomyosin cortex acting against intracellular pressure governs the mechanics of the mitotic cell\textsuperscript{20}. In this thesis, we find that cortical myosin II was enriched in mitotic rounding and correlated with increased intracellular pressure and tension (Fig. 2.7). However, it is still unclear whether the increase in intracellular pressure is a result of increased contractility of the actomyosin cortex acting against passive intracellular hydrostatic pressure or if the cell cortex is only balancing the rising osmotic pressure during the progression of mitosis. Theoretically, the volume changes required to achieve an osmotic gradient and elevate the intracellular pressure to \(\sim 500\) Pa would be almost undetectable\textsuperscript{84}. Hence, further work will be required to reveal the details of how intracellular pressure is generated and maintained by mitosis-specific osmotic regulation.
7. METHODS

Cells
HeLa cell lines were maintained in DMEM with 2 mM GlutaMAX (Life Technologies) supplemented with 10% fetal bovine serum (Life Technologies), 100 µg ml\(^{-1}\) penicillin and 100 µg ml\(^{-1}\) streptomycin (Life Technologies) at 37°C with 5% CO\(_2\).

To image F-actin and non-muscle myosin IIA, we used a HeLa cell line expressing human MYH9-GFP and Lifeact-mCherry. 0.5 mg ml\(^{-1}\) geneticin (Life Technologies) and 0.5 µg ml\(^{-1}\) puromycin (Life Technologies) were used for antibiotic selection. Myosin IIA assembles from three homodimer pairs: regulatory light chain, essential light chain, and myosin heavy chain 9 (MYH9), which was tagged with GFP\(^{144}\). Myosin IIB was not analyzed because it is not expressed in HeLa cells\(^{145}\). Lifeact, a well-characterized marker for F-actin was used to image actin\(^{29,146}\). For FRAP experiments, we used a HeLa cell line expressing β-actin-GFP and 5 mg ml\(^{-1}\) geneticin was used for antibiotic selection. To image tubulin and histones, we used a HeLa cell line expressing mouse tubulin-GFP and H2B-mCherry. To image myosin II and histones, we used a HeLa cell line expressing human MYH9-GFP and H2B-mCherry. HeLa cell lines transiently expressing mutant Rho GTPases were not supplemented with any antibiotic selection.

For AFM-based microcantilever experiments cells were grown in glass bottom Petri dishes (WPI FluoroDish) and the media was changed to DMEM (Life Technologies) with 4 mM Na\(_2\)CO\(_3\) buffered with 20 mM HEPES/NaOH at pH 7.2 (the AFM medium) supplemented with 10% fetal bovine serum. Where indicated, cells were arrested in mitosis with 4 µM S-trityl-L-cysteine (STC), an eg5 inhibitor that prevents bipolar spindle formation\(^{74}\) and, in contrast to agents interfering with microtubule assembly, does not alter mitotic intracellular pressure\(^{20}\). Rounded interphase cells were obtained by trypsinization and allowing the cells to partially spread for four hours in AFM medium.
Measuring cortex/cytoplasm ratio and FWHM of F-actin and myosin

Confocal images of a MYH9-GFP Lifeact-mCherry expressing HeLa cell were analyzed with a self-written Igor macro (Igor Pro). The center of the cell was determined by fitting a circle to the edge of the MYH9-GFP signal and 60 points were initially positioned 6° apart along the circle. Next, the 60 points were automatically repositioned to be at the cell periphery. Through each of these 60 points, intensity profiles from 0.6 µm thick radial line were obtained. The 60 fluorescence intensity profiles were aligned at the cell periphery (zero µm) and superimposed (Fig. 2.2A-C). The average of all fluorescent intensity profiles was subsequently plotted. Within each average profile, the peak intensity \( p \) between -2 and 0.5 µm from the cell edge and the mean intensity \( m \) between -2 and -1 µm from the cell edge were extracted. The cortex/cytoplasm ratio \( R \) for the fluorescence image was determined as the ratio of \( p \) and \( m \). The half peak height \( I \) is the intensity halfway between \( p \) and \( m \). FWHM was determined as the width (in µm) of the signal at \( I \) (Fig. 2.2D,E). The yellow markers from the MYH9-GFP image were copied into the Lifeact-mCherry fluorescence image (Fig. 2.2F). Subsequently, the cortex/cytoplasm ratio and FWHM for F-actin was also determined (Fig. 2.2F-J).

Measuring intracellular pressure and cortical tension

To determine the intracellular hydrostatic pressure \( \Delta P \) and the cortical tension \( T \) of a round mitotic cell, we applied an AFM based assay (Fig. 2.5B). A wedged AFM cantilever was used to uniaxially (two parallel plates) confine the height of the cell to 10 µm. The confined cell with an intracellular hydrostatic pressure \( \Delta P \) exerts a force against the cantilever\(^ {69} \). To determine \( \Delta P \) and \( T \), the cell was assumed to be a liquid core surrounded by a cortical shell having a uniform tension, a model shown to be applicable to mitotic or otherwise rounded cells. An optical section of the constrained cell was used to determine the maximal radius of the cell. For a cell of known height and maximal radius, the cell-cantilever contact area \( A_{\text{contact}} \) and cell surface mean curvature \( (1/R_1 + 1/R_2) \) was calculated using a linear combination of elliptical integrals that describe the shape of a cell compressed between parallel plates\(^ {147} \). \( \Delta P \) is \( F \) divided by \( A_{\text{contact}} \). Since, \( T \) is \( \Delta P \) divided by twice the mean
curvature (Laplace’s law), for a cell of known shape intracellular hydrostatic pressure can be converted to tension\textsuperscript{70}.

**Perturbants**

Latrunculin A, blebbistatin, nocodazole and Y27632 were purchased from Sigma-Aldrich, Cdk1/2 inhibitor III and SMIFH2 from Merck, MG132 from Calbiocem, C3-toxin (cell permeable) and calpeptin from Cytoskeleton, Ro3306, CK666 and IPA3 from Tocris and FRAX486 from ChemieTek. All reagents were dissolved in DMSO except cell permeable C3-toxin, which was dissolved in 50\% (v/v) glycerol. For chemical perturbation, the cells were incubated with the corresponding drug at the appropriate concentration for at least 30 minutes before start of measurements, except where mentioned otherwise.

**Plasmids, RNAi and transfections**

Effectene transfection reagent (Qiagen) was used to transfect plasmids into wild-type HeLa-Kyoto cells as per the reagent handbook. Plasmids encoding pGFP RhoA, Rac1 and Cdc42 variants were obtained from Jason Mercer (ETH Zurich). All siRNA transfections were performed using Lipofectamine RNAiMAX transfection reagent (LifeTechnologies) with AllStars siRNA (Qiagen) used as a negative control. DIAPH1\_1 (Qiagen) and DIAPH1\_2 siRNA (Qiagen) were used to target DIAPH1. Ect2 (Qiagen) and MYH9 RNAi (Qiagen) used in this study were used previously\textsuperscript{17,93}.

**Experimental setup**

An AFM (CellHesion 200, JPK Instruments) was mounted on an inverted confocal microscope (Observer.Z1, LSM 700, Zeiss)\textsuperscript{67}. Tipless microcantilevers (NSC12/tipless/noAl, Mikromasch) were modified with epoxy (ET302, Epoxy Technology) wedges to correct tilting\textsuperscript{69}. A 63x/1.3 LCI Plan-Neofluar water immersion objective (Zeiss) was used. Cells were kept at 37°C using a Petri dish heater (JPK Instruments).
**Image processing and analysis**

In all the representative images, contrast and brightness were adjusted to have similar cytosolic contrasts for visual comparison, using Zeiss AxioVision software (Rel. 4.8). Mann-Whitney statistical tests were carried out in Origin labs Origin 8.6.
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## APPENDIX

### Tables

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<td>-</td>
<td>-</td>
<td>Non-homogenously enriched (Fig. 6b)</td>
</tr>
<tr>
<td>Zang et al., 1997&lt;sup&gt;150&lt;/sup&gt;</td>
<td>HS1 (Human)</td>
<td>Live cell imaging. Labeling, GFP-myosin</td>
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<tr>
<td>O’Connell et al., 1999&lt;sup&gt;151&lt;/sup&gt;</td>
<td>NRK (Rat)</td>
<td>Fixed cells. Labeling, phalloidin and anti-myosin</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Paper</td>
<td>Cells/ Organism</td>
<td>Imaging method</td>
<td>Interphase</td>
<td>Mitosis</td>
<td>Comment</td>
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<tr>
<td>Wei and Adelstein, 2000²⁴</td>
<td>HeLa (Human)</td>
<td>Fixed cells. Labeling, non-muscle myosin II</td>
<td>Cortical F-actin -</td>
<td>Non- homogenously enriched (Fig. 8b)</td>
<td>Enriched at only at the cleavage furrow (Fig. 8c)</td>
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<tr>
<td>Straight et al., 2003²⁵²</td>
<td>HeLa (Human)</td>
<td>Fixed cells. Labeling, non-muscle myosin II</td>
<td>Cortical Myosin -</td>
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<td>Enriched at only at the cleavage furrow (Fig. 2A)</td>
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<tr>
<td>Maddox and Burridge, 2003²⁶</td>
<td>HeLa (human)</td>
<td>Fixed cells. Labeling, Alexa Phalloidin 594</td>
<td>Cortical F-actin -</td>
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<tr>
<td>Dean and Spudich, 2006²⁵¹</td>
<td>S2 (Drosophila)</td>
<td>Fixed cells. Labeling, phospho-regulatory light chain</td>
<td>Cortical Myosin -</td>
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<td></td>
</tr>
<tr>
<td>Uehara et al., 2010²⁵²</td>
<td>S2 (Drosophila)</td>
<td>Live cell imaging. Labeling, GFP-myosin II heavy chain</td>
<td>Cortical Myosin -</td>
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</table>

**Comment**

- N-terminal of myosin is essential for myosin to localize to the cleavage furrow.
- Used blebbistatin to understand control of cytokinesis.
- Show the importance of RhoA and Rho kinase in mitotic rounding.
- Illustrates the role of Rho kinase in myosin recruitment to cleavage furrow.
- Studies factors that determine myosin II localization to cleavage furrow during anaphase.
<table>
<thead>
<tr>
<th>Paper</th>
<th>Cells/Organism</th>
<th>Imaging method</th>
<th>Interphase</th>
<th>Mitosis</th>
<th>Comment</th>
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<td>Heng and Koh, 2010³</td>
<td>Unknown</td>
<td>Unknown. Labeling, actin and myosin IIA</td>
<td>-</td>
<td>-</td>
<td>Reviews actin cytoskeletal dynamics during cell division.</td>
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<tr>
<td>Luxenburg et al., 2011⁷</td>
<td>Primary epidermis (Mouse)</td>
<td>Whole tissue mount – fix and stain. Labeling, Alexa Phalloidin 546 and anti-MIIA</td>
<td>Enriched, same as interphase (Fig. 5)</td>
<td>Enriched throughout the cortex (Fig. S7)</td>
<td>Serum response factor (srf) in developing mouse epidermis is responsible for proper function of cortical actomyosin in mitosis</td>
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<tr>
<td>Meyer et al., 2011³</td>
<td>Wing disc epithelia. (Drosophila)</td>
<td>Whole tissue mount – fix and stain. Labeling, Alexa Phalloidin 546 and anti-phospho-myosin light chain.</td>
<td>Enriched, same as interphase. (Fig. 1)</td>
<td>Punctate enrichment phospho-regulatory light chain. (Fig. 1, S1F)</td>
<td>Shows interkinetic nuclear migration (IKNM) to be a feature of pseudostratified epithelial cells. Illustrated the role of mitotic rounding in IKNM.</td>
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<tr>
<td>Paper</td>
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<td>Imaging method</td>
<td>Interphase</td>
<td>Mitosis</td>
<td>Comment</td>
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<td>Matthews et al., 2012</td>
<td>HeLa (Human)</td>
<td>Fixed cells. Labeling, phalloidin-TRITC and anti-phospho-myosin light chain.</td>
<td>Not enriched. Cortex/cytoplasm ratio of ~1 (Fig. 2E, F)</td>
<td>Enriched. Cortex/cytoplasm ratio of ~4.5 (Fig. 2E, F)</td>
<td>No enrichment of phospho-myosin light chain (Fig. S3)</td>
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<tr>
<td>Nakajima et al., 2013</td>
<td>Wing disc epithelia. (Drosophila)</td>
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<td>Fritzsche et al., 2013</td>
<td>Filimin-deficient blebbing M2 melanoma cells (Human)</td>
<td>Live cell imaging. Labeling, Actin-GFP and MRLC-GFP</td>
<td>Enriched (Fig. 2A)</td>
<td>Enriched (Fig. S11)</td>
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<td>Clark et al., 2013</td>
<td>HeLa (Human)</td>
<td>Live cell imaging. Labeling, Actin-GFP and Lifeact-EGFP</td>
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<tr>
<td>Biro et al., 2013</td>
<td>HeLa (Human)</td>
<td>Live cell imaging. Labeling, MYH9-GFP</td>
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</table>
Table 1 Mitotic localizations of actin and myosin reported in the literature. While enrichment of actin and myosin to the cytokinetic cleavage furrow is a universal feature of mitosis, there are varied reports of cortical enrichment of actin and myosin prior to anaphase. Earlier studies tracking cortical myosin in fixed cells report little to no enrichment, while more recent studies, especially *in vivo*, indicate enrichment.
<table>
<thead>
<tr>
<th>Condition</th>
<th>Target/Action</th>
<th>Conc.</th>
<th>Transient pressure (Pa)</th>
<th>Persistent pressure (Pa)</th>
<th>Transient tension (mN/m)</th>
<th>Persistent tension (mN/m)</th>
<th>Cortex/cytoplasm ratio</th>
<th>Blebs (%)</th>
<th>n (#)</th>
<th>Fig.</th>
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</thead>
<tbody>
<tr>
<td>Control, mitosis</td>
<td></td>
<td>(N/A)</td>
<td>(N/A)</td>
<td>1.69±0.15</td>
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<td>3.63±0.27</td>
<td>(N/A)</td>
<td>11</td>
<td>2.6</td>
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<td>(Transmitotic assay, at maximum force)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>Control, mitosis</td>
<td></td>
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<td>747±29.8</td>
<td>505±17</td>
<td>2.45±0.10</td>
<td>1.66±0.06</td>
<td>2.38±0.03</td>
<td>2.99±0.09</td>
<td>45±6</td>
<td>82</td>
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<tr>
<td>Interphase</td>
<td></td>
<td>(N/A)</td>
<td>97±32.2</td>
<td>59±23</td>
<td>0.359±0.121</td>
<td>0.22±0.09</td>
<td>1.94±0.16</td>
<td>1.45±0.05</td>
<td>0±0.0</td>
<td>7</td>
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<tr>
<td>AllStars siRNA mitosis</td>
<td>Control siRNA</td>
<td>(N/A)</td>
<td>499±49.8</td>
<td>414±50</td>
<td>1.45±0.14</td>
<td>1.20±0.14</td>
<td>2.23±0.08</td>
<td>3.35±0.33</td>
<td>22±14.7</td>
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<tr>
<td>Ect2 siRNA mitosis</td>
<td>Ect2 depletion&lt;sup&gt;17&lt;/sup&gt;</td>
<td>(N/A)</td>
<td>0.957±0.114</td>
<td>173±28.6</td>
<td>0.957±0.114</td>
<td>0.490±0.08</td>
<td>1.91±0.11</td>
<td>1.45±0.13</td>
<td>0±0.0</td>
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</tr>
<tr>
<td>MYH9 siRNA mitosis</td>
<td>MYH9 depletion&lt;sup&gt;15&lt;/sup&gt;</td>
<td>(N/A)</td>
<td>558±53.9</td>
<td>207±41</td>
<td>1.61±0.147</td>
<td>0.60±0.11</td>
<td>2.18±0.02</td>
<td>2.88±0.41</td>
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<tr>
<td>DIAPH1&lt;sub&gt;1&lt;/sub&gt; siRNA (Qiagen) mitosis</td>
<td>DIAPH1 depletion&lt;sup&gt;15&lt;/sup&gt;</td>
<td>(N/A)</td>
<td>321.11±22.54</td>
<td>259.15±27.46</td>
<td>0.93±0.06</td>
<td>0.75±0.07</td>
<td>1.67±0.10</td>
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<tr>
<td>DIAPH1&lt;sub&gt;2&lt;/sub&gt; siRNA (Qiagen) mitosis</td>
<td>DIAPH1 depletion&lt;sup&gt;15&lt;/sup&gt;</td>
<td>(N/A)</td>
<td>285.83±62.13</td>
<td>209.98±40.49</td>
<td>0.81±0.20</td>
<td>0.59±0.13</td>
<td>1.86±0.11</td>
<td>2.18±0.31</td>
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<tr>
<td>Cdk1/2 III mitosis</td>
<td>Cdk1 inhibitor&lt;sup&gt;39&lt;/sup&gt;</td>
<td>3 µM</td>
<td>(N/A)</td>
<td>199.51±35.17</td>
<td>(N/A)</td>
<td>0.63±0.11</td>
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<tr>
<td>Ro3306</td>
<td>Cdk1 inhibitor&lt;sup&gt;38&lt;/sup&gt;</td>
<td>5 µM</td>
<td>(N/A)</td>
<td>176.91±21.85</td>
<td>(N/A)</td>
<td>0.51±0.06</td>
<td>2.00±0.01</td>
<td>1.49±0.13</td>
<td>(N/A)</td>
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<tr>
<td>Latrunculin A treatment, mitosis</td>
<td>F-actin depolymerizer&lt;sup&gt;157&lt;/sup&gt;</td>
<td>50 nM</td>
<td>532.9±79.3</td>
<td>389±47</td>
<td>1.79±0.28</td>
<td>1.31±0.17</td>
<td>2.26±0.13</td>
<td>2.12±0.14</td>
<td>45±16</td>
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<tr>
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<td>200 nM</td>
<td>26±10.2</td>
<td>12±2</td>
<td>0.08±0.03</td>
<td>0.04±0.01</td>
<td>1.83±0.20</td>
<td>1.01±0.00</td>
<td>29±18</td>
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<tr>
<td>Blebbistatin treatment, mitosis</td>
<td>Myosin II inhibitor&lt;sup&gt;152&lt;/sup&gt;</td>
<td>10 µM</td>
<td>597±83.1</td>
<td>210±73</td>
<td>1.96±0.29</td>
<td>0.70±0.25</td>
<td>2.45±0.10</td>
<td>2.85±0.22</td>
<td>10±10</td>
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<tr>
<td></td>
<td></td>
<td>100 µM</td>
<td>289±51.2</td>
<td>15±2</td>
<td>0.94±0.17</td>
<td>0.05±0.01</td>
<td>2.47±0.14</td>
<td>2.39±0.17</td>
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<td>NSC23766</td>
<td>Rac1 inhibitor&lt;sup&gt;99&lt;/sup&gt;</td>
<td>50 µM</td>
<td>(N/A)</td>
<td>291±18</td>
<td>(N/A)</td>
<td>0.87±0.06</td>
<td>(N/A)</td>
<td>(N/A)</td>
<td>(N/A)</td>
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<td>75 µM</td>
<td>(N/A)</td>
<td>394±48</td>
<td>(N/A)</td>
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<td>(N/A)</td>
<td>(N/A)</td>
<td>(N/A)</td>
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<tr>
<td>Condition</td>
<td>Target/Action</td>
<td>Conc.</td>
<td>Transient pressure (Pa)</td>
<td>Persistent pressure (Pa)</td>
<td>Transient tension (mN/m)</td>
<td>Persistent tension (mN/m)</td>
<td>Cortex/cytoplasm ratio</td>
<td>Blebs (%)</td>
<td>n (#)</td>
<td>Fig.</td>
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<td>EHT1864 treatment, mitosis</td>
<td>Rac1 inhibitor</td>
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<td>100 µM</td>
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<td>Pirl1 treatment, mitosis</td>
<td>Cdc42 inhibitor</td>
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<td>(N/A)</td>
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<td>(N/A)</td>
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<td>20 µM</td>
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<td>ML141 treatment, mitosis</td>
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<td>30 µM</td>
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<td>Calpeptin treatment, mitosis</td>
<td>RhoA activator</td>
<td>50 µg/ml</td>
<td>2.94±0.19</td>
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<td></td>
<td>RhoA inhibitor</td>
<td>100 µg/ml</td>
<td>2.13±0.17</td>
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<td>C3-toxin treatment, mitosis</td>
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<td>232.8±14.4</td>
<td>141±48</td>
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<td>20 µg/ml</td>
<td>189.2±47.5</td>
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<td>SMIFH2 treatment, mitosis</td>
<td>Formin inhibitor</td>
<td>20 µM</td>
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<td>396±52</td>
<td>2.21±0.30</td>
<td>1.37±0.18</td>
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<td>40 µM</td>
<td>107±13.1</td>
<td>37±5</td>
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<td>Rho kinase inhibitor</td>
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<td>540±79.3</td>
<td>210±30</td>
<td>1.73±0.26</td>
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<td>25 µM</td>
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<td>161±90</td>
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<td>2.57±0.08</td>
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<td>CK 666 treatment, mitosis</td>
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<td>950±62.5</td>
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<td>160 µM</td>
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<td>50 µM</td>
<td>844±62.8</td>
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<td>1.87±0.14</td>
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<td>200 µM</td>
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<td>4.04±0.33</td>
<td>70±15</td>
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</tbody>
</table>

Table 2. Inhibitors and perturbations used in single cell experiments in order of figure appearance. With the exception of the trans-mitotic measurements, all the cells in mitosis were arrested with 4 µM STC. Listed columns include the condition of the cell, target/action of perturbant used, (Table 2 continued in the next page)
(Table 2 continued)
the concentrations used (Conc.), the resulting transient and persistent pressure and tension, cortex/cytoplasm ratio of F-actin and myosin II, percentage of blebbing cells, number of cells (n) studied and the figure in which the condition appears. Values are mean ± s.e.m.
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AFM</td>
<td>Atomic force microscope</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>C3-toxin</td>
<td>Clostridium botulinum C3 exoenzyme</td>
</tr>
<tr>
<td>Cdk1</td>
<td>Cyclin-dependent kinase 1</td>
</tr>
<tr>
<td>DIC</td>
<td>Differential interference contrast (microscopy)</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle's Medium</td>
</tr>
<tr>
<td>DN</td>
<td>Dominant negative</td>
</tr>
<tr>
<td>DP</td>
<td>Dominant positive</td>
</tr>
<tr>
<td>ECR</td>
<td>Extracellular region</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-to-mesenchymal transition</td>
</tr>
<tr>
<td>ERM</td>
<td>Ezrin, radixin and moesin</td>
</tr>
<tr>
<td>F-actin</td>
<td>Filamentous-actin</td>
</tr>
<tr>
<td>FRAP</td>
<td>Fluorescence recovery after photobleaching</td>
</tr>
<tr>
<td>FWHM</td>
<td>Full width at half maximum</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>GDI</td>
<td>Guanosine nucleotide dissociation inhibitors</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescence protein</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>IKNM</td>
<td>Interkinetic nuclear migration</td>
</tr>
<tr>
<td>MLCK</td>
<td>Myosin light-chain kinase</td>
</tr>
<tr>
<td>MYH9</td>
<td>Myosin heavy-chain 9</td>
</tr>
<tr>
<td>NEBD</td>
<td>Nuclear envelope breakdown</td>
</tr>
<tr>
<td>PAK</td>
<td>p21-activated kinase</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>STC</td>
<td>S-trityl-L-cysteine</td>
</tr>
<tr>
<td>UT</td>
<td>Untreated</td>
</tr>
</tbody>
</table>
WT  Wild type

**Symbols**

\( \text{a.u.} \)  Arbitrary units
\( p \)  Peak intensity
\( m \)  Mean intensity
\( R \)  Cortex/cytoplasm ratio
\( I \)  Half peak height
\( \Delta D \)  Cantilever deflection
\( F \)  Force exerted by the cell against the cantilever
\( \Delta V \)  Voltage change
\( H \)  Height of a uniaxially confined cell
\( A_C \)  Cell-cantilever contact area
\( T \)  Cortex tension of cell
\( \Delta P \)  Intracellular pressure difference
\( P_i \)  Intercellular pressure
\( P_o \)  Extracellular pressure
\( R_1 \) and \( R_2 \)  The two radii of principle curvature at a specified point
\( s.e.m \)  Standard error of the mean
\( v/v \)  Volume fraction
\( \text{NS} \)  Not significant

**Equations**

\[ \Delta P = P_i - P_o = F / A_c \]
\[ T = \Delta P / (1/R_1 + 1/R_2) \]
\[ R = p/m \]
\[ I = (p + m)/2 \]
Acknowledgements

Firstly, thanks a ton Daniel for giving me the opportunity to take up a challenging scientific project and for pushing till I could give my very best. Your support and never-give-up approach has shaped my scientific outlook.

Loads of gratitude to Jonne, I could always depend on you for good advice at both scientific and personal levels. Thanks for your unwavering support, especially during a tough phase of my PhD right after the move from Dresden to Basel.

Thanks Martin, for showing me the ropes of using the AFM when I was a newbie and for your contribution whilst wrapping up my project.

Thank you Cédric, for your enthusiastic support and for taking that extra care to ensure that working in the lab is a pleasure.

I have always had the support of the Muller lab members during my incredible time here, I am thankful for this. I am also grateful to Tony Hyman, Yusuke Toyoda and Jason Mercer for their timely advice and inputs.

I take this opportunity to thank my father, P. S. Ramanathan; mother, Rama Ramanathan; and sister, Arundhati Ramanathan. Your constant encouragement and support from the first day of my life until now has made me what I am.

Finally, I wish to thank my good luck, because I was fortunate enough to find my partner, my azhaghi, Swathi Raghuraman. The last two years have been intense and I would be nowhere without you by my side.
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Master of Science: Molecular Bioengineering, TU Dresden, Germany.
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July-2002 to May-2006:
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RESEARCH EXPERIENCE

Oct-2008 until now:
The mechanism of mitotic rounding: Role of the actomyosin cortex (PhD thesis), at Prof. Daniel J. Mueller’s lab in TU Dresden, Germany and ETH Zurich, Switzerland

Mar-2008 to Sept-2008:
Single molecule studies of DEAD box helicases using magnetic tweezers (Master thesis), at Prof. Ralf Seidel’s lab, Biotechnological Center, TU Dresden, Germany
Nov-2006 to Mar-2008:
Single molecule studies of DNA translocating molecular motors, the Type III restrictions enzymes, using magnetic tweezers, at Prof. Ralf Seidel’s lab, Biotechnological Center, TU Dresden, Germany

Dec-2005 to May-2006:
Organic synthesis of prodigiosin derivatives (Bachelor Thesis), at Prof. P. Gautam’s lab, Centre for Biotechnology, Anna University, India

May-2005 to Sep-2005:
Studied trends in cytokine production in HIV+ patients using flow cytometry, at Dr. Soumya Swaminathan's lab, HIV Division-Tuberculosis Research Centre, India

TEACHING/MENTORING EXPERIENCE

Nov-Dec 2011:
Designed and supervised a short AFM based project for an undergraduate student of University of Basel

Nov-Dec 2010:
Designed and supervised a short cell biology project for an undergraduate student of University of Basel

Oct-Nov 2009:
Conducted an AFM introduction course for three undergraduate students of TU Dresden

SCIENTIFIC TALKS

Dec 2013:
Three talks titled “Actomyosin cortex in mitotic rounding” at the labs of Dr. Philipp Niethammer at Memorial Sloan Kettering, New York, Dr. Matthew Gibson at Stowers Institute, Kansas City and Dr. Orion Weiner at University of California, San Francisco

Oct 2009:
Talk titled “AFM studies of mitotic cell rounding” at “Cell Shape changes 2009”, Curie school, Institut Curie, Paris, France
PUBLICATIONS

Ramanathan S. P., J. Helenius, M. P. Stewart, C. J. Cattin, A.A. Hyman, D.J. Muller. 2014. Cdk1 Dependent Mitotic Enrichment of Cortical Myosin II Promotes Cell Rounding Against Confinement (Manuscript under revision).


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