The handle http://hdl.handle.net/1887/29816 holds various files of this Leiden University dissertation

Author: Hoorn, Hedde van
Title: Cellular forces: adhering, shaping, sensing and dividing
Issue Date: 2014-11-26
CHAPTER 5

OUTWARD FORCE EXERTION IS CRUCIAL FOR MITOSIS

\[1\]

\[1\]This chapter is based on: H. van Hoorn, M. de Valois, C. Backendorf and T. Schmidt, Outward force exertion is crucial for mitosis, to be submitted
abstract

Cell division is tightly regulated by the kinetochore, a hierarchical assembly of nearly 100 proteins that connects DNA in chromosomes to the mitotic spindle to complete the mitotic cycle. Biochemical regulation of mitosis has been extensively studied, while its physical implications are not well understood. Here we show that throughout mitosis cells are mechanically coupled to their environment, heavily deform the extracellular matrix and an outward pushing force is needed for successful division. We used fluorescently labeled HeLa cells to correlate chromosome- and microtubule dynamics directly to extracellular force exertion. Distinct successive hallmarks of force exertion correlated to well-known mitotic stages with an adaptation in force exertion consistently preceding cellular phenotype. These results illustrate the importance of cell mechanics next to biochemical interactions throughout a cellular lifecycle. Our novel experimental observations lead us to propose a force balance that proves crucial for successful division and the mechanosensory function of checkpoints regulating progression through mitosis.
5.1 Introduction

Proper control over progression through the cell cycle and orientation of division is ubiquitous in life and vital in development. The importance of cell cycle control is evident from the large number of checkpoint-mechanisms, for instance in the formation of kinetochores and the multifaceted spindle-assembly checkpoint mechanism [1–5]. The importance of cell cycle regulation is further exemplified from the observation that deregulation of the centrosome cycle can lead to chromosome instability and ultimately to the development of cancer [6, 7]. A multitude of proteins is involved in proper cell cycle progression and a great insight has developed over the past years [8, 9]. The main focus in past research has been on biochemical interactions and identification and localization of proteins. However, cell cycle progression is not just controlled biochemically, but is also greatly influenced by physical environmental cues.

How forces are transmitted through the mitotic spindle and progressively through the stages of cell division, has been investigated for decades [10, 11]. However, even in recent years it still remains a technical challenge to address the question of how cellular force exertion progresses throughout division [12, 13]. Tension-dependency in checkpoints was discovered nearly two decades ago, showing a direct tension-phosphorylation mechanosensitive mechanism [14, 15]. However, an inconsistent problem in this matter is the need for a physical disruption of cellular components in order to measure the forces exerted on them. Here, we try to gain insight in the force balance throughout mitosis by measuring extracellular forces simultaneously with internal re-organization of chromosomes and microtubules (MTs).

Several important extracellular insights during cell division in single eukaryotic cells have been discovered in recent years. The extracellular matrix (ECM) was modified to control the direction of cell division and it was shown that the ECM could guide the axis of cell division [16]. Further experiments using externally applied strain, ECM-patterning and laser-ablation showed that physical disruptions could also alter the direction of cell division [17]. An important role for retraction fibers was found for determination of the cell division axis. From these studies it became clear that externally applied spatial and mechanical cues (outside-in coupling) directly influenced cell division. However, the mechanical inside-out coupling due to forces generated by the cell transmitted onto the ECM is not well studied.
In previous studies, a large range of forces was quantified during cell division. Furthermore, force measurements throughout the full cycle with a functional correlation to local force exertion have not been reported. Local forces exerted directly on chromosomes were quantified using optical tweezers in the range of 1-10 pN [13]. The tension in retraction fibers was found to be in the order of 100-1000 pN [17]. A cell-wide, mitotic rounding pressure applied by the cell was found to be in the range of 100 nN, as measured by atomic force microscopy [18]. However, all previous measurements relied on an active perturbation, by impeding cellular behavior either with a bead or an AFM cantilever. To clarify the extracellular force balance, we aimed to investigate the locally exerted forces on the ECM dynamically with a passive measurement throughout the cell cycle.

We quantified extracellular forces using micropillar arrays of varying stiffness. Over time, we imaged fluorescently labeled HeLa cells and examined both chromosomes and tubulin using confocal microscopy. Inward pulling forces of 50-100 nN in interphase were released before chromosome condensation. Progression through prometaphase was accompanied by a build-up of outward pushing forces that culminated during metaphase. On a soft or hard substrate, outward pushing force amounted to 100-150 nN or 450-550 nN, respectively. Before successful cell division, a force plateau was present through anaphase leading up to a peak in outward pushing force during telophase. After physical separation, the two daughter cells spread and continued to exert inward pulling forces as is common for cells in interphase. However, not all cells exerted outward pushing forces throughout mitosis. Strikingly, a decrease in outward pushing forces during metaphase correlated to mitotic disturbance and the presence of three centrosomes instead of two leading to tripolar spindles. We thus conclude that the outward pushing force is crucial for spindle integrity and proper progression through cell division.

5.2 Methods

5.2.1 Cell culture

HeLa cells were cultured in high-glucose Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% calf serum (Thermo Scientific), 2 mM glutamine and 100 μg/ml penicillin/streptomycin at 37 °C with 5% CO2. Cells either expressed H2B-GFP stably or were trans-
duced with tubulin-GFP using baculovirus (BacMam 2.0, Invitrogen). Baculovirus transduction was performed overnight prior to the measurement at \(\sim 70\%\) confluency with 40 particles per cell. Cells were then seeded at single cell density directly on the micropillar array and allowed to spread \(~6\) hours. Micropillar arrays were subsequently inverted onto \(\#0\), 25 mm diameter, round coverslips (Menzel Glaser) for imaging.

### 5.2 Methods

#### 5.2.2 Micropillars

Hexagonal arrays of poly-di-methyl-siloxane (PDMS, Sylgard 184, Dow Corning) micropillars of 2 \(\mu\)m diameter, 2 \(\mu\)m spacing and with a height of 4.1 and 6.9 \(\mu\)m were produced using replica-molding from a silicon wafer into which the negative of the structure was etched by deep reactive-ion etching. The pillar arrays were flanked by integrated 50 \(\mu\)m high spacers (as described in [19] and shown in supplemental figure S2) such that pillar tops and hence cells attaching to them were within the limited working distance of our high-NA objective (<170 \(\mu\)m) on an inverted microscope. The use of a high-NA objective is a prerequisite for any high-resolution optical imaging. The micropillar arrays were kept from floating using a support weight of glass. Live-cell measurements were performed in overnight time-lapse measurements of 10-15 hours duration on a confocal spinning-disk setup with a home-built focus-hold system. The temperature was kept at 37 °C with constant 5% CO2 concentration in a stage-top incubator (Tokai Hit, Japan).

The tops of the micropillars were coated with a mixture of Alexa568-labeled and unlabeled fibronectin (1:5) using micro-contact printing. A 40 \(\mu\)l drop with 60 \(\mu\)g/ml fibronectin was incubated 1h on a flat piece of soft (1:30 PDMS, crosslinker:base ratio), washed with ultrapure water and left to dry under laminar flow. After 10 minutes UV-Ozone (Jelight) activation of the micropillars, micro-contact printing was performed 5 minutes and non-printed areas were blocked 1 hour with 0,2% Pluronic (F-127, Sigma) in PBS. Finite Alement Analysis (FEA) that was fed the exact micropillar dimensions as measured by in-situ scanning electron microscopy (SEM, FEI nanoSEM) allowed us to precisely calibrate the force-deflection relation. Short and tall micropillars on the array had a characteristic spring constant of 70.9 and 16.7 nN/\(\mu\)m, respectively.

In the analysis, we consistently used a third order polynomial fit to the FEA results to obtain the force for a given deflection. The position of the pillar tops was observed by fluorescence microscopy at 561 nm excitation.
From those fluorescence images the exact pillar-centroid positions were determined down to 30 nm accuracy. The deflection precision of 30 nm corresponded to a force accuracy of 2 and 0.5 nN for the short and tall pillars, respectively. All analysis was done using specifically designed software (Matlab, Mathworks).

5.2.3 Microscopy

Imaging was performed on an inverted microscope (Zeiss Axiovert 200) with a 100X, 1.4 NA oil objective (Zeiss). The setup was expanded with a Confocal Spinning Disk unit (Yokogawa CSU-X1), an emCCD camera (Andor iXon DU897) and a home-built focus-hold system at the rear port. The focus-hold system consisted of a 850 nm diode laser that was coupled into the rear port reflected into the objective using a dichroic that only reflected light with a wavelength >800 nm (Chroma). The reflected light on the glass-water interface was collected at the back-port and positioned onto a Si-amplified photodetector (PDA36A, Thorlabs). The relative signal was measured to a sample (90/10 splitter) of the signal of the incoupled diode laser to account for fluctuations of the diode laser. The position of the reflected light was a direct readout for the position of the glass-water interface relative to the objective over a range of 10-20 μm. A PID-controller programmed in Labview (National Instruments) controlled the objective positioning through its mounting on a PIFOC piezo (Physike Instrumente).

For each overnight measurement, multiple positions were recorded every 2 minutes. The positions were manually selected by moving the XY-stage (MarzHauser) and corresponding relative positions to the glass-water interface denoted the Z-position relative to the highest value for the derivative of the photodiode signal as a function of piezo-movement. Overnight, typically 8-14 positions were recorded. At each position, the focus-hold system determined the position of the coverslip-water interface and relative to that recorded the micropillar array with 561 nm laser light (Cobolt) and a z-stack 3-8 μm with 488 nm laser light (Coherent) below the micropillars for the chromosomes (for H2B-GFP cells) or microtubules (for tubulin-GFP cells). In all images shown, a maximum-intensity z-projection is constructed from this z-stack.
5.2 Methods

5.2.4 Radial force interpretation

A possible explanation for the apparent outward deflections of pillars would be an optical lensing effect caused by the rounding of the cell. We imaged the micropillars underneath the cell through the cell body, which has a different refractive index as compared to the medium surrounding the cell. A rounded dividing cell would then enable a lensing effect due to the higher refractive index inside the cell. This would indeed make the pillars appear deflected outward while they are are not deformed at all. In our measurements, however, this lensing effect does not play a significant role.

The refractive index of a HeLa cell has been accurately quantified \cite{20} and is highly inhomogeneous ranging from 1.34 - 1.4. When we take the an extreme case of a full lens, where the refractive index is 1.38 everywhere in the cell compared to the surrounding medium, a rounded cell of 10 µm height with radius 15 µm would yield an apparent deflection of at maximum $\sim 150$ nm (see supplemental figure S4). This is an upper limit, since the measurement in \cite{20} shows high uniformity in the cell, which would severely decrease the lensing ability. The actual deflections we measured were $\sim 300-400$ nm per pillar. In a very extreme case, we would thus overestimate the outward pushing forces by a factor of 2, so the outward pushing is still significant in our measurement.

Finally, we observed a lack of outward pushing but random significant deflections in the case of cells that showed a mitotic disturbance during mitosis. If the lensing effect would have been significant, we would have seen outward pushing also in this case. However, there were significant deflections, but in all directions (not just radially outward). The difference in refractive index is attributed to an increased protein concentration in the cell, which would also be the case for the triplet division. We thus conclude that the deflections are a real measure of outward pushing forces and not an effect of lensing by the rounded cell.

It is important to note that the quantified deflections are only in-plane and thus parallel to the axis of division. Importantly our method can vary the global stiffness of the extracellular environment. With this variation we show that the cell indeed pushes harder when the outside is stiffer. With a significant variation of stiffness in organisms, it indeed makes sense that the extracellular force exertion can be increased so a certain deformation can be obtained.
5.3 Results

Figure 5.1
Quantification of cellular forces during division. (A) Phases during mitosis were quantified by following a maximum z-projection of z-stacks over time of a HeLa nucleus with H2B-GFP (scalebar corresponds to 10 µm). (B) PDMS micropillars were used to measure cellular traction forces during cell division. The tops of these pillars (image taken with Scanning Electron Microscopy, SEM) were coated with Fibronectin. (Scalebar corresponds to 5 µm) (C) Radial forces were quantified relative to the nucleus center (H2B-GFP schematically depicted by green) or the center of the mitotic spindle (when tubulin-GFP was used). Positive radial forces are defined as outward forces and negative radial forces were defined as inward forces. (Dotted line marks cellular outline, x marks relative center for radial forces)

Our research question was aimed at the correlation between two dynamic processes; extracellular force exertion and cell division. A more extensive description of the methods is given in Methods. Briefly, we quantified cell division and force exertion simultaneously by performing time-lapse imaging of HeLa cells on micropillar arrays of varying stiffness. We observed the state of the cell cycle (see figure 5.1A) by labeling either alpha-tubulin or histone-core protein H2B with Green Fluorescent Protein (GFP). In both cases the cells could still complete a full cell-cycle
and seemed not to be hindered by the presence of GFP. The cell-cycle phase was quantified manually by comparing the time-lapse movies to the predicted visual state given by literature [1, 21]. The phases are consistently color-coded by green, red, magenta and purple for prophase, metaphase, anaphase and telophase, respectively. Timepoints of a given phase always denote the start of a phase.

Force exertion was quantified using micropillar arrays (see figure 5.1B), as previously described [19]. Micropillars with heights of 4.1 µm and 6.9 µm were used, both with a diameter of 2 µm. Live-cell high-resolution fluorescence microscopy was performed overnight using a spinning-disk confocal attached to a home-built microscope with a multi-positioning system and a home-built autofocus system. Z-stacks from 3 to 8 µm above the pillars were recorded with a 488 nm laser, while the pillar z-slice was imaged with a 561 nm laser (to image the Fibronectin labeled with fluorescent dye Alexa568). From the deflection map and the calibrated pillar stiffness [19], the forces cells exerted on the ECM were quantified using specifically designed algorithms and further analyzed in Matlab (Mathworks).

Finally, we noticed in our analysis that the forces exerted were primarily radially oriented relative to the nucleus center (or metaphaseplate center in the case of tubulin-GFP cells). The radial component of the force was much larger as compared to the perpendicular force (as quantified in supplemental figure S1). Upon progression through telophase, the dividing cells obtained two radial force centers. Relative to the radial force centers, inward (pulling) forces were defined as negative radial forces, while outward (pushing) forces were defined as positive forces (see figure 5.1C). For each cell, the radial forces were then summed to obtain the net radial force per cell (as given in figure 5.3 and 5.5B).

5.3.1 From pulling to pushing forces in prometaphase

It is known from previous research that cells in interphase typically exert inward pulling forces. Inward pulling forces have been observed for fibroblasts [19, 22, 23], epithelial cells [23, 24] and muscle cells [23, 25]. Similarly, we observed pulling forces exerted by HeLa cells in interphase when plated on micropillar arrays (see figure 5.2A and C, figure 5.4A and E and figure 5.5A). Pulling forces were present at the cell periphery, directed towards the cell center. The maximum force exerted was 10-50 nN per pillar, depending on the stiffness of the micropillars. Over the
During cell division, inward pulling forces decrease and outward pushing forces increase. (A-B) Timelapse of cell division forces on low-stiffness micropillars ($k_{\text{bend}} = 16.7 \text{ nN/\mu m}$). (C-D) Timelapse of cell division forces on high-stiffness micropillars ($k_{\text{bend}} = 70.9 \text{ nN/\mu m}$). Phases are consistently color-coded with prophase in green, metaphase in red, anaphase in magenta and telophase in purple. Force scalebar in lower left consistent for each stiffness, fluorescence scalebar in lower right.

We investigated force exertion on micropillar arrays of different heights and thus varied the stiffness. Micropillars with a bending modulus of $k_{\text{bend}}=70.9 \text{ nN/\mu m}$ and $k_{\text{bend}}=16.7 \text{ nN/\mu m}$ were used, calibrated as previously described [19] and discussed in Methods. On both stiffnesses, we observed radial outward force exertion during progression through mitosis (see figures 5.2A and C). Histograms of radial forces in figures 5.2B and D show inward-pulling (negative radial force) in interphase, as
expected. Going into mitosis, pulling was released and outward pushing forces (positive radial force) build up. In the histograms for metaphase and anaphase it is particularly clear that a force distribution around a positive radial force of 20 nN build up (figure 5.2D). For the low-stiffness pillars a broadening of the distribution towards positive forces was observed due to outward pushing forces as observed in figure 5.2A.

To get a clear picture of the progression of radial forces over the entire cell division cycle, the radial forces were summed to obtain a net radial force per cell. Figures 5.3A and B give the time-course for the net radial force for one cell on low- and high-stiffness pillars, respectively. Cells on micropillars progressed through mitosis over the time course of 1-2 hours, without a correlation between substrate stiffness and time to divide. Markedly, on both stiffnesses the initiation of prophase as observed by chromosome condensation was always preceded 5-20 minutes by the release of contractile forces.

Throughout cell division the micropillar arrays were inverted. We thus conclude that cells were continually physically connected to the fibronectin-ECM with enough strength to keep the cell attached to the substrate counteracting gravitational forces. Fluorescence imaging of tubulin-GFP (see figure 5.4) further confirmed that the cells were continuously spread on the substrate, rounding up in a dome-like shape. Using our methodology we were able to assess that ECM-binding undergoes severe remodelling and stress throughout the cell division cycle. Importantly, release of pulling forces preceded initial chromosome condensation and build-up of pushing forces coincided with progression through prometaphase. The change in force exertion thus consistently preceded the structural reorganization progressing into formation of the mitotic spindle.

5.3.2 Force plateau during anaphase and telophase

Chromosomes re-locate massively during cell division, but MTs guide this reorganization and the ultimate physical separation of the daughter cells. We localized MTs by labeling them with GFP. Baculovirus transduction with tubulin-GFP enabled us to simultaneously visualize MT dynamics and extracellular force exertion (see figure 5.4). As with the H2B-labeled cells, the cells showed the inward pulling-force phenotype (figure 5.4A) in interphase.

Proceeding into metaphase, the outward pushing force increased to
Figure 5.3
Outward pushing forces increase through mitosis. Net radial force over time on a low-stiffness substrate (A) and on a high-stiffness substrate (B) follow roughly the same trend. For multiple cells (at least 5 cells per condition) the inward pulling force is already released before prophase on both low-stiffness (C) and high-stiffness (D) micropillar arrays and a successive outward pushing force (positive radial force) builds up.
an approximate plateau. On the lower-stiffness micropillar arrays the outward force increased gradually into anaphase at a plateau force of $(120\pm30)$ nN (mean±s.d.). On the higher stiffness the plateau force was readily reached upon metaphase at $(490\pm50)$ nN. Larger contractile forces on substrates with a larger stiffness were previously observed for fibroblasts and other cell types [22, 23]. The outward pushing forces during cell division also increased with increasing substrate stiffness, showing that the dividing cell also adapts to the stiffness of the environment.

Next, we noticed that in the radial force curves that were able to capture the starting point of cytokinesis, 8 out of 9 cases showed a final peak increase in outward pushing forces. Just before physical separation of the cells, a brief additional outward pushing force was measured. Figure 5.3A shows an example of unresolved forces before force decrease, while in figure 5.3B the peak increase can be observed just before cytokinesis. Another peak in outward pushing upon cytokinesis is shown in supplemental figure S3.

Re-organization of MTs and final separation of the chromosomes coincided with the final pushing force increase upon cytokinesis (figure 5.4B-E). The point of peak outward pushing force corresponded to figure 5.4C. This timepoint in the mitotic cycle also corresponded to the start of contraction of the cleavage furrow through actomyosin contractility. It is likely that the additional outward push is caused by the final contraction of the actomyosin ring.

Increased outward pushing forces were consistently present at the MT base in anaphase and telophase at an approximate plateau. Outward pushing may be a vital part of the force balance which keeps the mitotic spindle together and chromosomes aligned. As the pushing forces decreased again after the peak in figure 5.4E, mitosis was almost completed and the contractile actomyosin ring proceeded to pinch of the two daughter cells. Pushing force decreased throughout this stage, as can be observed in figure 5.3 and S3. When the cells were fully separated, they spread again, exerting inward pulling forces as observed in figure 5.4F.

### 5.3.3 Outward pushing is vital for succesful mitosis

During our measurements, we encountered three cases in which the mitotic spindle spontaneously became disturbed and a third centrosome was observed. Figure 5.5A shows the timecourse of MTs and force over time for such a cell undergoing a mitotic disturbance. Coincidentally,
Figure 5.4
Outward pushing from two distinct bases during cytokinesis. (A) Cell in interphase with microtubules labeled with GFP (green) on fibronectin-coated micropillars (red). (B-E) During physical separation over the time course of several minutes, two bases attach to the substrate pushed radially outward. Physical seperation of a mitotic spindle in (B) to the pinching off of two individual cells in (E) is clearly visible. Total outward pushing forces peak in (C) during physical seperation. (F) After seperation, both daughter cells spread and start exerting pulling forces at the cell periphery again. (Arrow scalebar in lower left always corresponds to 20 nN and fluorescence image scalebar in lower right to 10 µm)
we observed a striking consistent force exertion hallmark. Initially, the inward pulling forces were released before the start of prophase, as was the case in successful division. However, upon initial spindle formation the outward pushing force did not increase immediately, but showed a delayed response. Outward pushing forces build up eventually, but could not maintain a plateau force. After several hours, the outward pushing force decreased and fluctuated around zero net radial force.

The evolution of the net radial force over time for a cell undergoing a mitotic disturbance is shown in figure 5.5B. The decrease in outward pushing force preceded the observation of one of the centrosomes splitting in two, creating a mitotic disturbance. At 166 minutes in figure 5.5A, the lack of a net outward pushing force can be seen as randomly oriented pillar deflections. The net radial force greatly fluctuated here, due to random orientation of all forces. Pillars were significantly deflected, but in a random fashion in all directions. The reason for this discrepancy became clear later when the centrosomes separated and the cell appeared to have 3 centrosomes (centrosomes duplicate in S-phase [1]). After the third centrosome separated, the structure again stabilized and the net radial force increased again up to a plateau of \(\sim 100\) nN.

In another example of mitotic disturbance inward pulling was again released, but further increase in outward pushing was not visible over the full measurement of 15 hours. A tripolar spindle was again visible, but the outward pushing force again did not increase. The mitotic disturbance in figure 5.5 delayed mitosis for several hours. In the previous prophase- and metaphase force response (figure 5.2 and 5.3), the force exertion characteristics also preceded a phenotypic response. This again indicates that the force balance is a precursor for molecular rearrangement. After formation of the tripolar spindle, the cell did proceed into three daughter cells in this case, but only after another increase in outward pushing forces.

The same characteristic force plateau and peak in outward forces build up at anaphase, with ultimately again a short force peak at telophase (352 minutes), as was observed during succesful mitosis. Large outward pushing forces were again observed at the MT base (figure 5.5A), with much larger forces at the lower left base compared to the other ends of the two spindles. The same structural reorganization of the MT structure could be observed as in figure 5.4. The increased forces here can be explained by the active outward pushing of two individual mitotic spindles,
Figure 5.5
Mitotic disturbance is preceded by a lack of outward pushing forces. (A) Time-lapse microscopy of microtubules (green) and micropillars (red) on micropillars ($k_{\text{bend}} = 16.7 \text{ nN/µm}$). (B) Net radial force over time for the cell in (A). (C-D) Schematic representation of microtubules (green), microtubule motors (kinesins, red), cell outline (dashes line) and transmission of forces (arrows). Larger arrows indicate larger forces. (C) Proposed outward pushing mechanism during successful cell division. (D) Mitotic disturbance shows an increased outward pushing where the forces from two mitotic spindles join.
causing larger forces in the lower left compared to the two other centrosomes. In both successful and disturbed division, the outward pushing force plateau was always present before anaphase, telophase and ultimately cytokinesis could take place. This observation provides further evidence for a spindle-mediated force balance.

Radial outward pushing forces during cell division preceded the various characteristic phases of mitosis (figure 5.3). The MT structure at the base of radial forces reorganized during a final peak of force exertion at cytokinesis (figure 5.4). A disturbance in force exertion preceded a mitotic disturbance leading to triplet division (figure 5.5A-B). We therefore propose a model describing a force balance consistent with our experimental observations in which different origins of force exertion contribute to outward pushing from the mitotic spindle to a more sparse MT network towards the cell edge (figure 5.5C-D). Forces are likely maintained by a multitude of interactions, varying from microtubule dynamics and pressure to kinesins and kinetochore complexes. The outward pushing force balance proved to be vital for mitotic spindle integrity and proper cell division.
5.4 Discussion and conclusion

The process of cell division is tightly regulated through numerous checkpoints. Here, we have shown that the mechanical integrity throughout mitosis is of vital importance to the equal division of chromosomes and proper division into two daughter cells. Using our methodology, we have shown that cells go from exerting inward pulling forces on the ECM in interphase through a progressive cycle of outward pushing forces in mitosis. Throughout this process, the cell is mechanically coupled to the ECM-coated substrate continuously. Through active force exertion throughout the division cycle, we demonstrated that the cell is not only outside-in [16, 17] but also inside-out mechanically coupled to the ECM. We measured outward force exertion build-up as the mitotic spindle organizes in prometaphase. Through anaphase and telophase outward pushing forces were needed for progression through the cell cycle. Finally, we assessed that a proper spindle-mediated force balance is crucial for successful cell division.

Assembly of the mitotic spindle is regulated by multiple checkpoints [3]. As chromosomes and microtubules form attachments, this is heavily regulated by the KMN (KNL1, MIS12 and NDC80) network connecting centromeres to microtubules on the dynamic plus end. The increase in outward pushing force coincided with the build-up of this complex where the NDC80 complex may guide microtubule-mediated forces [2]. Outward pushing forces could be further enhanced by multiple kinesins that have proven vital for spindle formation [26, 27]. A previously quantified balance between the actin network along the cell membrane and a build-up of hydrostatic pressure would again positively correlate to the extracellular force exertion we observed [18]. Force exertion carried by many MTs with forces in the order of 3-4 pN [28] per MT for elongating MTs from the two centrosomes contribute to the idea of cohesive outward pushing during mitosis as described in figure 5.5C and D. Furthermore, the most dynamic MTs can be found at the center of the mitotic spindle as well as at the cell edge [21]. These dynamics at the KMN network [3] at the metaphase plate and the actin network [18] at the cell membrane, respectively, correspond to the locations of force transmission in our model of a changing force balance throughout mitosis.

As the cell further proceeds into metaphase, kinetochore-based activation of the Spindle Assembly Checkpoint plays an important role. The Anaphase Promoting Complex (APC, also known as Cyclosome)
triggers further mitotic progression upon phosphorylation of KNL1 [3]. Biochemically these processes are regulated by MAD1/MAD2 complexes with CDC20 activating APC/C. Incorporation of the kinase Aurora B then plays an important role [5, 29]. A gradient in phosphorylation state has been directly observed as a function of distance from the metaphase-plate, regulated by tension [30]. Such a phosphorylation gradient would fit well with our notion of a force balance, since we observed a short outward force peak upon the transition from anaphase to telophase. That peak in force could be the final pull along the chromosomes that Aurora B needed to proceed. Outward pushing forces rapidly decreased after final separation. In previous studies it became clear that intracellular forces seem crucial for proper progression through all of these mechanisms, but the force balance with the ECM was never directly quantified.

We propose that this mechanosensitive response depends on the build-up of spindle-mediated outward pushing. If outward pushing is not build up, progression through mitosis is stalled. This is confirmed by the observation that if outward pushing was not present, centrosomes moved apart and a tripolar spindle formed. Our new methodology allowed us to quantify this force balance that couples the mitotic machinery from the inside-out to the extracellular matrix. These mechanical features related to progression through the cell cycle aid in understanding the biophysical coupling through kinetochores and checkpoints. In future work, it will be interesting to further dissect the roles of specific molecular players and their effect on force progression through mitosis.
5.5 Supplemental figures

**Figure S1**
Radial forces compared to perpendicular forces. The mean of all measured net radial forces exerted during the various stages of mitosis are much larger as compared to the perpendicular components (bars indicate s.e.m.).

**Figure S2**
SEM image of spacer next to micropillar array. Micropillars (left) are shown adjacent to a 50 µm high spacer to enable the inversion of the arrays to be able to perform high-resolution imaging with a high-NA objective.
Figure S3
The peak in outward pushing just before cytokinesis. A peak in outward pushing forces is visible just before physical division at 50 minutes (time relative to metaphase).

Figure S4
Lensing effect causes no more than apparent 150 nm deflection per pillar. (A) Curvature of the cell from 10 µm height along the radius of the cell. (B) When we assume a cell with refractive index of 1.38 everywhere around a medium with refractive index 1.34 this curve gives the apparent lensing effect along the radius.
**Figure S5**

Build-up of outward forces does not occur when the mitotic spindle is disturbed. Inward pulling forces from interphase are released, but pushing forces are not observed. This curve of net outward force exertion corresponds to another cell where the mitotic spindle is again disturbed and a tripolar spindle forms. Until the end of the timelapse measurement (900 minutes) the cell is stuck in metaphase.


