ABSTRACT

“Tears of the wine” is a common phenomenon caused by the surface tension gradient of alcohol and water interface. Since its discovery by physicist James Thomson in 1855, it indicates an important relationship between a stable interface and mechanical surface tension. Despite of its commonality and simplicity, such physical relation has been showing promising potential in cell biology. Human and animal cells, unlike bacterial and plant cells, do not have rigid cell walls to maintain their shapes. The extracellular environment is separated from the cell only by a thin lipid membrane. Therefore, mechanical forces exerted from both side of the lipid membrane plays a crucial role in determining the stability of the membrane surface. However, unlike passive water-wine interface, human and animal cells can actively exerted forces to its extracellular environment, in order to compensate and to adjust sudden change in extracellular environment, through varieties of mechanosensitive signal pathways.

My graduate studies start with a simple interfacial force balance condition, known as the “Young-Laplace Equation” in the field of fluid mechanics. Despite of its simplicity, this force balance equation couples the cell shape and membrane tension with internal and external, active and passive mechanical forces, such as hydrostatic pressure difference across the cell membrane and active cortical contractile stress. Membrane tension is believed to be the crucial mechanical que which triggers a series of biochemical signal pathways, such as Rho-MLC signal pathways, which subsequently adjust the cortical active stress and exerting forces on the extracellular environment. In addition, osmotic adjustment by opening and closing mechanosensitive ion channels on the membrane in response to membrane tension change also proves to be important in maintaining a steady state cell shapes and size.
This theoretical background enables us to model and to explain cellular behavior when cells are subjected to sudden osmotic shock and mechanical stretching or confinement at short time scale. At longer time scale, we further discover that active cortical contraction and hydrostatic pressure difference plays an important role throughout cell growth and cell cycle progression.

My theoretical works are backed up with biological experiments, which I have been working with my wonderful lab mates and collaborators from Professor Denis Wirtz’s lab. From collaboration, I am able to measure the volume and the amount of myosin expression (which directly related to the actin cortical stress) at the single cell level. Advanced image analysis methods help me to gain a quantitative understanding of what determines cell shapes and volume at both single cell and overall population level. From that, I further proved the close relationship between cortical active stress and cell shape and volume I purposed in the previous theoretical formulation. Furthermore, we discovered that active cortical stress can potentially serve as a mechanical checkpoint as cells existing G1 phase.

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# TABLE OF CONTENTS

ABSTRACT .................................................................................................................. ii

ACKNOWLEDGEMENT ............................................................................................... iv

TABLE OF CONTENTS ............................................................................................... vi

LIST OF TABLES .......................................................................................................... viii

LIST OF FIGURES ........................................................................................................ ix

CHAPTER 1: INTRODUCTION AND MATHEMATICAL FRAMEWORK ......................... 1
  1.1 An Introduction to Cell Mechanics .................................................................... 1
  1.2 Basic Force Balance ......................................................................................... 4
  1.3 Thesis Overview ............................................................................................... 9

CHAPTER 2: ACTIVE BIOCHEMICAL REGULATION OF CELL VOLUME AND SIMPLE
MODEL OF CELL TENSION RESPONSE .................................................................. 12
  2.1 Introduction ..................................................................................................... 12
  2.2 General Model Description ............................................................................. 16
  2.3 Results ............................................................................................................ 22
    2.3.1 Robust control of cell volume and membrane tension ...................... 22
    2.3.2 A simple model of cell tension homeostasis .................................. 24
    2.3.3 Cell tension depends on cantilever stiffness .................................. 26
    2.3.4 Cells during a step change in force ..................................................... 27
  2.4 Discussion ....................................................................................................... 29

CHAPTER 3: ROLE OF MEMBRANE-TENSION GATED Ca FLUX IN CELL
MECHANOSENSATION ......................................................................................... 39
  3.1 Introduction ..................................................................................................... 39
  3.2 Materials and Methods .................................................................................. 42
    3.2.1 Cell culture ......................................................................................... 42
    3.2.2 Preparation of microfluidic device ..................................................... 42
    3.2.3 Treatment of cells in compression device ........................................... 44
    3.2.4 Fixation and immunostaining cells in the compression device ....... 44
    3.2.5 Measurement of calcium content using calcium indicating dye ...... 44
    3.2.6 Image acquisition ............................................................................... 46
    3.2.7 Image analysis and data acquisition .................................................... 46
  3.3 Results ............................................................................................................ 47
Table 2-1. Parameters in the model. These parameters are obtained from estimates of typical eukaryotic tissue cells. The rate parameters \((a_1; a_2; d_1; d_2)\) are unknown and adjusted to match experiment .................................................................29

Table 3-1: Parameters used in the model .................................................................60
List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>Mechanistic models that describe cell dynamics must incorporate cell mechanics, biochemistry and gene expression together in a single picture. These systems interact with each other and give rise to complex cell behavior in biological context.</td>
</tr>
<tr>
<td>1-2</td>
<td>Mechanical models of the cell membrane</td>
</tr>
<tr>
<td>2-1</td>
<td>Illustration of Rho Signaling Pathway</td>
</tr>
<tr>
<td>2-2</td>
<td>Model calculations for a spherical cell during omotic shock</td>
</tr>
<tr>
<td>2-3</td>
<td>Biochemical control of contraction and ion permeation help to maintain cell volume and membrane tension</td>
</tr>
<tr>
<td>2-4</td>
<td>Contour plots of steady state behavior of the cell after 0.2 MPa hypo/hypertonic shock</td>
</tr>
<tr>
<td>2-5</td>
<td>Cylindrical cell responses to vertical displacements</td>
</tr>
<tr>
<td>2-6</td>
<td>Tensional homeostasis depends on cantilever stiffness</td>
</tr>
<tr>
<td>2-7</td>
<td>A cylindrical cell subject to a jump in pulling force</td>
</tr>
<tr>
<td>2-8</td>
<td>Ion flux effects when a cylindrical cell is subjected to a mechanical force jump</td>
</tr>
<tr>
<td>2-9</td>
<td>Comparison between models with and without Rho signaling and shear dependent myosin activity for cells with a force jump</td>
</tr>
<tr>
<td>3-1</td>
<td>Response of HT1080 cells to mechanical compression</td>
</tr>
<tr>
<td>3-2</td>
<td>Response of cells to mechanical compression when they are incubated in Ca(^{2+}) free medium</td>
</tr>
<tr>
<td>3-3</td>
<td>The response of cells to mechanical compression when they are incubated with TRPV4 inhibitor</td>
</tr>
<tr>
<td>3-4</td>
<td>Intracellular Ca(^{2+}) dye signal after cells are treated with TRPV4 inhibitor or incubated in Ca(^{2+}) free medium for 30 minutes.</td>
</tr>
<tr>
<td>3-5</td>
<td>Theoretical prediction of cells under mechanical compression</td>
</tr>
<tr>
<td>3-6</td>
<td>YAP expression level before and after compression</td>
</tr>
<tr>
<td>3-7</td>
<td>Model predations with realistic cell geometry</td>
</tr>
<tr>
<td>3-8</td>
<td>Fluoresce image example and intensity expressions</td>
</tr>
<tr>
<td>3-9</td>
<td>YAP/TAZ expression within a cell with and without compression and the adhesion shape dependence of mechanosensation of YAP.</td>
</tr>
<tr>
<td>3-10</td>
<td>Blebbistatin treatment does not significantly affect mechanosensation of RhoA when cells are subject to mechanical compression.</td>
</tr>
<tr>
<td>4-1</td>
<td>Cell volume is heterogeneous and depends on substrate stiffness</td>
</tr>
<tr>
<td>4-2</td>
<td>Cell volume in relation to cell adhesion area and cell shape.</td>
</tr>
</tbody>
</table>
Figure 4-3: Total level and spatial distribution of pMLC are predictors of cell volume. ..........93

Figure 4-4: Cell volume is correlated with nuclear YAP/TAZ level in 3T3s and NuFFs ..........94

Figure 4-5: MSCs show bifurcated behavior in YAP nuclear localization and pMLC level ......95

Figure 4-6. Nuclear YAP and pMLC relation suggests a late G1 checkpoint based on cell tension. ..........................................................................................................................................................................................96

Figure Appendix 1: Quantitative immunofluorescence analysis. .................................................................97

Figure 5-1: Mean stationary volume, peak volume and volume right after division. Cells with most nuclear YAP/TAZ has the highest volume of all three measurements. .................................100

Figure 5-2: Growth rate of the five cell lines. Cells with largest amount of Nuclear YAP grow fastest ........................................................................................................................................................................101

Figure 5-3: Immunostaininig result of Parental (293), YAPKO and LATS 1/2 KO lines. ........102
1.1 An introduction to cell mechanics

Despite of lacking rigid cell walls like plant cells and bacteria cells, animal and human cells can adopt a variety of shapes and sizes and can maintain a relatively stable shapes and sizes even under sudden changes of extracellular environment, through actively exerting forces on the lipid membrane and extracellular matrix to counter forces from extracellular environment (Discher, et. al. 2005; Fraley, et. al. 2010; Bloom, et. al. 2008; Nelson, et. al. 2005). The physical mechanism driving shapes and sizes adaptation plays a crucial role in cell biology, organismal development, tissue morphogenesis and disease biology (Engler, et. al., 2006; Li and Nicklas, 1995). At longer time scale, biologists have discovered such shapes and sizes adaptation is related to cell cycle dynamics, through some “checkpoint” behavior (Ginzerg et. al., 2015; Varsano, et. al., 2017). Henceforth, scientists have been looking for a proper way to relate active and passive mechanical forces with cell shapes and sizes.

The breakthrough has a humble beginning on a dinner table. About 150 years ago, when people started noticing the wine droplets continuously forming and dropping from a ring of clear liquid sticking on top of their glass, (known as the “Tears of the wine”), little do they know that such phenomenon (later known as Marangoni effect) is caused by mechanical surface tension gradient of the interface between alcohol and water, when these two liquids are not homogeneously mixed (Thomson 1855; Carlo 1865). This obscure physical phenomenon carries a powerful message: the relationship between mechanical surface tension and a stable shape of a droplet. The interfacial condition was then studied over the last 150 years, and, today, it finds its way in cell mechanics. Throughout my graduate studies, I show how does such normal physical
phenomenon plays an important role in building the bridge between cell shapes and size and active and passive mechanical forces.

At the early stage of my Ph.D. works, I discovered a simple force balance equation at cell membrane surface, known in the fluid mechanics field as the “Young-Laplace Equation”, which is well-studied in the non-biological applications, especially in calculating capillary pressure (Robert 1999) and determining stability of a soap bubble (Culick 1960). Human and animal cells have fluid-like lipid membrane and a cytoskeleton which behaves like viscoelastic fluid (Bausch, et. al., 1998; Joanny and Prost, 2009), it is quite appropriate to assume that Young-Laplace equation-based mechanical model can give a very accurate prediction on cell shapes at different conditions. Discussed in the next section, when using Young-Laplace equation to the cell mechanics, this force balance equation involves active cortical contractile stress from cell cortex, hydrostatic pressure difference across the cell lipid membrane, membrane tension, as well as local membrane curvature, which is local cell shape. Despite of its simplicity, it gives a very comprehensive picture of how mechanical forces from both sides of the cell membrane affect cell shape and size. Theoretically, through this force balance relations, we can calculate the cell shapes and volume at any given mechanical environments and boundary conditions, either in 2D or 3D, which we will discuss further in the later chapters. As a matter of fact, with the help of biological experiments, which gave a quantitative comparison of myosin content (which is proportional to the active cortical contractile stress) between cells at different conditions, we show that cells at steady state strictly obeys Young-Laplace equation, which I will discuss more in the subsequent chapters.

However, cells are much more complicated systems than the “tears of the wine” (water-alcohol interface), or a soap bubble (which is liquid-air interface). While this interfacial force
balance which works for wine droplets and soap bubbles should also works for the cell membrane at all time and at any given conditions, the cells can actively adjust forces through a variety of biochemical signal pathways, notably through Rho GTPase which controls the myosin contraction and pressure adjustment through active and passive ion fluxes across the cell membrane, in the time scales of minutes (Simoes et. al. 2010; Amano, et. al., 2010; Jilkine, et. al., 2007; Maddox, et. al., 2003; Zhao, et. al., 2007; Sokabe et. al., 2010; Seminario-Vidal, 2011; Pare, et. al., 2014; Kolesnikov, et. al. 2007). Sudden change in membrane surface tension is primary responsible in triggering a series of mechanosensitive pathways, which helps the cell to adapt the new environment (Zhao, et. al., 2007). This assumption is by compressing the cells in a microfluidic-based air-driven compression device. While cells are compressed, the membrane tension decreases immediately, I observed an obvious decrease in cellular Rho activity, which is the upstream of myosin contraction (I will show the results in detail in Chapter 3). Not only does this assumption is tested, I also found out the intracellular Calcium is the link between Rho activation and membrane tension. At time scale of hours or longer, biologists start to discover the relationships between mechanical forces and transcription factor moving in and out of cell nucleus, which potentially affects cell cycle progression and perhaps even the cell phenotype (Provenzano, et. al, 2009; Dupont, et. al., 2011; Halder, et. al., 2012). In summary, we can describe mechanics gens and biochemical signal pathways as a circular loop (Fig. 1-1). Transcriptional changes at longer time scale changes cell force generation. Such changes can alter cell shapes and sizes, which further affects transcriptional changes. The opposite can also be true: changes in mechanical environment can trigger changes in biochemical signal pathways which further changes the transcriptional changes.

Before we go any further, let me briefly describe the force balance in the next section.
1.2 Basic Force Balance

The purpose of developing a quantitative force balance at cell membrane surface is to examine the combined contribution of active cortical contraction and osmotic pressure difference.

At cell membrane surface, let us consider a surface element $S$, with unit normal vector, $n$, and bounded by contour $C$ (Fig. 1-2 (a)) with unit tangent vector, $t$, going counter-clockwise. $m$ is a unit vector that is perpendicular to both $t$ and $n$. If the membrane surface has surface tension, $T$, then the total force on the region bounded by $c$ from membrane tension is then:

$$ F_c = \int_C T \cdot m \, dl (1 - 1) $$

where $dl$ is a line element to the contour $c$ (Fig. 1-2). From Fig. 1-2, we have: $m = -n \times t$.

Then, Eq. 1-1 becomes: $F_c = -\int_C T n \times t \, dl$.

If we integrate an arbitrary vector field: $a \times b$ on the closed contour $c$, where $b$ is a constant vector field, the Stoke’s theorem tells us:

$$ \int_c (a \times b) \cdot t \, dl = \int_S (\nabla \times (a \times b)) \cdot n \, dS (1 - 2) $$

The left-hand side is: $\int_c (a \times b) \cdot t \, dl = \int_c -b \cdot (a \times t) \, dl$. And, since: $\nabla \times (a \times b) = b \cdot \nabla a - b(\nabla \cdot a)$, right-hand side has become: $\int_S [b \cdot \nabla a - b(\nabla \cdot a)] \cdot n \, dS$. Substituting these relations into Eq. 1-2 and cancel the constant vector $b$, we have: $-\int_C a \times t \, dl = \int_S (n \cdot \nabla a - n(\nabla \cdot a)) \, dS$.

Compare this final form with Eq. 1-1 by setting $a= Tn$, we have:

$$ F_c = \int_S -Tn(\nabla \cdot n) + \nabla T \, dS (1 - 3) $$
This relationship shows that there is a net force that acts perpendicular to the boundary \( c \), and is proportional to the local mean curvature, \( H \), since \( 2H = \nabla \cdot \mathbf{n} \) (Spivak 1966). If the tension is constant and the surface is flat, the line integral will be zero.

At equilibrium, the force from the tension, \( F_c \), has to be balanced by the net force acting on the membrane, \( F_f \). The total force on the surface is then:

\[
F_f = \int_S \left[ \mathbf{n} \cdot \sigma_2|_S - \mathbf{n} \cdot \sigma_1|_S \right] dS \quad (1 - 4)
\]

where \( \sigma_1|_S \) is the extracellular stress evaluated at membrane surface, and \( \sigma_2|_S \) is the intracellular stress evaluated at membrane surface. This force jump condition is a fundamental property of interfaces and does not depend on material properties. In our flowing discussion, we generally assume that there is no stress in the extracellular region, or, \( \sigma_1|_S = 0 \). Force balance gives: \( F_c + F_f = 0 \). Therefore, writing Eq. 1-4 and 1-3 on the normal and tangential direction at differential element level (under continuum mechanics framework):

\[
T(\nabla \cdot \mathbf{n}) = \mathbf{n} \cdot \sigma_2|_S \cdot \mathbf{n}\quad (1 - 5)
\]

\[
\nabla T \cdot \mathbf{t} = -\mathbf{t} \cdot \sigma_2|_S \cdot \mathbf{n}\quad (1 - 6)
\]

Eq. 1-5 and 1-6 are subject to modification if extra body forces are presented in the interface. For example, if the hydrostatic pressure difference, \( \Delta P \), will add an additional normal force term to Eq. 1-5, which becomes: \( (\nabla \cdot \mathbf{n})T = \mathbf{n} \cdot \sigma_2|_S \cdot \mathbf{n} + \Delta P \). At single cell level, we generally assume the share stress is small, and tension gradient can be ignorable. But it is not true when we study multicellular system within the epithelial tubes. Eq. 1-5 is commonly known as Young-Laplace equation.
Within the cytoskeleton network, actin actively polymerizes near the cell membrane and depolymerizes some distance from the membrane. This continuous mass flux generates an actin flow, and maintains a relatively constant cortical thickness, \( h \), which is around half micrometer (Clark et al., 2013). Within the cortex, actin filaments rapidly turn over and small myosin motor assemblies exert active contractile forces (Juilcher, et al., 2007; Luo, et al., 2012). Here, we generally assume the myosin-generated stress is isotropic, and is only along the two tangential directions of the membrane surface: \( \sigma_{active} = -\sigma^a (t \otimes t + m \otimes m) \). The negative sign indicates the reaction is contractile. In the next chapter, we will discuss how biochemical signal pathway controls \( \sigma^a \). In general, \( \sigma^a \) is proportional to the amount of activated or phosphorylated myosin. Since we model the cytoskeleton network as viscoelastic fluid, (Joanny and Prost, 2009) the intracellular stress is then:

\[
\sigma_2 = -pI + \mu(\nabla u + \nabla u^T) + \sigma_{active} (1 - 7)
\]

in which \( I = \delta_{ij} \); \( u \) is the spatial velocity field of actin network, and \( \mu \) is the shear viscosity of the network. The diagonal part of the stress tensor acts like a pressure, but the shear part (off-diagonal) vanishes when we assume the actin flow rate is small or is spatially independent. This is the long-time scale behavior of the actin networks. In the subsequent studies, we will hold this assumption. Within the actin network, force balance gives: \( \nabla \cdot \sigma_2 = 0 \). If we assume spatial variation of \( \sigma^a \) is ignorable and cytoskeleton network thickness, \( h \), is much smaller than local cell radius of curvature, \( H^{-1} \), the gradient operator only operates on the two base tangential vectors, which will result: \( \sigma^a h (\nabla \cdot n) n = pn \). Taking this result in Eq. 1-5 plus the hydrostatic pressure term, we have the final form of Young-Laplace Equation: (Normal force balance)

\[
\Delta P = (\sigma^a h + T)(\nabla \cdot n)(1 - 8)
\]
Notice here, $\sigma^a h$ acts as a tension term. Therefore, in the subsequent text, we may frequently referred it as “active cortical tension”.

From this force balance condition, we see that the active stress in the cortex and the membrane tension both balance the hydrostatic pressure difference. At steady state, membrane tension, $T$ is very small compare to cortical tension ($T$ is in the order of $0.01$ to $0.1$ pN/nm (Safran 1994), which is about $1\%$ of $\sigma^a h$), and, therefore, we often ignore membrane tension at longer time scale studies in the subsequent chapters. However, at shorter time scale, a sudden change in osmotic environment or an external mechanical force can potentially increase $T$ by $20$ times or even more, and, therefore, its role can no longer be ignored.

The local mean curvature, $2H = \nabla \cdot n$, determines the local cell shape. Therefore, Eq. 1-8 is sufficient to determine overall cell shape, with correct boundary conditions. For spherical, suspended cells, $2H = 2/R$, in which $R$ is the radius of spherical cell.

For a symmetrical cell spreading in a flat circular pattern (axisymmetric), as shown in Figure 1-2(b), a cell surface point has the coordinate: $r = (R(\theta) \cos \theta \cos \phi, R(\theta) \cos \theta \sin \phi, R(\theta) \sin \theta)$; in which $R$ and $\theta$ are defined in Fig. 1-2(b). From this parameterization, the metric tensor is:

$$ g = \begin{bmatrix} R^2 + \left( \frac{dR}{d\theta} \right)^2 & 0 \\ 0 & R^2 + \cos^2 \theta \end{bmatrix} $$ (1-9)

Therefore, the local areal element is: $dA = \sqrt{\det g} \, d\theta d\phi = R \cos \theta \sqrt{R^2 + \left( \frac{dR}{d\theta} \right)^2} \, d\theta d\phi$. For a prefect spherical surface, this expression becomes $dA = R^2 \cos \theta$. The unit normal vector is: $n = \left( \det g \right)^{-\frac{1}{2}} \left( \frac{\partial r}{\partial \theta} \times \frac{\partial r}{\partial \phi} \right)$, and the curvature tensor is:
\[
\Theta = \left[ \frac{\partial \theta}{\partial \theta} \frac{\partial r(1)}{\partial \phi} \quad \frac{\partial \phi}{\partial \theta} \frac{\partial r(1)}{\partial \phi} \right] n(1) + \left[ \frac{\partial \theta}{\partial \theta} \frac{\partial r(2)}{\partial \phi} \quad \frac{\partial \phi}{\partial \theta} \frac{\partial r(2)}{\partial \phi} \right] n(2) + \left[ \frac{\partial \theta}{\partial \theta} \frac{\partial r(3)}{\partial \phi} \quad \frac{\partial \phi}{\partial \theta} \frac{\partial r(3)}{\partial \phi} \right] n
\]

\[(1 - 10)\]

in which \( r(1), r(2) \) and \( r(3) \) are the first, second and third component of position vector \( r \); and \( n(1), n(2) \) and \( n(3) \) are the first, second and third component of local unit normal vector \( n \).

With this parameterization, the off-diagonal part of the curvature tensor is zero and the two principle curvatures are: \( c_1 = g_{11}^{-1} \theta_{11} \) and \( c_2 = g_{22}^{-1} \theta_{22} \). The mean curvature is, therefore: \( 2H = c_1 + c_2 \).

\[
2H = \frac{2 \left( \frac{dR}{d\theta} \right)^2 + R^2 - R \frac{d^2 R}{d\theta^2} + 1 + \frac{dR}{d\theta} R^{-1} \tan \theta}{\sqrt{R^2 + \left( \frac{dR}{d\theta} \right)^2}} \quad (1 - 11)
\]

For more complicated geometries, obtaining an analytical solution is impossible, and numerical method is required. One method I am going to use to approximate the local mean curvature is by triangulating the cell membrane surface shown in Fig. 1-2(c). The mean curvature at \( i \) th vortex is then (Noguchi and Gompper 2005; Atilgan and Sun 2007)

\[
H_i = \frac{1}{4a_i} \sum_j \left[ \tan \frac{\theta_{ij}}{2} + \sin \frac{\theta_{ij}}{2} \right] l_{ij} \quad (1 - 12)
\]

where \( a_i \) is the area around the vertex, and the sum is over \( j \) vertices connected to the \( i \) th vertex. \( l_{ij} \) and \( \theta_{ij} \) are the length of the connecting edge and the angle between triangles sharing the edge, respectively.
1.3 Thesis Overview

As mentioned in the previous section, I purpose a simple model which describes the relationship between hydrostatic pressure difference, active myosin contraction and membrane tension and local mean cell curvature. Such force balance serves as a bridge between mechanical forces and internal cellular biochemical pathway. In this work, I examined how cells adjust itself to different mechanical environment at the time scale of minutes, as well as how mechanical tension relate to cell growth and cell cycle progression. At shorter time scale, I develop a model which couples the force balance condition with active regulation of hydrostatic pressure difference as well as biochemical regulation of cortical tension. Such model of how membrane tension directly relates to Rho activation, which is the upstream of myosin activation, is examined through directly compressing the cells in the microfluidic device. To be more concrete, we purpose that calcium is the key component of such mehcanosensation. At longer time scale, with the help of my lab mates, we discover a crucial checkpoint behavior of cortical tension. Here, I will give a quick summary on the subsequent chapters and how they connect together.

In Chapter 2, I modeled how cells with simple geometry (suspended spherical cell and adherent cylindrical-shaped cell between substrate and cantilever) under osmotic shock and uniaxial mechanical force. I showed the cells are able to recover to pre-osmotic shock size, due to the active regulation of cortical stress through Rho GTPase and osmotic adjustment through active and passive ion fluxes.

The model described in Chapter 2 is under the assumption that sudden change in membrane tension directly triggers the Rho activation. But is that really true, and if it is, how could this happen? Studies have suggested that mechanosensitive Calcium channel, such as TRPV4 (Sokabe, et. al. 2010), plays a role in such process. Therefore, with the help from
Professor Wirtz’s lab, we developed an air-driven microfluidic device, which mechanically compress the cell. To monitor Rho activity under mechanical compression, we used HT1080 cells which stably express FRET-based sensor from Dr. Yi Wu at University of Connecticut. This process and results are described in Chapter 3, and we concluded that calcium plays an important role in mechanosensation.

Both Chapter 2 and Chapter 3 describe cellular behavior at short time scale (time scale shorter than 30 minutes). But what happen to the cell shapes and size changes at time scale of hours? I am particularly interested in the relationship between mechanical forces and cell cycle progression. With the help of my lab mates, we make a connection between mechanical tension and mechanosensitive Hippo pathway. At the end of Chapter 3, I mentioned about how mechanical compression “forces” YAP/TAZ, which is believed to play a crucial role in cell cycle progression at longer time scale, out of cell nucleus. The nuclear YAP/TAZ remains low as long as the confinement is in place, even after 13 hours. This indicates a potential relationship between mechanical forces and cell cycle progression. Henceforth, in Chapter 4, I first demonstrate the steady state cell shapes strictly obey Young-Laplace Equation, by experimentally measuring cell volume at different ECM stiffness and analyzing fix and stain data, which comparing cortical tension of cells under different conditions by quantifying the total Phosphorylated myosin light chain, or, pMLC, level. This is done with the help of my lab mates, Nico Perez and Nash Rochman. I further investigate how cortical tension affect the cell cycle progression, through quantifying the amount of YAP/TAZ in the cell nucleus. With the help of my lab mates Nash Rochman and Nico Perez, together, we are able to make a connection between pMLC and nucleus YAP/TAZ expression and identify cortical tension as a cell cycle checkpoint when cells are leaving G1 to S phase.
Finally, in Chapter 5, I summarize what I find and current and future work I am currently and will be doing after this thesis.

![Mechanical Forces: Active and Passive Forces in Cytoplasm, Cortex and Membrane](image)

![Chemistry: Enzymatic Process and Signaling Pathways](image)

![Genes: Transcription Regulation and 3D Chromosome Structure](image)

Figure 1-1: Mechanistic models that describe cell dynamics must incorporate cell mechanics, biochemistry and gene expression together in a single picture. These systems interact with each other and give rise to complex cell behavior in biological context.

Figure 1-2: **Mechanical models of the cell membrane.** (a) A typical picture of the membrane surface (blue) which is adhered to actin filaments through transmembrane proteins such as formins (red insert). We consider the membrane as a single fluid layer and the actomyosin cortex is modeled as a fluid gel. (b) Side-view of a cell adhered to a circular pattern. Here we parametrize the surface using spherical coordinates (local radius, \( R \), and azimuthal angle, \( \theta \), as showing the panel. We assume the cell geometry is symmetric about \( \theta = \frac{\pi}{2} \). (c) Illustration of surface triangulation. For all the triangular elements sharing the vertex \( I \) (colored in orange), \( j \) labels vertices connected to the vertex \( i \) (1 to 6). \( a_i \) is the area enclosed by the green dotted lines which perpendicularly dissect the \( ij \) edges through the center.
Starting from the “Tear of the wine”, people noticed the important relationship between mechanical surface tension and a stable droplet. Today, this concept has been further studied in the field of cellular physics. Discussed in Chapter 1, I developed a force balance condition which couples the hydrostatic pressure difference, cortical active contractile stress and cell shape. Active contractile forces exerted by eukaryotic cells play significant roles during embryonic development, tissue formation and cell motility. At the molecular level, small GTPases in signaling pathways can regulate active cell contraction. Here, starting with the assumption that membrane tension directly activates small Rho GTPase, which is the upstream effector of myosin contraction, I show mathematically that this active regulation of cellular contractility together with osmotic regulation can robustly control the cell size and membrane tension against external mechanical or osmotic shocks. We find that the magnitude of active contraction depends on the rate of mechanical pulling, but the cell tension can recover. The model also predicts that the cell exerts stronger contraction against stiffer cantilevers, and therefore exhibits features of mechanosensation. These results suggest that a simple system for maintaining homeostatic values of cell volume and membrane tension could explain cell tension response in different environments.

2.1 Introduction

It has been long studied that, in response to sudden change of extracellular environment, eukaryotic cells can actively exert mechanical forces to maintain a homeostatic shape and size. Because of its significance, scientists have been trying to measure the forces cell exerted in both 2 dimensional and 3 dimensional environments (Discher, et. al., 2005; Fraley, et. al., 2010;
Bloom, et. al., 2008; Nelson, et. al., 2005). The results have been shown that such active force exertion by the cell is not only important during cell migration, tissue and organ formation and development, but also important during cell volume control in response to osmotic changes (Stewart, et. al., 2011). Many experiments have noted that cells on stiffer substrates apply stronger contractile forces (Discher, et. al., 2005; Ingber 2002; Geiger, et. al., 2009).

Biochemical signaling pathways have been implicated in this active force generation. Notably, GTPases such as the Rho family of proteins, are part of the signaling pathway that controls myosin II assembly and force generation. The active form of Rho phosphorylates ROCK, which then activates myosin light chain (MLC) (Simoes, et. al., 2010; Amano, et. al., 1997; Jilkine, et. al., 2007; Maddox, et. al., 2003; Zhao, et. al., 2007). This leads to the assembly of myosin mini filaments and an increase in contractile forces. Remarkably, Rho itself also responds to externally applied mechanical forces. When cells are mechanically pulled by attached magnetic beads, the active form of Rho also increases and then diminishes in time, presumably correlating with changes in contractile force (Zhao, et. al., 2007). Related phenomena are seen when cells are subjected to pipet suction. Here, an increase in myosin accumulation is observed at the location of suction force (Luo, et. al., 2013; Fernandez-Gonzalez, et. al. 2009), although Rho activation was not directly measured in those experiments. Lastly, when cells are subjected to osmotic shocks, which changes the mechanical tension across the cell membrane cortex, myosin contraction has been implicated in restoring the cell volume back to the pre-shock values (Stewart, et. al., 2011; Hoffman, et. al., 2009). More recent studies have shown that mechanosensitive (MS) ion channels can regulate the activity of Rho and catalyze the conversion from the inactive form to the active form (Simoes, et. al., 2010; Sokabe, et. al., 2010; Seminario-
Vida, et. al., 2011; Pare, et. al., 2014; Kolesnikov, et. al. 2007). These experiments suggest that there is an important feedback loop between active cell force generation and mechanical tension.

In this Chapter, we coupled the force balance condition discussed in Chapter 1 to a biochemical network by considering simple suspended or mitotic cells where they are spherical, and cylindrical cells between a flat cantilever and a fixed adhesion area. The latter situation has been elegantly examined recently in experiments (Webster, et. al., 2014; Chaudhuri, et. al., 2009). As discussed in Chapter 1, in this chapter and subsequent chapters, the actomyosin cortex can be modeled as an active viscoelastic fluid with rapid actin turnover (Joanny, et. al., 2009; Julicher, et. al., 2007; Mayer, et. al., 2010). Force balance condition described in Chapter 1 suggests that membrane tension could serve as a signal for the control of active contraction and osmotic pressure. Indeed, cell osmotic pressure is partially controlled by mechanosensitive ion channels and ion transporters in the membrane (Jiang and Sun 2013; Hoffmann, et. al., 2009). Recent studies have shown that MS channel TRPIV is involved in activating Rho in response to osmotic pressure changes (Sokabe, et. al., 2010; Seminario-Vidal, 2011). Related experiments in Drosophila cells indicate that the transmembrane protein Toll can activate Rho and contraction (Pare, et. al., 2014; Kolesnikov, et. al., 2007; Simoes, sde, et. al., 2010). Further evidence also suggests that membrane tension is a global signal that controls cell polarization (Houk, et. al., 2012). Labeling of the active form of Rho in live cells showed that Rho is preferentially activated near the cell leading edge, where membrane tension is like high (Pertz, et. al., 2006; Tkachenko, et. al., 2011). Here, we demonstrate that how to model this system mathematically and compute the cell response to external changes in osmolarity as well as externally applied forces.
The coupling between mechanical force balance and biochemical signal pathways purposed in this chapter unify a number of related phenomena in cell mechanics. First, the purposed system is able to maintain a relatively constant cell volume in response to osmotic changes. Osmotic shocks lead to changes in the hydrostatic pressure difference and membrane tension and cause water flow across the cell membrane. In our model, this leads to ion flows across the membrane and changes in active contraction. The result is a robust adjustment of cell volume and membrane tension back to the pre-shock values, in accordance with single-cell experiment shown in Stewart, et. al. 2011. We also show that neither ion flow nor active contraction alone leads to robust adaptation to osmotic shocks. Both systems are needed to obtain robust volume control. Second, when a cell is stretched between two cantilevers, external mechanical pulling also leads to water flow and membrane tension changes. This increases active contractile forces that again try to restore cell volume and membrane tension. Cell active contraction therefore changes over time. The contraction dynamics of the cell depends on the rate of pulling, and the final cell tension depends on the total amount of deformation. Third, when a cell is subjected to a jump in externally applied mechanical force, Rho becomes activated and there is a membrane tension jump. However, Rho activation recedes over time because of ion flows, and the membrane tension is restored to the pre-jump values, in accordanced with the dynamics observed by Zhao, et. al. 2007. Fourth, our model is able to predict that the cell will exert larger steady contractile force against a stiffer substrate. The steady-state cell volume also varies depending on cantilever stiffness. This result indicates that our model can explain some features of cell mechanosensation where stiffness of the cell substrate influences cell contractility. It also indicates that active control of cell contraction can explain cell volume dynamics as well as cellular response to externally applied forces.
We begin by considering mechanics of the cell cortex and membrane subjected to excess osmotic pressure in the cell. We then describe the regulation of osmotic pressure by membrane channels and ion pumps, and a simple model of Rho regulation of myosin contraction. Model predictions for cells subjected to osmotic shocks or mechanical shocks, such as a sudden application of pulling force, are analyzed. Detailed predictions of cell responses to mechanical forces and the mechanism of strain-rate-dependent force response are discussed. We also demonstrate that the model predicts increasing levels of myosin activation when the cell contracts against substrates of increasing stiffness. In comparison with our previous work on cell volume control (Jiang and Sun, 2013), which considered an elastic constitutive relation for the cell cortex, this work focuses on dynamics for a liquid-like cortex and active control of myosin contraction. The liquid-like cortex is highly the correct description for most tissue cells under normal circumstance. Therefore, our model suggests that the cell volume exists as a stable steady state of a dynamically controlled biochemical system.

2.2 General Model Description

The general force balance is discussed in Chapter 1, section 1.2. Despite the general agreement of modeling actin cortex as active viscoelastic gel as shown in Eq. 1-7, there are also dynamic actin cross-linking proteins that can potentially alter the mechanics of the cortex (Luo, et. al., 2012). Using a simple active gel model, force balance can be solved as shown in Eq. 1-8. When cells have a spherical geometry, Eq. 1-8 is simplified as:

\[
\frac{\Delta PR}{2} = T + \sigma^a h \ (2 - 1)
\]

in which \( R \) is the radius of the sphere.

The cytoplasm is crowded with proteins, RNA and ions; the osmotic pressure inside the cell is therefore generally higher than that of the extracellular milieu. Therefore, there is an
osmotic pressure difference $\Delta \Pi = \Pi_{\text{in}} - \Pi_{\text{out}}$ between inside and outside of the cell, due to the difference in mixing entropy. Since osmotic pressure is an entropic force, $\Pi_{\text{in}}$ is only related to the total osmolyte concentration in the cell, or $\Pi_{\text{in}} \sim RTc_{\text{in}} \propto \frac{n}{V}$; where $V$ is the cell volume (Fig. 4-2A); and $R$ is gas constant; and $c_{\text{in}} = \frac{n}{V}$ is the osmolyte concentration. This means, the osmotic pressure is independent to the permeability of the membrane, and the types of osmolyte, despite the fact that some ions, such as Calcium, may play an important role in triggering biochemical signal pathways, which we will discuss in Chapter 3. In this model, we assume all solute particles are indistinguishable. Water will flow in response to this osmotic pressure difference, but at static equilibrium, the osmotic pressure difference equals the hydrostatic pressure difference: $\Delta \Pi = \Delta P$. The hydrostatic pressure difference is balanced by tension in the membrane and mechanical stress in the cortex and surface curvature, as described in Eq. 2-1.

The force-balance relation in Eq. 2-1 alone does not determine global cell shape and size. This is achieved by dynamic myosin contraction, i.e., $\sigma^a$ is not a constant, but changes in response to mechanical forces applied to the cell. From the force balance relation in Eq. (2-1), we see that osmotic pressure, hydrostatic pressure or any mechanical forces on the cell will change both membrane tension and tension in the cortex. Therefore, we propose that membrane tension could be the upstream signal that catalyzes the activation of Rho. All these models suggest a feedback mechanism where increases in membrane tension and mechanical stress in the cortex lead to increasing myosin contraction and $\sigma^a$. This then restores the membrane tension and passive mechanical stress. Indeed, our model predicts that the cell can maintain essentially a constant membrane tension with this mechanism.

The chemical signaling network we purpose to examine is shown in Fig. 4-. Rho activation is triggered by membrane tension, $T$. Rho activates the MLC, which increases the
fraction of myosin minifilaments, $M$. The concentration of myosin minifilaments is directly
proportional to the active stress:

$$\sigma^a = K_{max} M (2 - 2)$$

in which $K_{max}$ is a maximum contractile stress cell can exert if 100% of the available myosin is
activate. It is a function of protein expression and therefore, can be assumed to be a constant over
short time scale (~minutes).

The biochemical equations are then:

$$\frac{d\rho}{dt} = a_1 \Lambda(T) (1 - \rho) - d_1 \rho$$

$$\frac{dM}{dt} = a_2 (1 - M) \rho - d_2 M (2 - 3)$$

where $a_1, d_1$ are the activation and deactivation rates for Rho, respectively, of Rho; $\rho, M$ are
percentages of activated Rho and myosin, respectively; $a_2, d_2$ are the activation and deactivation
rates for myosin, respectively. Here, for simplicity, we do not explicitly include ROCK in the
pathway. Instead, ROCK dynamics is included in the equation for Rho by using effective rate
constant $a_1, d_1$. $\Lambda$ is the Hill function for Rho activation, which depends nonlinearly on
membrane tension, $T$. We estimate the activation function as: $\Lambda = \frac{T - T_c}{(T_s - T_c)}$ when $T_c < T < T_s$.

$\Lambda = 1$ if $T > T_s$ and $\Lambda = 0$ if $T < T_c$. $T_c$ is the critical membrane tension at which Rho activation
starts and $T_s$ is a saturating tension (Fig. 2-1). The functional form of $\Lambda$ is essentially same as a
Michaelis-Menten type of enzymatic kinetics. Note the assumption of Eq. 2-3 is that the total
amount of Rho and MLC (which determines $K_{max}$) is a constant, which is only true at short time
scale.

Studies have suggested that transient cortical stress could change myosin binding to actin
and power stroke kinetics (Fernandez-Gonzalez and Zallen 2009; Luo, et. al., 2013). This
complex behavior would influence myosin assembly and contraction. These results suggest that myosin assembly and disassembly rates, $a_2$ and $d_2$, depend on transient passive stress in the cortex. One possible way to incorporate this effect is to write:

$$a_2 = a_{20}(1 + f(T_{\text{shear}}))(2 - 4)$$

where $a_{20}$ is constant and $T_{\text{shear}}$ is a passive transient force per unit length in the cortex. $f(T_{\text{shear}})$ is an activation function. For Newtonian fluids, $T_{\text{shear}}$ is proportional to the flow rate in the cortex. This phenomenological model is consistent with the idea that myosin assembly and force production depend on the shear stress (rate of deformation) in the cortex:

$$T_{\text{shear}} = \frac{4\mu v H}{h} (2 - 5)$$

in which $v$ is the general velocity scale of cortical velocity. For example, when the cylindrical cells are subject to uniaxial stretching or compressing, $v$ equals to the velocity at the boundary. The dimensionless activation function, $f(T_{\text{shear}})$ can be assumed as:

$$f = \frac{T_{\text{shear}}}{T_r} (2 - 6)$$

in which $T_r$ is a scale parameter, in units of force per unit length. This represents the tension scale above which shear stress will have a significant effect on myosin activation and force generation. This model also incorporates possible effects of actin cross linkers because changes in cross linker density also leads to change in viscosity, and therefore, affect myosin force production.

This model is also related to the strain-rate-dependent force change (Fig. 2-5). However, the details of the model will have to depend on a better understanding of the relationship between cortex mechanics and myosin assembly and contraction. Other mechanisms that regulate myosin contraction are also possible; for example, calcium influx from tension change can regulate...
myosin contraction directly (Matthews, et. al., 2006; Salbreux, et. al., 2007). It is likely that multiple mechanisms are at play to different degrees in different kinds of cells.

In addition to active regulation of myosin contraction, the cell can also adjust its internal osmotic pressure, $\Pi_{in}$, leading to cell-volume adaptation to osmotic shocks (Hoffmann, et. al., 2009; Stewart, et. al., 2011). Equations for water and ion fluxes were discussed in the recent article (Jiang and Sun 2013). They are:

$$\frac{dV}{dt} = -\alpha A(\Delta P - \Delta \Pi)$$
$$\frac{dn}{dt} = A(J_1 + J_2)(2 - 7)$$

in which $V$ and $A$ are cell volume and surface area, respectively. $\Delta P$ and $\Delta \Pi$ are hydrostatic pressure and osmotic pressure difference across the cell membrane. $dV/dt$ is the rate of cell-volume change due to water flow. $n$ is the total number of osmolytes in the cell. $J_1$ is the net ion flow rate out of the cell through passive mechanosensitive channels, which opens and closes in response to change in membrane tension: $J_1 = -\beta \Lambda'(T) \Delta \Pi$. The negative sign indicates the ion flows according to the ion concentration gradient. $\Lambda'(T)$ is similar Hill function of $\Lambda(T)$ shown in Fig. 2-1, and depends on different set of critical and saturation tension, $T_1$ and $T_2$, respectively.

$J_2$ describes ion flow through active ion pump which pump against the concentration gradients. $J_2$ can be computed from a simple channel model. Assuming that the pump uses energy input, $\Delta G_a$, to generate a pump force of $\frac{\Delta G_a}{\delta}$, in which $\delta$ is the membrane thickness, the steady state flux through a single channel is in the order of: $j_2 = -D \frac{\partial c}{\partial x} - \frac{D}{k_B T} c \left( \frac{\Delta G_a}{\delta} \right)$, where $c$ is ion concentration profile within the channel and $D$ is an effective diffusion constant. The ion
concentration satisfy boundary condition: \( c(0) = c_{in} \) and \( c(\delta) = c_{out} \). This gives the single pump flux as:

\[
j_2 = \frac{D \Delta G_a}{k_B T \delta} \left( \frac{\Delta \Pi \exp \left( -\frac{\Delta G_a}{k_B T} \right)}{RT \left( 1 - \exp \left( -\frac{\Delta G_a}{k_B T} \right) \right)} - c_{out} \right)
\]  (2-8)

From this expression, we can obtain critical osmotic pressure difference, \( \Delta \Pi_c = \Pi_{out} \left[ \exp \left( -\frac{\Delta G_a}{k_B T} \right) - 1 \right] \). When the osmotic pressure difference equals to the critical osmotic pressure difference, the flow rate of ion through active ion pump is zero (which means the ion pump can no longer pump against higher osmotic gradient than this).

The total flow rate through the ion pump is then:

\[
J_2 = Nj_2 = -\gamma (\Delta \Pi - \Delta \Pi_c) \quad (2-9)
\]

where \( \gamma \) is another effective permeation constant that contains the number density of the ion pumps, \( N \). In general, depending on the molecular mechanism of the ion pump, the flux would depend on individual ion concentrations. Eq. 2-9 is the simplest model. More complex models, such as those in (Gao et. al. 1995) and (Armstrong 2003) can be explored as well.

Eq. 2-1, 2-2, 2-3, 2-7 are set of equations that describe cell-volume changes in response to changes in osmotic pressure, mechanical forces and active motor activity. For a spherical cell, the unknowns are \( \rho, M, T, \Delta P, \sigma^a, R \) and \( n \). These equations are not closed, and they require one other relationships—the constitutive law for the cell membrane, which relates the membrane tension to overall area changes of the membrane. A simple linear relationship (Safran 1994) is:

\[
T = \frac{\kappa (A - A_0)}{A_0} \quad (2-10)
\]

where \( \kappa \) is an effective elastic modulus and \( A_0 \) is the reference membrane area where it is not under tension. \( A_0 \) is set by total number of lipid molecules, and can depend on lipid trafficking,
which may be also triggered by membrane tension (Upadhyaya and Sheetz 2004; Alberts, et. al., 2002). In addition, $A_0$ includes possible entropic properties of the membrane and reflects the fact that the membrane is typically highly folded in the cell (Peliti and Leibler 1985). However, lipid trafficking occurs at time scales of hours, and therefore, we can regard reference area is a constant.

The parameters we used in this set of system can be found in table 2-1, at the end of this chapter.

2.3 Results

2.3.1 Robust control of cell volume and membrane tension

Using this model, we can mathematically describe the dynamics of the cell volume during osmotic shock (Fig. 2-2). Results show the behavior of a stable dynamical system arising from active control, where a stable volume is determined not by any reference geometry but by cell parameters such as $K_{max}$ and $n$. A sudden decrease in $\Pi_{out}$ causes water influx into the cell across the membrane, decreasing $\Delta P$, which leads to an increase in membrane tension. Membrane tension changes trigger chemical activation of the Rho-MLC pathway, as well as opening of ion channels at the cell surface. This active contraction and the ion fluxes help membrane tension to recover from the initial changes. Because the cell cortex is a viscoelastic fluid without any reference shape, active contractile stress must adjust to maintain a constant cell volume. If there is no active control, the cell volume cannot adapt properly to osmotic shocks (Fig. 2-3 a). Under hypertonic shock, ion pumps are essential in regulating cell volume, since myosin contraction does not play a role. Without ion fluxes, the cell is unable to recover after hypertonic shock (Fig. 2-3). Without myosin active contraction, the cell is still able to recover, but there is a large overshoot in the cell-volume change after recovery.
Critical parameters in this volume adaptation system are $\Delta \Pi_c$, permeation constants $\gamma$ and $\beta$, and maximum contractile stress, $K_{\text{max}}$. These parameters all can influence the final steady state volume of the cell (Fig. 2-3 b). As we noted, the critical osmotic pressure difference is a function of $\Pi_{\text{out}}$; therefore, the cell volume after recovery is not exactly the same as before the osmotic shock (Fig. 2-2 c). Instead, assuming everything is constant within the cell, the steady-state cell volume decreases as $\Pi_{\text{out}}$ decreases, even though volume initially increases transiently after $\Pi_{\text{out}}$ decreases. $\gamma$ and $\beta$ are permeation constants of the passive and active ion channels. These parameters are related to overall expression levels of these membrane proteins and their molecular properties. We see that these parameters determine the overall steady-state solute content in the cell, and, therefore, steady-state volume (Fig. 2-4).

$K_{\text{max}}$ is a parameter that depends on total available myosin. Myosin active stress also depends on an intact cell cortex, so, depolymerizing actin would impact $K_{\text{max}}$. We see that the steady state cell volume and membrane tension declines as $K_{\text{max}}$ increases (Fig. 2-3). Larger $K_{\text{max}}$ also increase the hydrostatic pressure difference at steady state. However, larger $K_{\text{max}}$ provides stronger control of cell volume after osmotic shocks (Fig. 2-3 b), where the cell volume change after osmotic shock is minimized with respect before the shock.

The rate of cell-volume adaptation depends on the water permeation parameter, $a$, and the chemical rate constants $a_1, a_2, d_1$ and $d_2$ that govern the speed of Rho and myosin activation. In this work, we have set these rate constants such that adaptation occurs within 10 min. This is consistent with results seen in Stewart et al. 2011 and Zhao et al. 2007, where activation of Rho occurred around 5 min after cells were pulled by magnetic beads. This is also the same timescale as for myosin activation after pipet aspiration, which changes the hydrostatic pressure difference across the membrane (Luo et. al. 2013).
As mentioned earlier, we have assumed that the lipid reference area, \( A_0 \), is a constant and that cell volume changes from fluxes through membrane channels such as aquaporins. Cells can also change volume through endocytosis, whose rates should also depend on membrane tension. At longer timescales of hours, lipid trafficking from the Golgi to the cell surface can occur. This would potentially change \( A_0 \), and it suggests a third mechanism of cell membrane-tension control. Previous work suggests that membrane tension can be controlled if the rates of lipid addition and subtraction are functions of membrane tension (Sens and Turner, 2006). This phenomenon is likely if the membrane tension has changed persistently for long periods, and myosin contraction and ion transport are unable to restore membrane tension. Lipid trafficking can provide the final mechanism of restoring cell integrity.

### 2.3.2 A simple model of cell tension homeostasis

The proposed model not only can describe how cells respond to osmotic changes, it can also predict cell response to external applied mechanical forces. Many experiments using different techniques have examined how cells respond to mechanical forces. Here we focus on a simple geometry where the cell is between two plates. One of the plates is actuated vertically at velocity \( v \), leading to an overall change in cell height, \( H \) (Fig. 2-5). Such an experiment was performed recently, where the cell adhesion areas at the two plates are fixed (Webster, et. al. 2014; Webster, et. al., 2011). This implies that there is negligible change in cell adhesion during pulling. We model the mechanics of the cell within this experiment. The goal is to explain changes in mechanical tension on the cantilever.

For a cylindrical cell, the cell volume and surface area are determined by the overall height \( H \) and cell radius, \( R \). During fast pulling or large strain, strictly speaking the cell no longer maintains a cylindrical shape, and the cell radius will vary along the \( z \) direction (Fig. 2-5). The
full geometry is mechanically complicated to analyze. For simplicity, we limit our discussion to
the regime where the pulling rate is slower than the water permeation rate and approximate the
cell as a cylinder throughout. The cell adhesion areas at the two plates are fixed, \( z = 0 \) and \( z = H \).
Therefore we assume \( R \) remains constant through the pulling/compressing process (\( \partial R/\partial t \sim 0 \)).
In this case the volume change is simply related to a change in \( H \), i.e., \( \partial V/\partial t = \pi R^2 \partial H/\partial t \).

Under this condition, the tension can be expressed as:

\[
T = \left( \Delta P + \frac{F}{\pi R^2 - \pi (R - h)^2} \right) R - \sigma a h \left( 1 + \frac{h}{R} \right) \left( 1 - \frac{h}{2R} \right) \left( 2 - \frac{11}{2} \right) (2 - 11)
\]

which is obtained by adding the extracellular force, \( F \), to the Young-Laplace equation. This
result relies on the fact that \( h/H \) is a small parameter and the pulling velocity is slow, and
therefore, the rate of cortical volume change is slow. We see that the membrane tension is again
a combination of hydrostatic pressure, pulling force, cell dimensions, and active contractile
stress. Together with the constitutive relation in Eq. 2-10, water and ion permeation, and
biochemical regulation, we again have a close set of equations. From these, we can predict how
cells respond to external dimensional change and mechanical force, as well as osmotic changes.

Some model results are shown in Fig. 2-5, where we compute the force response of the
cell following the methods used in the experiments of Webster et al 2014. Direct comparisons
between our model results and experimental data from Webster et al 2014 are shown in Fig. 2-5
c and d. We first apply a vertical displacement of 1 micrometer at \( z=H \). This displacement is
applied at different speeds, as illustrated in Fig. 2-5. We find that the force response of the cell
depends on the rate of the external displacement. A faster displacement drives a stronger
development of active contractile force. When a tensile displacement takes place, \( \Delta \Pi \) decreases
due to the volume expansion, which also changes \( \Delta P \). Tension also increases from stretching in
the membrane. These are the initial passive mechanical events upon a sudden tensile
displacement. Subsequently, ions start to flow inside the cell \( (J_2) \) and myosin contraction becomes activated. This leads to contractile stress changes and adaptation of osmotic pressure.

As the osmotic pressure difference recovers, the tensile force also decreases. Our model shows features that are consistent with experiments on cell tension. When cells are pulled, the force jumps significantly but then recovers to a smaller value. The final steady-state force depends on the overall deformation and the strain rate, matching experimental results. As the strain becomes larger, the cell activates a larger portion of myosin in response to pulling and this force eventually saturates. However, the cell pressure is not dependent on strain rate and depends only on ion flux rates. The model also shows that in the negative strain direction, the cell adjusts osmotic pressure but generally resists compression. Here, active contraction does not play a significant role. In previous experimental studies, the cell was also subjected to a step-function-like displacement (with the strain rate approaching infinity), with the result that the final force is smaller than the final force when the pulling rate is smaller: 1 micrometer/min. Currently, our model does not predict such nonmonotonic strain response, because we assume that the cell maintains cylindrical shape and water permeation is fast.

2.3.3 Cell tension depends on cantilever stiffness

Remarkably, this model predicts that the cell should be sensitive to the stiffness of the cell environment. The following experiment can be modeled by our equations. A flexible cantilever is placed at one end of the cell and the cell is allowed to contract against it (Fig. 2-6). When the force exerted by the cell is equal to the cantilever force, \( F = -K\Delta H \) where \( K \) is the cantilever stiffness, the system will reach mechanical equilibrium. Graphically, mechanical equilibrium is equivalent to the intersection of the \( F \)-versus- \( \Delta H \) curve and \( F = -K\Delta H \). The result shows that the cell contractile force increases with increasing cantilever stiffness.
Experimental results from traction-force microscopy have shown a similar trend (Discher, et. al. 2005; Lo, et. al. 2000). Here, the cell volume and membrane tension are both higher when the cantilever is stiffer, and the cell exerts stronger contractile force to try to reduce volume and membrane tension. Note that adhesion in this problem is kept fixed, so cell adhesion changes do not play a role in our model.

In addition to cell tension, the steady-state cell volume also increases with cantilever stiffness (Fig. 2-6 b). These results suggest that while trying to maintain cell volume and membrane tension, the cell will exert forces that depend on the stiffness of the environment or the surrounding extracellular matrix. Although our model does not currently describe cells on a flat substrate, it is possible that the cell on flat surfaces may increase adhesions by spreading more broadly, thus increasing overall cell volume and membrane tension. Contractile forces are then increased to compensate for volume and tension changes. In addition, the strain-rate-dependent force response results in a different force-strain curve, as shown in Fig. 2-6 b. This adds a strain-rate-dependent dimension to the cell’s response to changing cantilever stiffness (Fig. 2-6, c).

In addition, the model predicts that the steady-state amount of active myosin also depends on overall strain and the stiffness of the cantilever. The amount of myosin activation increases with increasing cantilever stiffness. With higher overall myosin activation and active tension in the cortex, the effective stiffness of the actin network in the cortex should be higher.

2.3.4 Cells during a step change in force

Figs. 2-5 and 6 show the cell response under a step change in displacement. Alternatively, one can apply a step change in force, and the results are shown in Fig. 2-7. Here, a jump in applied force of $F=300$ nN is applied. The model predicts that the force jump leads to a
rapid increase in membrane tension and lowering of osmotic pressure. This leads to activation of Rho and contraction. Concomitantly, the cell also adjusts the osmotic content, but this is slower. As the osmotic content rises, the contractile force falls and the membrane tension adjusts to close to its value before the force jump. The increase and decrease in Rho predicted by the model are in agreement with data from Zhao et al. 2007, where cells were pulled by magnetic beads while the active form of Rho was measured. Note that our model assumes that water flow is relatively fast, and that the cell volume increases at the same rate as the force application. This is in general not realistic. Cell volume would slowly increase, which means that the initial jump in Rho activation should be slower. The full problem where water permeation rate is slow is complex and requires sophisticated computational approaches. It is beyond the scope of this article.

Nevertheless, the model predicts an increase and subsequent decrease in Rho activation from cell osmotic adaptation. Also, notice that the intracellular pressure does not recover completely after application of force. This is because pressure at steady state is determined by osmolyte concentrate, and transient ion fluxes may lead to a lower steady-state osmotic pressure.

I also find out that the recovery process from a constant mechanical tensile force, $F=300\text{nN}$ is also highly dependent on both Rho signal pathway and osmotic adjustment. In Fig. 2-8, I showed when a 300 nN uniaxial pulling force is applied to the cylindrical-shaped cell, both $\gamma/\beta T_2$ and $\Delta \Pi_c/\Pi_{out}$ affect the steady state pressure difference and Rho activation level. Ion fluxes become larger when we increase these parameters. Increasing ion fluxes increases the steady state pressure and decreases the permanent pressure change after pulling. However, increasing pressure also results in higher membrane tension and cortical contraction.

If Rho signal network is disabled (Fig. 2-9), under the same myosin assembly parameter (same $T_r$), turning off Rho network disables cell tension recovery, unless $a_2$ is a stronger
function of \( T_{\text{shear}} \). Physically speaking, myosin assembly and contraction have to be more sensitive to transient stress in order for tension recovery if Rho signal network is disabled. This suggests that the cell tension response system is more robust if both mechanisms are involved. However, this also suggests that cytoskeletal structures alone may be able to maintain cell volume and tension without Rho activation in some situations. These redundant mechanisms may explain robustness of cell shape and mechanics in varying environments.

### 2.4 Discussion

Unlike plant and bacteria cells, animal cells lack a stiff outer cell wall to maintain its shape. Thus, active processes must compensate for changes in mechanical tension to maintain the cell shape and volume. Membrane tension and cell volume are both important for many cellular processes. Therefore, a key question is how these variables are maintained. In this paper, we relate cell membrane and cortical tension with the osmotic pressure difference. We unify water and ion permeation, cell cortical mechanics and myosin active contraction in a single model of cell mechanical and osmotic response, and show that osmoregulation and regulation of myosin contraction can work together to maintain cell volume and membrane tension.

Biochemical pathways that can adjust cell contractility have been identified. We study a model where membrane tension directly signals Rho activation and active contraction, and show that such a model can maintain cell volume and membrane tension for variety of environmental perturbations. Of course, other feedback mechanisms are possible. In particular, it has been noted that myosin II accumulation and contraction itself is tension sensitive (Luo, et. al., 2013).

Transient tension in the actin network may also activate Rho and myosin contraction. However, the actin cortex is highly dynamic and turns over quickly on time scales of seconds. Sustained signaling from actin or myosin alone would require complex coordination. Nevertheless, it is
possible multiple feedback mechanisms are at play, and further experiments are needed. Although detailed kinetics of the biochemical pathway is currently not available, we have used generic forms of activations and deactivation. Therefore we expect qualitative agreement with experimental observations.

In this model, the cell membrane has a reference size, described by the constant $A_0$. This reference size is the equilibrium area of the membrane when there are no forces acting on it. It is determined by the total amount of lipid in the membrane and any possible thermal fluctuations that can generate folds. In the cell, $A_0$ is further regulated by lipid trafficking from the Golgi (Tang and Edidin. 2002). If the membrane is under high tension, addition of lipid may become more likely than subtraction (Sens and Turner. 2006). Therefore, there is a third regulatory mechanism that controls $A_0$. Lipid trafficking, however, is likely quite slow, occurring on a timescale of hours, although rapid vesicle fusion has been observed in some situations (Groulx, et. al. 2006). Therefore, in our current model, $A_0$ is assumed to be constant. It is also possible to develop a more comprehensive model incorporating dynamics of. The actual quantitative predictions on homeostatic membrane tension depend on parameters such as $T_c$ and $T_s$, and detailed measurements are needed to determine these parameters in MS channel activation. In our model, we assume that the total protein content in the cell is constant and there is no active production of osmolytes (described by $n$) in the cytoplasm. In a live cell during the G1 phase of the cell cycle, $n$ of course changes due to transcription and translation, and the overall cell volume also increases. How the total protein content is controlled remains to be studied. There appear to be other signaling networks that control the total protein content, possibly by coupling to the active cell-volume control system.
In addition, our model currently does not consider charges and voltage effects. This would require a more detailed model where Na, K, and Cl ions, and their respective channels, pumps, and exchangers are considered. This more detailed model requires other unknown parameters, and is beyond the scope of this article. Results of our model show that a feedback control algorithm governing active cell contraction and osmotic regulation can maintain cell volume and control cellular force generation. Multiple feedback systems are potentially at play in maintaining cell volume and tension. Our model can be extended to consider cells at the multicellular tissue scale as well. At this larger scale, our model exhibits behaviors that are somewhat similar to those observed in the cellular Potts model used in tissue mechanics, although the details are not completely the same (Maree, et. al. 2007; Honda 1978; Farhadifar, et. al. 2007). In addition, with changes in parameters, the model can also exhibit oscillatory behavior (Koride et. al. 2014). The missing model elements are signals that govern actin polymerization (through the Rac pathway) and signaling from cell adhesion. These elements are critical for understanding cell mechanics during migration and interaction with extracellular matrices. Cell adhesion and subsequent signaling affect both actin polymerization and myosin contraction. Once again, it is possible that membrane tension also plays a role. Experiments with beads have shown that integrin engagement alone can trigger actin polymerization (Bun, et. al. 2014). Modeling of these different pathways will yield new predictions about cell behavior in a wide variety of settings.
Table 2-1. Parameters in the model. These parameters are obtained from estimates of typical eukaryotic tissue cells. The rate parameters \((a_1; a_2; d_1; d_2)\) are unknown and adjusted to match experiments.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value (unit)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a_1)</td>
<td>5 (/min)</td>
<td>Activation rate of Rho</td>
</tr>
<tr>
<td>(a_1)</td>
<td>5 (/min)</td>
<td>Activation rate of MLC</td>
</tr>
<tr>
<td>(d_1)</td>
<td>5 (/min)</td>
<td>Deactivation rate of Rho</td>
</tr>
<tr>
<td>(d_2)</td>
<td>5 (/min)</td>
<td>Deactivation rate of MLC</td>
</tr>
<tr>
<td>(A_0)</td>
<td>((4\sim10) \times 10^3 (\mu m^2))</td>
<td>Reference (unstretched) cell membrane area</td>
</tr>
<tr>
<td>(T_c)</td>
<td>0 (pN/nm)</td>
<td>Critical tension for Rho activation</td>
</tr>
<tr>
<td>(T_s)</td>
<td>0.8\sim2 (pN/nm)</td>
<td>Saturation tension for Rho Activation</td>
</tr>
<tr>
<td>(T_1)</td>
<td>0 (pN/nm)</td>
<td>Critical tension for MS ion channels</td>
</tr>
<tr>
<td>(T_2)</td>
<td>0.8\sim2 (pN/nm)</td>
<td>Saturation tension for MS ion channels</td>
</tr>
<tr>
<td>(\Pi_{out})</td>
<td>0.3\sim0.5 (MPa)</td>
<td>Osmotic pressure in the extracellular environment</td>
</tr>
<tr>
<td>(\Delta\Pi_c)</td>
<td>0.1\sim2 (\Pi_{out})</td>
<td>Critical osmotic pressure for active ion pump</td>
</tr>
<tr>
<td>(h)</td>
<td>0.5 ((\mu m))</td>
<td>Cortical layer thickness</td>
</tr>
<tr>
<td>(K_{\text{max}})</td>
<td>10\sim500 (KPa)</td>
<td>Maximum active contractile stress</td>
</tr>
<tr>
<td>(\kappa)</td>
<td>0.2 (pN/nm)</td>
<td>Effective elastic modulus for the membrane</td>
</tr>
<tr>
<td>(\alpha)</td>
<td>30,000 (nm(^3)/(pN \cdot s))</td>
<td>Permeation constant for water flux</td>
</tr>
<tr>
<td>(\beta)</td>
<td>(10^{-23} (\text{mol} \frac{\text{nm}}{\text{pN}^2 \cdot s}))</td>
<td>Permeation constant for MS ion channel</td>
</tr>
<tr>
<td>(\gamma)</td>
<td>(0.05\sim1.5 \times 10^{-3} \beta T_2 \left(\frac{\text{mol}}{s \cdot \text{pN}}\right))</td>
<td>Permeation constant for ion pump flux</td>
</tr>
</tbody>
</table>
Figure 2-1: (a) Illustration of the Rho signaling pathway that activates myosin assembly and active contraction in the cortex. At mechanical equilibrium, the membrane tension must balance both osmotic pressure in the cell and active contraction in the cortex (Eq. 2-1). (b) In our model, we consider membrane-tension changes activates MS channels, which then activate Rho and myosin contraction. The contractile force negatively feedback to membrane tension. The probability of Rho activation, $\Lambda(T)$ starts to increase at critical tension, $T_c$ and saturates at $T_s$.

Figure 2-2: Model calculations for a spherical cell during osmotic shock. (a) Cartoon of the cell showing components important in our model. For the active ion pumps, we have used $\Delta \Pi_c =$
(b) The cell is subjected to a hypotonic shock and then a hypertonic shock. The shock magnitudes are 0.5 \( \Pi_{out} \) and then 0.75 \( \Pi_{out} \). The cell volume can recover to close to the pre-shock value. Indeed, volume, membrane tension, Rho-MLC activation level, and pressure difference all can recover, meaning that the cell can adapt to the new osmotic environment. There is a slight overshoot after recovery, because \( \Delta \Pi_c \) is proportional to \( \Pi_{out} \). (c) The steady-state cell volume depends on \( P_{out} \). The model predicts that the steady volume after recovery is smaller after a hypotonic shock.

![Graphs showing cell volume, membrane tension, and hydrostatic pressure](image)

**Figure 2-3: Biochemical control of contraction and ion permeation help to maintain cell volume and membrane tension.** (a) The model predicts that when Rho signaling control of myosin is turned off and \( \sigma^a \) is a constant (red line), or ion fluxes are turned off and \( J_1 = J_2 = 0 \) (Black line), the cell does not recover cell volume or membrane tension effectively. When both systems are active (green line), the cell volume and membrane tension can effectively maintain a homeostatic value. Note that the initial cell volume before shock is kept the same in the model, but different initial starting points for \( n \) and \( \sigma^a \) are needed to achieve the same initial volume. (b) Steady-state cell volume, membrane tension, and hydrostatic pressure are determined by maximum possible myosin active stress \( K_{max} \) and \( \Pi_{out} \). As \( K_{max} \) increases, the cell volume decreases and pressure increases. The membrane tension decreases. Decreasing \( P_{out} \) decreases steady-state cell volume, although the volume does transiently increase at first. The degree of volume overshoot, \( \Delta V/V_{initial} \), after osmotic shock also depends on \( K_{max} \).
Figure 2-4: Contour plots of stead state behavior of the cell after a 0.2 MPa hypo/hypertonic shock. Labels indicate values of contours. Two critical parameters: $\gamma/\beta T_2$ and $\Delta \Pi_c/\Pi_{out}$ determine the steady state behavior. The contour plots for cell volume, membrane tension, and osmotic pressure difference, which equals to the hydrostatic pressure difference at steady state, and precent change in volume after the shock: $\Delta V/V$. The contour values are labeled.

Figure 2-5: Cylindrical cell response to vertical displacements. (a) The vertical dimension of the cell, $H$, is changed at different velocities. The cortical thickness is $h$ and the contact radius with the cantilevers is $R$. The model is used to compute the necessary pulling force, $F$. (b) As $H$ is changed, the cell force response goes through several phases. Here, red and blue curves
represent two different velocities of vertical displacement. The velocity of vertical displacement affects the transient force developed by the cell. If vertical displacement is fast, there is a large jump in force, because osmotic pressure and active stress cannot adapt quickly. The final steady state force also depends on the displacement velocity. The final steady-state $F$ depends on the rate of mechanical pulling, or strain rate. During pulling, the model also can compute the changes in cell osmotic pressure, $\Delta P$, and the level of myosin active stress. (c) Comparisons of the model results with experimental results from **Webster et al. 2014**. The final steady-state force agrees well with experimental results. The model can also fit a short-term transient force jump. (d) Model prediction of steady-state force as a function of vertical strain for a slow pulling case (1 micrometer/min). Curves at faster pulling rates are shown in Fig. 2-6. Note that the force is not zero at zero strain. Zero force is reached when the strain is $\sim$10%.

**Figure 2-6** (a) A cell contracting against a flexible cantilever. The force of the cantilever is $F = -K\Delta H$, where $K$ is the cantilever stiffness. (b) Mechanical equilibrium is reached when the cantilever force is equal to the cell contractile force. This represents the intersection between the $F$-versus-strain curve and $F = -K\Delta H$ (red line). Our model predictions for $F$ versus strain are taken from Fig. 2-5 d. Different $F$ versus-strain curves represent results from different strain rates. Results show that the cell increases contractile force as $K$ becomes larger (green curve). Here, for an adhesion radius of 20 micrometer, $K = 10$ nN/μm is equivalent to a Young’s modulus of 500 Pa. (c) The model also predicts that cell volume is larger with a stiffer cantilever (blue curve). The contractile force generated by the cell also increases with increasing cantilever stiffness. This is because the activated form of myosin increases with increasing cantilever stiffness, which results from the increasing activation of Rho.
Figure 2-7 A cylindrical cell subjected to a jump in pulling force (300 nN) at $t = 0$ with $K_{\text{max}} = 100$ kPa. The jump in applied force increases the activation of Rho and active stress. The vertical strain therefore decreases slowly after a sudden increase. Water and ion flows also help to adjust the overall tension in the membrane. After a transient jump in pressure, tension and Rho activation, the cell eventually recovers to the steady state Rho and pressure.

Figure 2-8: Ion flux effects when a cylindrical cell is subjected to a mechanical force jump of 300 nN. Rho activation and pressure increases with higher ion inflow, but the unrecovered ratios of Rho and pressure differences also become larger.
Figure 2-9: Comparisons between models with and without Rho signaling and shear dependent myosin activity for cells with a force jump of 300nN at $t = 350$ min. $T_r$ is the parameter describing the level of shear stress dependence in myosin activity. If Rho signaling is turned off, $T_r$ has to decrease by a factor of 5 in order to obtain similar recovery dynamics.
In Chapter 2, I demonstrate, from a theoretical point of view, how animal and human cells are able to adopt to osmotic shock and the sudden change in mechanical environment. This adaptation process involves elements such as cytoskeleton and active contraction from myosin. The theory purposed in Chapter 2 is based on the coupling between force balance equation (Young-Laplace) and a set of models which describes the tension-triggered Rho GTPase signal network, as well as active and passive osmotic regulation. But how does mechanical tension directly affect RhoGTPase and myosin contraction? In this chapter, we demonstrate that calcium currents and membrane tension-sensitive ion channels directly signal to the Rho GTPase and myosin contraction. In response to membrane tension changes, cells actively regulate cortical myosin contraction to balance external forces. The process is captured by a mechanochemical model where membrane tension, myosin contraction and the osmotic pressure difference between the cytoplasm and extracellular environment are connected by mechanical force-balance. Finally, to complete the picture of mechanotransduction, we find that the tension-sensitive transcription factor, YAP, translocates from the nucleus to the cytoplasm in response to mechanical compression.

3.1 Introduction

Mechanotransduction, the conversion of physical force into biochemical information inside the cell, is a complex process that regulates a large variety of physiological processes such as embryogenesis and tissue growth (Ingber 2003; Orr et. al., 2006). Dysregulation of mechanotransduction is implicated in the development of major human diseases, such as cancer.
and arteriosclerosis (Jaalouk et. al., 2009; Paszek et. al., 2005; Etienne-Manneville and Hall 2002; Garcia-Cardena et. al., 2001; Haga et. al., 2007). The most upstream process in mechanotransduction is mechanosensation: the earliest step in which the cell senses changes in the external mechanical environmental and/or forces. It is known that the actin cytoskeleton and myosin contractility are required for mechanosensation and mechanotransduction. Though there are many studies that examine how forces regulate cytoskeletal dynamics in vitro (Parekh et. al., 2005; Kovar and Pollard 2004), precisely how the F-actin network and myosin are signaled in live cells by external forces during mechanosensation is unclear. In this paper, by mechanically compressing live cells, we identify that transmembrane calcium currents and membrane tension-sensitive cation channels are responsible for activating RhoA GTPase, which regulate non-muscle myosin II assemblies in the cell cortex and cytoplasm. These experimental results, together with a mechanical model of the cell cortex, suggest that the cell maintains a homeostatic value of membrane tension, and activates myosin contraction in response to tension changes. This feedback loop leads to a dynamic adjustment of active stress generated by the cell, and ultimately can explain main features of mechanosensation.

Cortical tension and myosin contraction in tissue cells are biochemically controlled by the Rho family of small GTPases, especially RhoA (Etienne-Manneville and Hall 2002). RhoA switches between a GTP-bound, active state and a GDP-bound, inactive state, which signals to the Rho-associated kinase, ROCK. ROCK phosphorylates myosin light chain, which then controls mini-filament assembly and generation of active contractile stress. Externally applied mechanical forces can trigger the response of RhoA. For example, the active form of RhoA increases when cells are mechanically pulled by magnetic tweezers (Zao et. al., 2007; Scott et. al. 2016). High shear stress (65 dyn/cm²) on bovine aortic endothelial cells leads to a decrease in
RhoA activity (Liu et. al., 2014), whereas low shear stress induces an initial increase in RhoA activity, which is followed by returning to control levels after 10 minutes (Wojciak-Stothard and Ridley 2003).

To investigate the mechanosensation in live cells in real time, we established a microfluidic-based mechanical compression system, in which the live cells can dynamically switch between a confined and un-confined status. A FRET based sensor is used to monitor the real-time response of RhoA activity in cells when they are subjected to different environments. We find that the mechanical compression leads to an immediate drop in RhoA activity as indicated by the RhoA FRET sensor. The decreased RhoA activity is maintained while the cell is compressed. Upon decompression, RhoA activity resumes to the original level. Either depriving cells of calcium or blocking transient receptor potential cation channel subfamily V member 4 (TRPV4) significantly decreases the change in RhoA activity in response to the mechanical shock. Moreover, inhibiting myosin activity by blebbistatin does not affect RhoA activity change during compression. These results can be recapitulated in a computational mechanical model of cell mechanosensation where membrane and cortical tensions are explicitly connected to an externally applied force. Conceptually, the results and the model suggest that mechanosensation partly arises from a negative feedback control system that maintains a homeostatic membrane tension. To connect mechanosensation with downstream mechanotransduction, we further reveal that the Yes-associated protein (YAP) transcription factor largely left the nucleus and distributed more in the cytoplasm upon compression. This suggests that there is a direct link between physical forces, cell cortical tension and YAP transcriptional activity, as revealed by parallel studies in other settings (Dupont et. al., 2011; Low et. al, 2014).
Our results are relevant for understanding how cells respond to external mechanical forces, and interact with physically confined environments. When metastatic cancer cells leave their primary tumor sites and migrate form a metastasis tumor, they move within and between three-dimensional tissues, capillaries and lymph nodes, the properties of which cannot be fully recapitulated by 2D petri dishes (Fraley et. al., 2010; Giri et. al., 2013; Cukierman et. al., 2001; Lu et. al., 2012). Similarly, cells of the immune system, such as dendritic cells, also migrate within tissues to sample different environments (Alvarez et. al., 2008). Cells also experience mechanical forces from the surrounding matrix (Wirtz et al., 2011) as well as from other cells in different environments (Humphrey et. al., 2014; Swartz et. al., 2001; Thiam et. al., 2016; Berre et. al., 2012). Our work identifies the most upstream signals that allow cells to respond to mechanical forces and physical changes. Identification of calcium as the primary signal is also consistent with observations in cells under mechanical stretch (Kim and Sun et. al., 2014), and opens doors to manipulate mechanosensation properties of live cells.

3.2 Materials and Methods

3.2.1 Cell culture

Human fibrosarcoma HT1080 (ATCC) cells were cultured in Dulbecco's modified Eagle's medium (Mediatech Inc.), high glucose (4.5 g/L), supplemented with 10% fetal bovine serum (Hyclone) and 1 % Pen/Strep (Sigma). The cells were maintained in an incubator with 5% CO2 at 37 °C. HT1080 cells stably expressing RhoA FRET sensor was developed by Dr. Yi Wu at the University of Connecticut, (van Unen et. al., 2015) recently checked for contamination.

3.2.2 Preparation of microfluidic devices
Molds to print the cell culture chamber and air chamber were fabricated by negative photoresist (SU8-2100, MicroChem Corp.). Typical soft lithography procedure was applied to fabricate our microfluidic devices. Briefly, a 200 μm-thick layer of SU8-2100 photoresist (MicroChem Corporation, Newton, MA, USA) was spun-coated onto a silicon wafer and cross-linked by UV light exposure through a photomask. Developer was used to remove non-crosslinked photoresist. Then 200 μm thick layer of PDMS (1:10 of agent to base, Sylgard 184, Dow Corning Corp.) was spun onto the mold of culture chamber and 7 mm thick PDMS was poured onto the mold of air chamber. Both layers with half-cured PDMS were carefully aligned and then baked until completely cured. Mold for micropillars of 4 μm height was fabricated by patterning negative photoresist (SU8-2005, MicroChem Corp.) following similar procedures as described above. Then 100 μm thick layer of PDMS was spun onto the mold and baked until fully cured. The PDMS layer with pillars was carefully peeled off the mold and assembled onto a coverglass, with pillars facing the air. The height of micropillars was measured by profilometer (Dektak IIA). Channel device containing both air chamber and cell culture chamber, 5-mm circular coverglasses and the micropillar PDMS on glass were treated with plasma system. The circular cover glass was then put in the middle of the air chamber. Finally, the channel device with the cover glass was assembled with the micropillar PDMS, with pillar side facing the air chamber.

Before the experiment, 0.2 ug/ml collagen I solution was added into culture chamber, left incubated at 37 °C for 1 hour for coating. Cells were then injected into culture chamber through tubing. After cells adhered and spread onto the collagen-coated PDMS surface, a moderate pressure (~10 psi or 68 kPa) was applied through the tubing to the air chamber. The pressure was kept constant by a pressure regulator.
3.2.3 Treatment of cells in compression device

After cells were perfused into the culture chamber of the device, they were allowed to attach and spread to the collagen-coated PDMS bottom for 2 hours. Then calcium free medium or normal cell culture medium with drug was perfused into the cell culture chamber through tubing. At least 3ml of medium was used to ensure the existing medium was all replaced. Cells were returned to incubator. After 10 to 30 minutes, the compression device containing cells was transferred to the live-cell unit mounted on the microscope, followed by imaging and compression. The concentration of TPRV4 inhibitor was 5 μM. The concentration of blebbistatin was 25 μM.

3.2.4 Fixation and immunostaining cells in the compression device

After cells were compressed for 30 minutes or overnight, the live-cell unit was turned off. PBS was perfused into the device slowly at room temperature. 3.7% PFA was then perfused in at 1ml/hr using a syringe pump for 30 minutes. Mechanical compression on the cells was released by removing air pressure. After permealizing cells using 0.1% Triton-X in PBS, primary antibody detecting YAP/TAZ (1:100, Santa Cruz Techonology) was perfused into the device. Cells in the device was incubated with the primary antibody at 4°C overnight and then incubated with fluorescent secondary antibody for 2 hours at room temperature. Unbound antibodies were removed by continuously perfusing PBS through the device for 30 minutes at 3ml/hr using a syringe pump.

3.2.5 Measurement of calcium content using calcium indicating dye
The Calcium indicating dye we use is Fluo-4 Direct Calcium Assay Kit (from Thermo Fisher). A 2X stock was prepared according to the manufacturer’s recommended protocol. The 2X stock was then diluted to 1X working concentration with normal cell culture medium, normal cell culture medium with TRPV4 inhibitor or Calcium free medium according to the ensuing experiments.

Before the mediums with Calcium indicating dye were used, the cells were pre-incubated in the normal medium for 4 hours. During the 4 hours of incubation, some cells were treated with TRPV4 inhibitor or incubated under Calcium free medium for 30 minutes, according to the planned experiments. The method of treating cells with TRPV4 inhibitor and Calcium free medium is described above. Then, we replaced the media with respective media together with Calcium indicating dye. After incubating cells with Calcium indicating dye for further 2 minutes, we washed out the Calcium dye with respective media. Then, the cell culture is mounted on the microscope, ready for imaging. For each experiment, we image the cell for 15 minutes with 1 second exposure time. Pictures are taken for every 2 minutes. Example of a cell under differential interference contrast (DIC) channel and Calcium dye channel is shown in Fig. 3-4.

The approximate cell boundary was obtained from the DIC channel by measuring the local contrast, which is shown as the blue line in Fig. 3-4 (a). The approximated cell boundary is dilated for 10 pixels, in order to capture all the scattered fluorescent light (red line in Fig. 3-4 (a)). The cell boundary is further dilated for 50 pixels (black line in Fig. 3-4 (a)). The mean pixel intensity between the red line and the black line is used as approximated background intensity. Then, the total Calcium dye signal within one cell is calculated by summing up the pixel intensities within the red contour after subtracting the approximated mean background intensity. The data is normalized by the mean Calcium dye signal of the control set at initial time point.
The intracellular Calcium dye signal is shown in Fig. 3-4 (b). Cells stably express Calcium dye during the period of 15 minutes of imaging. The cells being treated with TRPV4 inhibitor or incubated under Calcium free media for 30 minutes have significant lower total intracellular Calcium dye expression compared to the control set (Fig. 3-4 (b) and (c)).

3.2.6 Image acquisition

All images were acquired with a Nikon TE2000E epifluorescence microscope (Nikon) equipped with Luca-R CCD camera (Andor Technology), an X-cite illuminator (Excelitas Technologies) and 40 x water immersion objective, NA=1.2, WD=200 μm (Nikon). Each cell was imaged using three scans: donor, FRET and acceptor, using the following band-pass filters: CFP (EX: 436/20, EM: 480/40); YFP (EX: 470/40, EM: 525/50); FRET (EX:436/20, EM:535/40). All images were taken at one-second exposure time. The pixel to length ratio is 1 pixel equals to 0.2 μm.

3.2.7 Image analysis and data acquisition

Since the pixel intensities within the cell region were much higher than the pixel intensities in the background region, a binary image based on the pixel intensities can be generated for each field of view, where pixels with high intensities in the cell region were marked with 1 and pixels elsewhere were marked with 0. We then used Matlab routine, “bwboundaries”, to trace the cell boundary from the binary image. Every traced region with area that was 1,500 pixels square (~60 micrometer square) or lower was considered as debris or cell fragment, and, therefore, was ignored.
For HT1080 live cells stably expressing RhoA FRET sensor, we used the YFP channel to trace the cell boundary, because of its relatively stable fluorescence regardless whether the cells were compressed (Fig. 3-8). For fix and stain images, we used the YAP channel to trace the cell boundary and H2B channel to trace the nuclear boundary. The actual cell boundaries were then dilated 10 pixels away from the original boundaries, in order to capture all the scattered light from the epifluorescence source. The nuclear boundaries, however, are only dilated two pixels away from the traced nuclear boundary, in order to avoid excessive overestimation of nuclear YAP (Examples are shown in Fig. 3-8).

The total fluorescent intensity for one cell was computed by adding all the pixel intensities within a traced boundary, after subtracting the mean background intensity. We repeated each set of experiments three times for statistical analysis. For each experiment, we scaled each integrated fluorescent intensity by the mean integrated intensity of the uncompressed control counterpart. The custom software was written in Matlab, and is available upon request.

3.3 Results

3.3.1 The air-driven microfluidic device to compress mammalian cells

To investigate the effects of mechanical compression on RhoA activity in mammalian cells, we designed an air-driven microfluidic compression device. Similar device was previously used to compress bacterial cells to investigate cell growth (Si et. al., 2015). As illustrated in Fig. 3-1a, the device is comprised of an upper and a lower chamber separated by a PDMS layer of about 200 μm in thickness. The upper chamber can be inflated by air pressure, which deforms the PDMS membrane downwards and applies mechanical compression on the mammalian cells
cultured in the lower chamber. To precisely control the compression on the cells, micropillars were introduced to the device. Micropillars were made of PDMS and assembled onto the bottom of the lower chamber. The pillars support the PDMS membrane, and provide a maximum limit to the downward membrane movement, and thereby control the degree of compression of the mammalian cells. The height of the pillar is about 4 µm, which is smaller than the typical height of the adherent HT1080 cells on 2D substrates (about 8-15 µm). The size of the pillar is 500 µm by 500 µm, with 200 µm distance between them.

To investigate the effects of mechanical compression on RhoA activity in mammalian cells, we used HT1080 cells stably expressing a RhoA FRET sensor. The intracellular sensor is comprised of two fluorescent proteins, RhoA GTPase, and a RhoA binding domain of the Rho effector PKN1 (van Unen et. al., 2015). When RhoA-GDP is converted to RhoA-GTP, a PKN1 moiety binds RhoA-GTP, giving a high FRET state that is detected as an increase in sensitized emission over CFP ratio. We tested the sensor by applying a Rho activator and inhibitor. Results showed that the activity of RhoA sensor changed correspondingly as the activator or inhibitor was added to the cells (Fig. 3-8(b)).

Before loading cells to the compression device, the cell culture chamber of the device was coated by 0.2 ug/ml collagen I to ensure the proper adherence and spreading of the cells onto the PDMS substrate. Then the cells were flown into the culture chamber through tubing. After cells adhered and spread onto the collagen-coated PDMS surface, a moderate pressure (~10 psi or 68 kPa) was applied through the tubing to the air chamber. The pressure was kept constant by a pressure regulator. The downward movement of PDMS layer between the air chamber and the cell culture chamber stopped when the layer contacted micropillars, which applied a defined
mechanical deformation on the HT1080 cells (Fig. 3-1a and b). During compression, a temperature of 37 °C was maintained and fresh medium was supplied by a constant flow.

3.3.2 Instantaneous and reversible RhoA activity change upon compression

Previous work showed that RhoA activity increases and then decreases when cells are pulled by magnetic beads mechanically (Zhao et. al., 2007). It is similarly interesting to probe the change of RhoA activity when live single cells are compressed vertically. We observed that the morphology of HT1080 cells changed instantaneously upon compression, as shown by a sudden increase in the observable size of the cell (Fig. 3-1b, middle panel, Fig. 3-1c). The ratio of the FRET channel and the donor CFP channel, which is an indicator of the RhoA activity, dropped significantly after the cells are compressed by the air-driven PDMS layer (Fig. 3-1c and d). The decrease in Rho activity is maintained constant throughout compression. To investigate whether such changes are reversible, we released the compression by turning off the air flow after 30 minutes of compression. We found that RhoA activity increased quickly back to the original value prior to compression, while there is also a simultaneous recovery in cell size (Fig.3-1 b-d). These results showed that changes in cell size and the RhoA activity are both instantaneous and reversible under compressive mechanical deformation. We also monitored and quantified the fluorescence intensity of YFP channel during the experiment, which should remain constant as long as the expression of RhoA sensor remains unchanged. Results showed that compression and release of compression did not affect the YFP intensity (Fig. 3-8(c)), which further validates that changes in the ratio between the fluorescence intensities of FRET and CFP channel during compression and de-compression are not due to optical artifacts.

3.3.3 Response of RhoA activity to mechanical compression is dependent on Calcium
In the carboxyl-terminal region of RhoA, there is a binding site for calmodulin, which is a ubiquitous transducer of calcium second messenger. A fusion protein was previously designed in which the activity of RhoA was controlled by calcium through calmodulin (Mills and Truong 2010). Moreover, previous research showed that Ca\(^{2+}\) influx is necessary for RhoA activation during human umbilical vein endothelial cell spreading on type IV collagen (Masiero et. al., 1999). To investigate whether calcium is required for the response of RhoA activity to vertical compression, we incubated cells in calcium free medium after they attached and spread onto the substrate in the compression device.

After cells were incubated in calcium free culture medium for 10 minutes, they were subjected to compression within the device. We observed that cells in calcium free medium for 10 minutes had slightly reduced RhoA activity (Fig. 3-2b). After the cells were compressed, RhoA activity still was reduced significantly. However, the relative decrease in RhoA activity for cells incubated in calcium free medium was smaller compared to the change for cells incubated in normal culture medium (Fig. 3-2b and c). We also treated cells with calcium free medium for 30 minutes before compression. We find that longer incubation of cells in calcium free medium significantly decreased the activity of RhoA in cells without compression (Fig. 3-2b and c). The relative decrease in RhoA activity after compression was much smaller compared with cells in control medium and in calcium free medium for shorter incubation time (Fig. 3-2b). To check that these changes are indeed related to reduced intracellular Ca\(^{2+}\), we imaged HT1080 cells treated with a fluorescent Ca\(^{2+}\) dye (Fig. 3-4). Results show that cells in calcium free medium indeed has a lower total intracellular calcium. Taken together, our results of HT1080-RhoA cells in the compression device suggest that both the baseline RhoA activity and the response of RhoA to mechanical compression are calcium dependent.
3.3.4 TRPV4 channels mediate the change of RhoA activity during mechanical compression

We then set out to identify the mechanosenstive element that mediates the response of RhoA activity to mechanical compression. Recent studies showed that mechanosensitive membrane ion channels can regulate the activity of RhoA (Simoes Sde et. al., 2010; Pare et. al., 2014; Kolesnikov and Beckendorf 2007). We are especially interested in transient receptor potential vanilloid 4 (TRPV4), a member of the TRP nonselective cation channel superfamily, because of its calcium permeability (Sokabe et. al., 2010; Seminario-Vidal et. al., 2011). TRPV4 channels are expressed in both neuronal and non-neuronal cells, including HT1080 cells. Channel activation allows cation influx into cells, leading to various Ca\(^{2+}\) dependent processes. We have identified the role of calcium in the response of RhoA activity to vertical compression, which suggests the potential involvement of TRPV4 channel in regulating RhoA activity.

To investigate the role of TRPV4 in RhoA activity and the response to mechanical compression, we treated cells with a TRPV4 inhibitor before compressing them. We observed that incubating cells in TRPV4 inhibitor for 10 minutes did not affect RhoA activity before compression (Fig. 3-3b and c). Compressing the cells in the presence of TRPV4 inhibitor also led to an instantaneous drop in RhoA activity, however, the magnitude of the change in RhoA activity due to compression was significantly smaller than that in control cells (Fig. 3-3b and c). RhoA activity in cells was reduced before compression after treated with TRPV4 inhibitor for a longer period of time, i.e. 30 minutes (Fig. 3-3b and c). There was essentially no change in RhoA activity after the cells are compressed in the presence of TRPV4 inhibitor. Imaging these cells treated with Ca\(^{2+}\) dye shows that they have reduced intracellular calcium (Fig. 3-4). These results are consistent with those from the cells incubated in calcium free medium, suggesting that
TRPV4 channels are indeed regulating the change of RhoA activity to mechanical compression through regulating calcium influx.

3.3.5 *Mathematical Model of cell response to mechanical compression*

The observed changes in Rho activation and the role of Ca flux can be recapitulated in a mathematical model of cell response to mechanical compression. When an external compression force, $F_{\text{ext}}$, is applied to the cell, the applied force alters overall force balance at the cell surface ($F_{\text{ext}}$ is negative if it is a compressive force). Mathematically, this force balance in the surface normal direction is expressed as (Chapter 2; Tao et. al., 2017):

$$2(\sigma_a h + T)H - \Delta P = F_{\text{ext}}/A,$$

(3-1)

where $\sigma_a$ is the active contractile stress in the cell cortex, $h$ is the cortical thickness, $T$ is the membrane tension, $\Delta P$ is the hydrostatic pressure difference across the membrane, $H$ is the cell surface mean curvature, and $A$ is the area over which the external force is applied. $F_{\text{ext}}$ is the external mechanical force experienced by the cell, which is negative if it is a compressive force. When the force is negative, the membrane tension is immediately lowered because $F_{\text{ext}}$ subtracts from $\Delta P$, especially at the apical surface. Therefore myosin tension and membrane tension needed to balance the reduced pressure is lowered. Mechanically, compressive $F_{\text{ext}}$ directly influences membrane tension, $T$, and results in reduced opening of TRPV4 and activity of RhoA. Changes in active RhoA leads to changes in $\sigma_a$, which re-adjusts membrane tension back to the homeostatic value. The opposite scenario is when a pulling force is applied to the cell as in Zhao et. al., 2007 and Kim and Sun, et. al. 2014. In this case, the membrane tension must increase, results in an increase in opening of TRPV4 and activity of RhoA. This feedback control loop can be expressed using kinetic equations as
\[
\frac{d\rho}{dt} = k_1 \frac{T^n}{T^n + \tau} (1 - \rho) - k_2 \rho (3 - 2)
\]

where \( \rho \) is the proportion of active RhoA, and \( k_{1,2} \) are rate constants modeling activation and deactivation. The RhoA activation rate also depend on membrane tension and calcium through a Hill-function, and \( \tau \) is an activation threshold parameter. This Hill-function explicitly describes the activity of TRPV4 as a function of membrane tension. As shown in Fig. 3-5(a) insert, the Hill function, \( \Lambda(T) = \frac{T^n}{T^n + \tau} \), can be approximated by a piecewise linear function. Activation threshold parameter, \( \tau \), determines the critical membrane tension, \( T_s \), above which the channel is fully open. The active contraction is modeled as directly proportional to the amount of active RhoA: \( \sigma_a(t) = \Gamma \rho(t) \), in which \( \Gamma \) is the contractile stress in the situation where RhoA is fully activated. This minimal model can approximately capture the behavior seen in experiments. Moreover, since mammalian cells can actively control the cytoplasmic pressure by adjusting their osmolyte and water content, water flows out of the cell when the hydrostatic pressure is increased, which reduces volume and membrane tension (Tao and Sun 2015; Tao et. al., 2017; Jiang and Sun 2013). When active control of \( \sigma_a \) and \( \Delta P \) are both incorporated the model is able to capture rate-dependent response of cells to external mechanical forces. Note that the force-balance condition in Eq. 1 assumes that the membrane does not detach from the cortex and there is no membrane blebbing. If there is blebbing, membrane tension changes are more dramatic, and the dynamic remodeling of the cortex and adjustment in contractility are complex.

Based on this model, we can examine two extreme scenarios when the cell is under mechanical compression: cells that maintain a constant cross-sectional area and cells that maintain a constant volume (i.e. constant\( \Delta P \), no water flow across the membrane). When cells maintain a constant cross-sectional area, the lateral membrane tension decreases as the volume of
the cell decreases. In addition, apical membrane surface of the cell in contact with the compression surface will experience lower tension. These factors combined suggest that RhoA activity goes down. When cells maintain a constant volume, lateral membrane tension increases as the cell is compressed in the vertical direction. Therefore, RhoA activity would increase in the lateral surfaces. The overall measured RhoA activity would increase.

In Fig. 3-5, we compare and contrast these 2 scenarios for a model cylindrical cell under compression. Different degrees of compression and computed RhoA activities are plotted as functions time. We find that the experimental results are best explained by the case where water can flow out of the cell (Fig. 3-7). In the Appendix, we also use the same model to compute the cell response for a more realistic cell geometry and find a similar result. We can conclude that when the cell is under vertical compression, the behavior is somewhere in between the model extremes discussed above: the overall cell volume decreased, despite the fact that the cell cross sectional area increased, leading to a lower membrane tension and lower RhoA activity. In the Appendix, we also discuss the case where the cell surface area remains constant during compression. In this case, the model predicts that Rho activity would remain constant and the cell internal hydrostatic pressure would adjust to mechanical compression through osmotic regulation (Fig. 3-7).

3.3.6 Change of YAP/TAZ subcellular localization upon mechanical compression

Recent research revealed the significant role that YAP (Yes-associated protein) plays in relaying the mechanical signal in extracellular matrix (ECM) to nucleus (Dupont et. al., 2011). Previous work also showed that the subcellular location of YAP/TAZ is regulated by cell tension and cell geometry, i.e. the cell spreading imposed by ECM  (Dupont et. al., 2011; Wada et. al., 2011; Aragona et. al., 2013). In our compression device, we observed that the observed area of
the compressed cells changes significantly in morphology. Thus, we hypothesized that the subcellular localization of YAP also changes upon mechanical compression.

To test our hypothesis, we fixed the compressed HT1080 cells 30 minutes or 13 hours after subjecting the cells to compression, and then we permeabilized the cells and stained them for YAP. Cells in uncompressed area showed predominant expression of YAP in cell nucleus (Fig. 3-6a, top panel). On the other hand, cells under compression showed much more signal of YAP staining in the cytoplasm (Fig. 3-6a, bottom panel). With the help of H2B-mCherry, a fluorescence labeled histone protein, we were able to quantify the amount of YAP staining in the nucleus and in the cytoplasm. Results showed that there was a dramatic and quick change in YAP subcellular localization upon mechanical compression. The ratio between YAP in nucleus and in cytoplasmic decreased by more than 60% after compression, i.e. most of the YAP “leaked out” the nucleus. There is no difference in cells compressed for 30 minutes or 13 hours (Fig. 3-6(b)). Interestingly, we also find that, while YAP “leaks out” of the nucleus when cells are compressed, cells are expressing more overall YAP, as shown in Fig. 3-9.

We further investigated the response of YAP localization in calcium free medium. Results showed that export of YAP from the nucleus to the cytoplasm during compression was suppressed when the cells were deprived of calcium, suggesting that the translocation of YAP during mechanical compression is dependent on calcium (Fig. 3-6(c)).

3.4 Discussion and Conclusions

Physical environment of the cell can influence many aspects of cell function, and cells have developed sensory systems to respond to environmental changes. By examining cells under mechanical compression, we discover that the activity of RhoA changes quickly in response to the externally applied force. Under compression, RhoA activity decreases, and recovers if the
compression is removed. We found that RhoA activity is regulated by Ca\(^{2+}\) and mechanosensitive cation channel TRPV4. When TRPV4 is blocked or Ca currents are diminished, RhoA activity decreased before mechanical compression, and the response to compression is also less pronounced. This behavior suggests that under normal conditions, the cell membrane is a tension sensor, and possibly through small tension fluctuations, TRPV4 is constantly signaling to RhoA to maintain the appropriate level of contraction. When TRPV4 is partially blocked or Ca currents are absent, the ability to maintain and sense tension changes is reduced. Since RhoA is directly involved in phosphorylation of myosin light-chain and generation of active contractile stress, these results implicate Ca\(^{2+}\) as a major regulator of cellular mechanosensation, in agreement with evidence from other lines of investigation (Thiam et. al., 2016; Hung et. al., 2016). Moreover, these results suggest that the cell membrane and associated channels are major elements in mechanosensation, in agreement with previous suggestions (Haswell et. al., 2011; Venkatachalam and Montell 2007). Our results indicate that on short time scales (~mins), the RhoA sensor FRET activity decreases first, mediated by a reduction in calcium influx.

The results also suggest that myosin contraction is involved in regulating cell membrane tension. This is consistent with a theoretical model of the cell cortex where cortical contraction and membrane tension are together balancing excess hydrostatic pressure in the cell. The excess hydrostatic pressure, which is on the order of 100-1000Pa, is generated from excess osmotic pressure inside the cell. Most of this excess pressure is balanced by cortical contraction, and membrane tension is maintained at a low value. During rapid changes in osmolarity or externally applied mechanical force, the membrane tension changes rapidly, which changes the Ca\(^{2+}\) currents and myosin contraction, so that the membrane tension can be restored back to the
homeostatic value. Therefore, the cortex acts as an active mechanical surface that dynamically adjusts its tension to changing external osmotic or mechanical perturbations. The active adjustment of cortical contraction is mediated by RhoA activity, signaled by $\text{Ca}^{2+}$ currents. The theoretical model of the cell cortex can fully explain the observed data, and predicts many features of active responses of cells to external forces and varying stiffnesses of the cell substrate.

If we attempt to alter myosin activity through non-calcium related pathways, for example by incubating cells in 25uM blebbistatin for 1 hour, our experimental results show that, on average, blebbistatin only decreases RhoA activity slightly, and the relative drop in RhoA activity due to mechanical compression is similar to that of control experiment (Fig. 3-10). This means that blebbistatin, although suppresses myosin contractility, does very little to influence mechanosensitivity of RhoA that is upstream to myosin. We also find that mechanical compression also directly impacts YAP transcription nuclear localization on the time scale of hours. YAP has been identified as a mechanosensitive transcription factor directly involved in mechanotransduction. Our results are consistent with the idea that YAP is sensitive to mechanical state of the cell. In addition, our data reveal that RhoA and myosin contraction should change rapidly (~mins) in response to changes in mechanical force. Therefore, YAP activity may be directly regulated by Rho or myosin activity.

As an additional observation, we find that the mechanosensitivity of YAP may also depend on the cell shape, as more rounded cells have more YAP leaking out of nucleus compared to more elongated cells (Fig. 3-9 (b) and (c) ). However, this may be due to the fact that more rounded cells are taller, and, therefore, are more compressed than the elongated cells.
Taken together, our mechanical compression experiments and model reveal that cells sense mechanical changes from changing membrane tension and calcium currents, and dynamically adjust contractile forces to balance external forces. The active regulation of cell contractile forces can also serve as a signal to change transcription factor localization and protein expression changes that completes the process of mechanotransduction.

3.5: Appendix

3.5.1 Model with realistic cell geometry

In the compression device, most of the cells are not cylindrical in shape. Instead, they form an adhesion with the substrate and the shape of the apical surface can vary. Therefore, we examine the model with a general assumption on cell geometry. Because of the complexity in the cell shape during the dynamic compression-release process, we are only able to compute the steady state results before and after compression. Before compression takes place, we assume the cell is a hemisphere, as shown in Fig. 3-7 (a). Therefore, the force balance equation (Eq. 3-1, Section 3.3) becomes:

$$\frac{\Delta P r_0}{2} = \sigma h + T (3 - 3)$$

where $r_0$ is the radius of the cell. To close the system, we can assume a linear constitutive relation for the membrane tension:

$$T = \kappa \frac{A - A_0}{A_0} (3 - 4)$$

where $\kappa$ is effective stretching modulus of plasma membrane, and $A_0$ is the reference membrane area when the membrane is not under any forces.
When compression takes place, we assume the cell, with height $L$, is symmetric about both $x$ (horizontal) and $y$ (vertical) axis (shown in Fig. 3-7 (a)). The top and bottom surfaces ($z=L/2, z=-L/2$) are almost flat, and therefore, the membrane tension at top and bottom surfaces is zero. As a result, what really contributes to RhoA signal pathway is the membrane tension at apical surface. Based on these assumptions, we can parametrize cell surface using cross-sectional radius, $r$, and vertical axis, $y$ (in which $y$ is ranging from $-L/2$ to $L/2$). Force balance equation (Eq. 3-1.), then becomes:

$$\Delta P + \frac{F_{\text{ext}} \cos \theta}{\pi r^2} = (\sigma_a h + T) \left[ \frac{r''}{(1 + r'^2)^{1.5}} - \frac{1}{r \sqrt{1 + r'^2}} \right]$$

in which $r' = \frac{dr}{dy}$ and $r'' = \frac{d^2r}{dy^2}$. At contact area ($y=L/2$), $F_{\text{ext}} = -\Delta P \pi a_a^2$, in which $a_a$ is the radius of contact area between the cell and PDMS layer. $a_a$ may depend on the externally applied vertical strain, as discussed in the previous study (Jiang and Sun 2013). $\theta$ is the angle between cell’s local normal unit vector and $y$ axis: $\theta = \arctan \left( \frac{L}{y} \right)$ (Fig. 3-7), and $\frac{F_{\text{ext}} \cos \theta}{\pi r^2}$ gives the normal component of vertical compression. One boundary condition for Eq. S3 is: $r' = 0$ at $y = 0$, because of symmetry. Based on the experimental findings, the maximum cross-sectional area increased about 50% after the cell is compressed, or, $r = \sqrt{1.5} r_0$, at $y = 0$ (Reference coordinate is shown in Fig. 3-7 (a)).

Theoretically, the exact cell geometry, membrane tension and proportion of active RhoA can be solved by coupling Eq. 3-5, with Eq. 3-4 and Eq. (3-2). (Eq. 3-4 becomes: $T(y) = \kappa \frac{dA(y) - dA_0}{dA_0}$, for spatially varying mean curvature; where $dA$ is local area element as a function of $y$: $dA = 2\pi r \sqrt{1 + r'^2} \, dy$; and $dA_0$ is local area element of stress-free membrane: $dA_0 = \ldots$)
However, solving these coupled equations proves to be difficult. This degree of complexity is added when we are dealing with spatially varying membrane tension and $\sigma_a$. We can simplify the model by assuming membrane tension and active contraction is spatially independent. With this assumption, we can examine how cells are going to behave under a 50% vertical compressive strain. We find that the overall cell volume and membrane area decreased, even though the maximum cross-sectional area increased by about 40%, as we observed in the experiment (Fig. 3-1). Therefore, membrane tension decreased and RhoA activity goes down as a result.

Table 3-1: Parameters used in the model

<table>
<thead>
<tr>
<th>Parameter Name</th>
<th>Value (unit)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrostatic pressure difference ($\Delta P$)</td>
<td>500 (Pa)</td>
</tr>
<tr>
<td>Maximum Contractility ($\Gamma$)</td>
<td>5,000 (Pa)</td>
</tr>
<tr>
<td>Saturation Tension ($T_s$)</td>
<td>0.1 (pN/nm)</td>
</tr>
<tr>
<td>Rho Activation Rate ($k_1$)</td>
<td>15 (/min)</td>
</tr>
<tr>
<td>Rho Deactivation Rate ($k_2$)</td>
<td>1 (/min)</td>
</tr>
<tr>
<td>Membrane Elastic Modulus ($\kappa$)</td>
<td>0.1 (pN/nm)</td>
</tr>
<tr>
<td>Stress-free Membrane Area ($A_0$)</td>
<td>$1.2\pi r_0^2$ (micrometer square)</td>
</tr>
<tr>
<td>Critical Tension ($T_s$)</td>
<td>0.1 (pN/nm)</td>
</tr>
<tr>
<td>Cortical Layer Thickness ($h$)</td>
<td>500 (nm)</td>
</tr>
<tr>
<td>Cell Radius before compression ($r_0$)</td>
<td>10 (micrometer)</td>
</tr>
</tbody>
</table>

3.5.2 Cylindrical Cells with Constant Surface Area

In the main text, we discussed the behavior of cylindrically shaped cells with constant cross-sectional area and constant volume during compression and release. In both cases, we
assumed that the membrane tension is linearly proportional to areal strain. There is, however, another possibility where cells keep a constant surface area. In this scenario, the membrane tension must be a constant during compression and therefore, the active contraction remains unchanged. If we assume cells are cylindrical in shape both before and after compression, keeping a constant surface area implies an increase in cell volume when the cells are compressed. According to our previous studies (Chapter 2), the pressure difference across the cell membrane: $\Delta P = \Delta \Pi - \alpha \frac{dv}{dt}$; where $\Delta \Pi$, the osmotic pressure difference across the cell membrane, is proportional to solute concentration difference, and $\alpha$ is membrane permeation constant. Since immediately after compression, the solute content does not change, a sudden increase in cell volume means a decrease hydrostatic pressure difference. The mechanical force balance condition, $\Delta P = (\sigma_a h + T)/R$, predicts that the radius of curvature, $R$, will increase to compensate the decrease in hydrostatic pressure difference. Active and passive ion flows across the cell membrane (Chapter 2) will occur to adapt to the sudden change in pressure difference. At steady state, the pressure difference recovers to the pre-compression state, as shown in Fig, 3-7.
Figure 3-1: Response of HT1080 cells to mechanical compression. (a) Experimental setup for the compression device. Fabrication of the device follows (Si et. al., 2015). Cells in the device are compressed by the application of air pressure above the PDMS layer. The compression depth is limited by the support pillars with height 4-6µm. (b) Epifluorescence image of cell (YFP channel) before and after compression, and after the release of compression. The cross-section area of the cell increases under compression, and decreases after the release of compression. (c) Scaled FRET/CFP and cell spreading area over time. Compression takes place at t = 0 min (Indicated by black arrow). Cells are then released at t = 38 min (indicated by red arrow) (n = 40; (d) Summary of average FRET/CFP when cell is uncompressed, compressed and released from compression. (All results are scaled with the mean value of uncompressed cells. Biological replicates = 3. For each biological replicate, there is one technical replicate. P Value: *** P<0.001. Scale Bar = 20 µm. n=40 cells. Error bar stands for standard error).
Figure 3-2: Response of cells to mechanical compression when they are incubated in Ca\(^{2+}\) free medium. (a) Epifluorescence images (YFP channel) of cells before and after compression. When cells are incubated in Ca\(^{2+}\) free medium, there is a significant increase in membrane blebs even without compression. (b) The scaled FRET/CFP ratio before and during the mechanical compression, for the three conditions in (a). Compression takes place at t = 0 min. The FRET/CFP ratio is scaled by the average FRET/CFP before compression for each respective control experiment. (c) Time averaged plots of FRET/CFP corresponding to panel (b). (Each set of results are scaled by its respective control, uncompressed mean FRET/CFP value. For each set, biological replicates = 3. For each biological replicate, there is one technical replicate. P Value: ***P<0.0001; ** P< 0.001; *P<0.01. Scale Bar = 20 µm. n=50 cells for the control data, n=47 cells for 10min Ca free medium, n=27 cells for 30min Ca free medium. Error bar stands for standard error).
Figure 3-3: The response of cells to mechanical compression when they are incubated with TRPV4 inhibitor. (a) Epifluorescence images (YFP channel) of cells before and during compression. (b) The scaled FRET/CFP ratio before and during mechanical compression, for all three conditions in (a). Compression takes place at t = 0 min. The FRET/CFP ratio is scaled by the average FRET/CFP before compression for each respective control experiment. (c) Time averaged plot of FRET/CFP corresponding to panel (b). (Each set of results are scaled by its respective control, uncompressed mean FRET/CFP value. For each set, biological replicates = 3. For each biological replicate, there is one technical replicate. P Value: ***P<0.0001. Scale Bar = 20 µm. n =57 cells for the control data, n = 34 cells for 10 min TRPV4 inhibitor; and n = 30 cells 30 min TRPV4 inhibitor. Error bar stands for standard error)
Figure 3-4: Intracellular Ca\textsuperscript{2+} dye signal after cells are treated with TRPV4 inhibitor or incubated in Ca\textsuperscript{2+} free medium for 30 minutes. (a) Image of a cell in the DIC channel and the fluorescence channel showing Ca dye in the cytoplasm. (b) Total intracellular Ca\textsuperscript{2+} dye signal over time for HT1080 cells with no treatment (control), TRPV4 inhibitor treatment and Ca\textsuperscript{2+} free medium treatment. The Ca\textsuperscript{2+} dye signal is generally stable for 15 minutes after imaging. The slight decrease in signal is due to dye leakage out of the cell, and occurs for all conditions. (c) Population and time average of total intracellular Ca\textsuperscript{2+} dye signal for control, TRPV4 inhibitor treatment and Ca\textsuperscript{2+} Free medium treatment. (Scale Bar = 20 μm. P Value: *** P<10\textsuperscript{-9}. Number of cells in each set of experiment is labeled in panel (c); Biological repeats = 2 for all sets; Technical repeats = 2 for control set of both biological repeats; Technical repeats = 2 for TRPV4 inhibitor treatment of both biological repeats; Technical repeats = 2 for Ca Free treatment of both biological repeats. Error bar stands for standard error).
Figure 3-5: Theoretical prediction of cells under mechanical compression. (a) Mechanistic cartoon of our model: Membrane tension, $T$, and the active contractile stress from myosin contraction, $\sigma^a$, are combined to balance the hydrostatic pressure difference across the cell membrane $\Delta P$. Here, $H$ is local mean curvature and $F_{\text{ext}}$ is an externally applied force over the apical region of the cell, $A$. Membrane tension changes influence Ca influx through TRPV4 channel, which influences Rho activity. The opening probability of TRPV4 channel is described by a Hill function, which can be approximated by a piecewise linear function; (b) Model prediction of RhoA activity in a cylindrical cell in response to vertical compression when the cell maintains a constant cross-sectional area and constant volume. Depending on the extent of vertical compression, reduction of RhoA activity is observed when the cell reduces volume. If the volume remains constant, RhoA activity generally increases. In the experiment, there is a wide range of vertical compression. The observed average RhoA activity decrease suggests that there is a slight reduction in cell volume.
Figure 3-6: YAP expression level before and after compression. (a) Fix and stain images of the cells before (top panel) and after (bottom panel) mechanical compression. (b) Ratio between the nucleus YAP and cytoplasmic YAP before and after compression. Around 70% of nucleus YAP leaked out into cytoplasm after compression. The equilibrium is reached at around 30 minutes after compression takes place. (n=60 cells before compression, n=70 cells for post-compression after 30 min, n=84 cells for post-compression after 13 hours.) (c) The change in the localization of YFP during mechanical compression reduces in the Ca\(^{2+}\) deficit condition, such that the mechanosensation of YAP is suppressed. (Scale bar = 20 micrometer. n = 23 cells for control, pre-compression data, n = 30 cells for control, post-compression data. n = 27 cells for Ca free, pre-compression data and n = 36 for Ca free, post-compression data. Biological replicates = 3. For each biological replicate, there is one technical replicate. P Values: ***P<0.0001; **P<0.01, *P<0.05. Error bar stands for standard error.)
Figure 3-7: Model predictions with realistic cell geometry. (a) Schematic description of the cell geometry before and after compression; (b) Corresponding RhoA activity, overall membrane area and membrane tension in response to vertical compression from the model. With realistic cell geometry, results are similar to the predictions from the simplified model described in the main text. (c) Model prediction of corresponding hydrostatic pressure difference in response to vertical compression, when the cell surface area is assumed to be constant. Here, osmotic regulation by the cell ensures hydrostatic pressure difference remains a constant at steady state before and after compression. Compression takes place at t = 0 min and the cell is released from compression at t = 17 min.
Figure 3-8: (a) Computational tracing of cell boundary from fluorescence images of cells stably express FRET sensor (YFP Channel) and fix and stain images; (b) HT1080 cells stably expressing RhoA FRET sensor were treated with 1 unit/ml Rho activator (Cytoskeleton, CN01) for 30 minutes or 2 μg/ml inhibitor (Cytoskeleton, CT04) for 4 hours, respectively. RhoA inhibitor and activator significantly increases or decreases the activity of the RhoA sensor after respective treatment. (c) Intensity changes in three fluorescence channels when cell is under compression: fluorescence intensities are scaled with precompression intensity value. The overall CFP intensity increased by 10 to 15% when compression is applied; overall YFP intensity remains more or less a constant (within 1% variation); while FRET intensity decreases about 15 to 20 % when compression took place. (Scale Bar = 20 micrometer; P Values: *P<0.05; ** P<0.01. Number of cells and biological and technical repeats are consistent with description in Fig. 1 in the main text. Error bar stands for standard error).
Figure 3-9: YAP/TAZ expression within a cell with and without compression and the adhesion shape dependence of mechanosensation of YAP. (a) YAP/TAZ transcription factor is labeled using immunofluorescence with and without compression. When the cell is under compression, the nuclear portion of YAP decreases, but the total YAP expression significantly increases. (b) Examples of rounded cell and elongated cell before and after compression; (c) Ratio of nuclear vs. cytoplasmic YAP in response to mechanical compression. For elongated cells, YAP is less sensitive to mechanical compression. (P values: ** P<0.01, *** P<0.001. Scale Bar = 20 micrometer. Here, the shape factor is defined as: \[ \frac{4\pi \times \text{Adhesion Area}}{\text{Adhesion Perimeter}^2} \]; cells are more rounded if the shape factor is closer to 1 and are more elongated if the shape factor is closer to zero. Number of cells and biological and technical repeats are consistent with the description at Fig. 5. Error bar stands for standard error.)
Figure 3-10: Blebbistatin treatment does not significantly affect mechanosensation of RhoA when cells are subject to mechanical compression. HT1080 cells stably expressing RhoA FRET sensor was incubated in 25 μM blebbistatin for one hour before imaging took place. (a) Overall FRET/CFP ratio over time. Compression takes place at t = 0 min. (b) Time average FRET/CFP in terms of mechanical compression. Before compression, blebbistatin does slightly decrease overall RhoA activity, but it does not affect the change in RhoA when compression takes place. (P Values: *P <0.01*** P<0.000 001 . n = 34 cells for control data and n = 28 cells for blebbistatin data. Biological Repeats = 3. For each biological repeat, technical repeat = 1).
Previous two chapters describe the active response of a cell to the sudden change in extracellular environment. We have found out cortical stress and hydrostatic pressure difference are crucial in cellular adaptation and to maintain a homeostatic shape and size. This means that at steady state, cells maintain, not only a constant size and shape, but also a constant hydrostatic pressure difference and cortical contraction. To keep the steady state, the membrane tension should remain relatively low and active cortical contraction alone should mostly balance the pressure difference, to avoid sudden activation of ion channels at global cell level. Since cell shape and size are such important parameters in studying cell cycle progression, we believed that at longer time scale, cortical stress and pressure, too, may also play an important role in cell cycle progression and cell size.

In this chapter, using the fluorescence exclusion method, we quantitatively measure cell volume for adherent cells on substrates of varying stiffness. We discover that the cell volume has a complex dependence on substrate stiffness, and is positively correlated with the size of the cell adhesion to the substrate. From the simple Young-Laplace Equation applied to adherent cells described in Chapter 1 (Eq. 1-8, Eq. 1-11, Eq. 1-12), we find the observed cell volume variation can be quantitatively predicted from the distribution of active myosin throughout the cell cortex.

To connect cell mechanical tension with cell size homeostasis, we quantified the nuclear localization of YAP/TAZ, a transcription factor involved in cell growth and proliferation. From Chapter 3, we have already shown that the level of nuclear YAP/TAZ is sensitive to extracellular forces, a sudden decrease in tension will “force” YAP/TAZ coming out of nucleus almost immediately. And, as long as compression is still in place, the nuclear level of YAP/TAZ remains unchanged even after 13 hours (Chapter 3, Fig. 3-6). In this chapter, we also show that
the level of nuclear YAP/TAZ is positively correlated with the average cell volume. Moreover, the level of nuclear YAP/TAZ is also connected to cell tension, as measured by the amount of phosphorylated myosin. Cells with greater apical tension tend to have higher levels of nuclear YAP/TAZ and a larger cell volume. These results point to a size-sensing mechanism based on mechanical tension: the cell tension increases as the cell grows and increasing tension biochemically feeds back to growth and proliferation control.

4.1 Introduction

Over the decades, biologists designed a number of experiments to study the growth dynamics in mammalian cells (Killander and Zetterberg 1965; Fox and Pardee 1970; Yen, et. al. 1975; Brooks and Shields 1985; Hola and Riley 1987 Conlon and Raff 2003; Godin, et. al., 2010; Son et. al., 2012). However, the mechanisms behind cell volume regulation are not well understood (Ginzberg et. al., 2015). It is known that different cell types from the same organism can have dramatically different volumes (Ginzberg et. al., 2015), but how cells sense and control growth/division rates in different conditions is not clear. From genetic studies, several pathways have been implicated in cell volume control: the mTor signaling pathway is known to regulate cell size by stimulating anabolism and inhibiting catabolism (Lloyd 2013; Schmelzle and Hall 2000). Similarly, the mammalian version of the Hippo pathway and its downstream effector YAP/TAZ are important in controlling tissue and organ size, and have been implicated in cell volume regulation (Zhao et.al., 2011; Yu, et. al., 2015; Dong, et. al., 2007; Saucedo and Edgar, 2007). While studies have suggested there is a cell size checkpoint within the cell cycle which determines the added cell volume (Ginzberg et. al., 2015; Varsano, et. al. 2017) , exactly how and what signaling pathways are connected with the size checkpoint is still unclear. Working from a different perspective, cells are active mechanical objects that form adhesions with the
extracellular matrix, and balance forces in the cytoplasm with the extracellular environment (Tao, et. al., 2017). Mechanical properties of the microenvironment have been shown to influence cell growth and cycle related phenomena including differentiation (Engler, et. al. 2006), and may impact cell volume as well. Indeed, YAP/TAZ has been shown to be sensitive to mechanical forces and the stiffness of the environment (Dupont et. al., 2011; Codelia, et. al. 2014; Ellosegui-Artola, et. al. 2016; Piccolo et. al., 2014; Low, et. al., 2014), which suggests the mechanical state of the cell could influence cell growth and volume. In this Chapter, we explore how cytoskeletal tension is related to cell volume and how substrate stiffness influences cell size through the measurement of single cell volumes for several different cell types. We show that how cells distribute their tension in different regions of the cell surface can explain the observed cell volume in different conditions. Moreover, we explore how single cell tension (reported with phosphorylated myosin light chain (pMLC)) is related to YAP/TAZ nuclear localization, and discover that the amount of nuclear YAP/TAZ, which is also the active form, is correlated with the amount of myosin in the apical region of the adhered cell. This is consistent with suggestions that YAP is sensitive to cytoskeletal tension (Dupont, et. al., 2011; Ellosegui-Artola, et. al., 2016). The level of nuclear YAP/TAZ also increases with increasing cell volume suggesting that as the cell grows, it increases myosin activity to maintain force balance and the change in the myosin level can serve as a signal for YAP/TAZ activity which influences the observed cell size.

Several methods have been used to quantitatively measure cell volume (Hurley 1970; Tzur, et. al., 2009; Cadart, et. al., 2017; Sung, et. al., 2013). Here we are interested in a high throughput measurement of live cell volume for single adhered cells. We use the fluorescence exclusion method developed by Bottier et al. and others (Cadart, et. al., 2017; Bottier, et. al., 2011). This method was able to reveal that mitotic cells swell before cytokinesis (Son, et. al.,
Here we simultaneously measure cell volume, cell adhered area, and cell shape factor for three different cell types on substrates varying from 3kPa to GPa (glass) in stiffness. We find that the mean cell volume depends on the substrate stiffness, but that dependence varies across different cell types. For all cells, the measured volume is strongly correlated with cell adhered area, but the slope of this correlation depends on the adhesion shape and the substrate stiffness. For the same adhesion area, more elongated cells have a smaller volume when compared with more circular cells. This result can be explained by a mechanical model of the cell where cortical tension developed by myosin is proportional to the mean curvature of the cell surface. In addition, from quantitative immunofluorescence measurements, we find that the total pMLC content and the spatial distribution of pMLC can predict cell volume. Using the measured pMLC levels as inputs, our mechanical model can be used to predict cell volume across all cell types on all substrates.

Cytoskeletal tension and substrate stiffness have been shown to influence the nuclear localization of YAP/TAZ (Dupont et. al., 2011; Codelia, et. al. 2014; Ellosegui-Artola, et. al. 2016; Piccolo et. al., 2014; Low, et. al., 2014), which in turn influences cell proliferation and growth (Shen, and Stanger 2015). The nuclear portion of YAP/TAZ is a cofactor with TEAD and regulates the transcription of a large group of proteins (Zhao et. al., 2008). To explore how cell tension is related to YAP/TAZ nuclear localization, we performed quantitative immunofluorescence measurements. While the results show dependence on cell type, for the terminally differentiated cells tested, we observed that the average cell volume is positively correlated with the level of nuclear YAP/TAZ. But the nuclear to cytoplasmic ratio of YAP/TAZ is not a predictor of cell volume. The nuclear YAP/TAZ level is also positively correlated with the amount of apical pMLC, a readout of apical cell tension. In mesenchymal stem cells (MSCs),
the behavior of YAP and tension is more complex, but the correlation between nuclear YAP/TAZ and apical pMLC persists. These results suggest that cell tension can potentially serve as a checkpoint signal which allows the cell to sense its volume and control the cell cycle progression in late G1.

4.2 Materials and Methods

4.2.1 Cell Culture

NIH 3T3 fibroblasts were a gift from D. Wirtz (Johns Hopkins University, Baltimore, MD), neonatal foreskin fibroblasts (NuFF) were a gift from S. Gerecht (Johns Hopkins University, Baltimore, MD) and mouse Mesenchymal Stem Cells were a gift from Xu Cao Lab (Johns Hopkins Medical School, Baltimore, MD). The cells were cultured in Dulbecco's modified Eagle's media (Corning) supplemented with 10% fetal bovine serum (FBS; Sigma) and 1% antibiotics solution [penicillin (10,000 units/mL) + streptomycin (10,000 µg/mL); Gibco] at 37 °C and 5% CO₂.

4.2.2 Micro-fluidic device fabrication

Silicon molds were fabricated using standard photolithography procedures. Masks were designed using AutoCAD and ordered from FineLineImaging. Molds were made by following manufacturer’s instruction for SU8-3000 photoresist. Two layers of photoresist were spin coated on a silicon wafer (IWS) at 500 rpm for 7 seconds with acceleration of 100 rpm/s and 2000 rpm for 30 seconds with acceleration of 300 rpm/s respectively. After a soft bake of 4 minutes at 95 °C UV light was used to etch the desired patterns from negative photoresist to yield feature heights that were approximately 15 µm. The length of the abovementioned channels is 16.88 mm and the width is 1.46 mm.
A 10:1 ratio of PDMS Sylgard 184 silicone elastomer and curing agent were vigorously stirred, vacuum degassed, poured onto each silicon wafer and cured in an oven at 80 °C for 45 minutes. Razor blades were then used to cut the devices into the proper dimensions, inlet and outlet ports were punched using a blunt-tipped 21 Gauge needle (McMaster Carr, 76165A679). The devices were then sonicated in 100% EtOH for 15 min, rinsed with water and dried using a compressed air gun.

50 mm glass bottom petri-dishes (FlouroDish Cell Culture Dish, World Precision Instruments) were rinsed with water and then dried using a compressed air gun. The petri-dishes and PMDS devices were then exposed to oxygen plasma for 1 minute for bonding. Finally, the bonded devices were placed in an oven at 80 °C for 45 minutes to further ensure enhanced bonding.

4.2.3 Synchronization and stiffness experiments

Cells were treated with 2 or 5 µg/mL of aphidicolin (Sigma-Aldrich, A0781) for 24 hrs to synchronize them in the G1 phase of cell cycle (Mittnacht et al, 1991). The optimal concentration and incubation period was determined using standard flow cytometry techniques (Fig. S8). To serum-starve cells, the media within the chambers were removed and the chambers were rinsed with 1X PBS 3 times. Serum-free media supplemented with 1% Pen/Strep was then injected into the chambers and left on the cells for 24 hrs.

For stiffness experiments, silicone elastomer was prepared by mixing a 1:1 weight ratio of CY52-276A and CY52-276B (Dow Corning Toray) for 3 kPa (Style et al. 2014), a 0.9:1 weight ratio of CY52-276A and CY52-276B for 12.6 kPa (Berget et al. 2016), a 1:1 weight ratio of QGel 920A and QGel 920B (Quantum Silicones) for 0.4 kPa (Gutierrez et al. 2011). In all
cases the elastomer was vacuum degassed for ~5 min to eliminate bubbles, the polymer was then spin-coated onto the micro-well of the dish at 1,000 rpm for 60 s. The dish was cured overnight and resulted in a ~50 µm thick layer of silicone. The devices were then rinsed with water, dried using compressed air, plasma treated and bonded to the cell volume PDMS devices. The final devices were again placed in an 80 °C for 45 minutes to enhance the bonding.

4.2.4 Cell volume measurements

Micro-fluidic chambers were exposed to 30s oxygen plasma before being incubated with 50 µg/mL of type I rat-tail collagen (Corning; 354236) for 1 hr at 37 °C. The chambers were washed with 1X PBS before approximately 50,000 cells were injected into them. The dishes were then immersed in media to prevent evaporation. The cells were allowed to adhere for 12-18 hrs in the incubator at 37 °C with 5% CO₂ and 90% relative humidity. On the day of the experiment, 0.5 µg/mL of Alexa Fluor 555 Dextran (MW 70 kD; ThermoFisher) dissolved in media was injected into the chambers and the devices were imaged within 1-2 hrs after injection.

The cells were imaged using a Zeiss Axio Observer inverted, wide-field microscope using a 20x air, 0.8 numerical aperture (NA) objective equipped with an Axiocam 560 mono charged-coupled device (CCD) camera. The microscope was equipped with a CO₂ Module S (Zeiss) and TempModule S (Zeiss) stage-top incubator (Pecon) that was set to 37 °C with 5% CO₂ for long-time imaging. Differential interference contrast (DIC) microscopy was used to accurately capture the cell area and shape and Epifluorescent microscopy was used to measure volume. Individual cells were traced using the following algorithm.

Cell contours were segmented from the DIC and epifluorescence (Volume) channels. First, a rough contour is generated from a smoothed copy of the Epi channel where pixels darker
than the background intensity are identified. Next a measure of the local contrast of the DIC channel (here high contrast regions are identified) is used to expand the contour to include small features (small lamellipodia etc.) which have low contrast in the Volume channel and may be missed. This expanded contour is used to identify the cell boundary. Inner and outer annuli are created by dilating this contour 10 and 25 pixels away from the cell. (See Figure Appendix 1) The mean fluorescence intensity of the pixels between inner and outer annulus, or mean background intensity, \( I_{\text{annulus}} \), is related to the total channel height. The volume boundary, shown as purple line in Figure Appendix 1(a), is created by dilating the cell contour 20 pixels away from the cell. The local fluorescence intensity enclosed by the volume boundary, \( I_V \), corresponds to the local height above the cell (\( h_2 \), shown in Figure 4-1(a)). Henceforth, \( \frac{h_2}{\text{Channel Height}} = \frac{I_V}{I_{\text{annulus}}} \). The volume of the cell is then calculated as follows:

\[
V = \int_A h_1 dA = \int_A (\text{Channel Height} - h_2) dA
\]

\[
\sim \text{Channel Height} \sum_{\text{pixels within volume boundary}} \left(1 - \frac{I_V}{I_{\text{annulus}}}\right) \delta A (4 - 1)
\]

in which \( \delta A = 1 \text{ pixels}^2 \). Under 20 X magnification, 1 pixel = 0.23 micrometer.

4.2.5 Immunofluorescence

Immunofluorescence was carried out as described as in (Aifuwa et al, 2015). Briefly, cells were seeded at either single cell density (12,000 cells/cm\(^2\) for 3T3s, 7,500 cells/cm\(^2\) for NuFFs) or confluent density (75,000 cells/cm\(^2\) for 3T3s, 60,000 cells/cm\(^2\) for NuFFs) for 12-18 hrs and then fixed with 4\% paraformaldehyde (Company) for 10 minutes. Samples were then rinsed 3 times with 1X PBS. 0.1\% Triton X (Company) dissolved in PBS is then added for 10
minutes, washed 3 times with 1X PBS and then the fixed cells are blocked with 1% bovine serum albumin (company) for 1 hr at room temperature. Primary antibodies are incubated overnight in 1% BSA. Antibodies used included: YAP 63.7 (1:100; ms; SC/101199), Phospho-Myosin Light Chain 2 Thr18/Ser19 (1:100; rb; Cell Signaling Technology #3674), Anti-CD105 (1:100, Rb, Abcam ab21224), Anti-Cd90/Thy1 (1:100, Rb, Abcam ab 133350). The next day the dishes are rinsed 3 times with 1X PBS and incubated for 2 hrs in secondary antibodies with the following secondary antibodies Mouse Alexa Fluor 488, Rabbit 568, and DNA was stained using 20 ug/mL of Hoechst 33342.

Wide-field microscopy using the set-up described above was used to measure the total pMLC, YAP/TAZ, and DNA content of the cells. To obtain spatial information about pMLC we used a Zeiss LSM 800 confocal microscope equipped with a 63X oil-immersion, 1.2 (NA) objective. A 567nm laser was used to image the stained cells. Images were acquired with a resolution of 1024 x 1024, which gives a field of view of 10485.76 µm². We imaged the cells with confocal image stacks of total thickness of 20 µm to cover the entire height of the cells. Confocal image slices were spaced 2 µm apart and the pinhole size was 1 µm.

For each fluorescence image, we subtract the pixel intensities with mean background intensity. A binary mask is generated based on the pixel intensities of fluorescence image (for the pixel intensities within the cell region is much higher than the intensities of anywhere else), where pixels within the cell/nucleus region are marked with “1” and pixels outside the cell/nucleus are marked with “0”. By multiplying the binary mask with actual fluorescence image, we can identify all the pixel values that is within the cell/nucleus. The total intensities within cell/nucleus boundary is calculated by summing up all the intensity values. The cell and nucleus
boundary is then traced by Matlab routine “bwboundaries”. Every traced region with total area of 1,500 pixels square or less is considered as debris or cell fragments, and, therefore, is ignored.

We utilized the pMLC channel to generate the binary mask for the cell. The traced boundary is then dilated 15 pixels away from the cell, to capture all the scattered light from epifluorescence image. The binary mask for the cell nucleus is generated based on Hoechst channel. No dilation is made on nucleus mask, to avoid overestimation of total nucleus YAP. We multiply the nucleus mask with every cell mask, to exclude all the nuclei from other cells within the same field of view (Example is shown in Fig. Appendix 1).

For confocal z stacks, the basal layer of the cell is identified when clear stress fibers are seen. All stacks that are below the basal layer are neglected. We identified the first apical slide when the stress fibers disappear. The traced boundary of every apical slides is dilated 5 pixels (~1 micrometer) inside the cell, to mark the inner boundary of cortical layer. Cortical pMLC of one apical slide is calculated by subtracting the total PMLC intensities that within the inner boundary from the total pMLC intensity. Fig. 3-4(c) shows pMLC are mainly cortical, except for basal layer, where clear stress fibers can be seen. Therefore, the pMLC within the cell cytoplasm is very minimal compared to cortical pMLC.

4.3 Results

4.3.1 Cell volume is heterogeneous and depends on substrate stiffness

To quantify cell volume in different physical and biochemical environments, we use the fluorescence exclusion method to simultaneously measure single cell volume, adhered area and cell shape for three different fibroblastic cell types (Fig. 4-1a). We compare common mouse fibroblasts (3T3) with human-isolated fibroblasts (NuFF) and mouse-isolated mesenchymal stem cells.
cells (MSC). 3T3 fibroblasts are from the standard NIH line. NuFFs are neonatal foreskin fibroblasts obtained from Global Stem (Rockville, MD) at passage 9 and used up to passage 28. MSCs were isolated from bone marrow of 6-week-old mice. Volume measurements were performed for cells at low density, and on substrates of 3kPa PDMS, 12.6kPa PDMS and glass (~GPa). We also tried 0.4kPa PDMS substrates, but found it to be too soft to form stable microfluidic channels for volume measurement. In addition, we measured cell volume during cell cycle arrest achieved through serum starvation or treatment with aphidicolin on glass substrates. The resultant cell volume measurements displayed in Fig. 4- show several striking features. 1) Individual cell volumes in each condition always show significant heterogeneity, with high proportion of smaller cells. This is in accord with previous results using a different method of measurement (Tzur, et. al., 2009). This heterogeneity is partly explained by the fact that cells are in different stages of the cell cycle, and cells divide symmetrically producing 2 daughter cells. Therefore there are more young cells than old cells. The shape of the volume distributions can be roughly explained theoretically from cell aging dynamics (Stukalin, et. al., 2013). 2) The average cell volume varies significantly across cell types, the largest line tested being the MSCs. The average cell volume also depends on the substrate stiffness. In particular, the 12.6kPa substrate always shows a significant deviation indicating unusual behavior at intermediate stiffness. For 3T3s and MSCs, the average cell volume at 12.6kPa is 32% and 50% higher than on 3kPa and glass, respectively. For NuFFs, it is 40% and 15% less than on glass and 3kPa, respectively. The sharp variation around intermediate stiffness is surprising, but parallels previous work that showed a similar change in cell adhesion shape (Rehfeldt, et. al., 2012) and traction force (Han, et. al., 2012) at intermediate stiffness. 3) Cell cycle arrest using serum starvation and aphidicolin produced significant changes in average cell volume as well. Cells
after serum starvation are generally smaller, while aphidicolin treated cells are significantly larger. Aphidicolin inhibits DNA polymerase and arrests cells in late G1 and early S (Krokan, et. al., 1981). Our results suggest that these cells do not copy their DNA (Fig. S1 and S8), but perhaps continue to accumulate cell mass.

Cell 2D adhered area is often used as a proxy for cell volume. Since we simultaneously measure cell area, cell shape, and cell volume, we can examine the correlation between cell area and volume. Indeed, in all conditions the cell area is positively correlated with cell volume (Fig. 4-2a); however, the slope of the area-volume correlation varies among different conditions. Moreover, the area-volume correlation depends on the 2D adhesion shape factor, defined as $S = \frac{4\pi \text{ Adhered Area}}{\text{perimeter}^2}$. Cells with circular adhesions ($S \approx 1$) are consistently larger in volume (Fig. 4-2a, and c), although there is significant noise. While cells with small adhesion areas do tend to have smaller volumes, adhesion area does not uniquely determine cell volume. For example, NuFFs generally have a larger spread area than 3T3s, but they have similar volumes.

**4.3.2 Cortical contractility and tension distribution can predict cell volume**

To further understand the connection between cell area and volume, we turn to a theoretical model of cell volume based on cell cortical tension balance. When cells are adhered to a flat substrate (Fig. 4-2b), the cell volume is defined by the geometric shape of the apical cell surface. The cortex of mammalian cells consists of an actomyosin network that dynamically adjusts to the hydrostatic pressure difference between the inside and outside of the cell (Tao, et. al., 2017). This force balance condition is described in Eq. 1-8, which can be re-whitened as:

$$H^{-1} = \frac{2a^h + T}{\Delta P}.$$

We write in this way, because $H^{-1}$ is directly related to the global cell size.
For a given pressure difference, cells can actively adjust cortical tension by activating different amounts of myosin contraction through the Rho signaling pathway (Zhao, et. al., 2007; Tao, et. al., 2017). However, under the condition of steady state, where there is no significant osmotic change, \( T \ll \sigma_a h \), and Eq. 1-8 is further simplified to \( H^{-1} = \frac{2\sigma_a h}{\Delta P} \), where \( \frac{2\sigma_a h}{\Delta P} \equiv \lambda \) has dimensions of length. \( H \) is a geometric property of cell, and is related to the apical cell shape \( R(\theta) \) (Fig. 4-2b, Eq. 1-11). If the cell adhesion size, shape, and \( \lambda \) are known, then the volume of the cell can be computed (Eq. 1-11 and 1-12). Theoretical results predict that for the same level of \( \lambda \), the volume is a monotonically increasing function of the adhesion area (Fig. 4-2c). Moreover, for the same adhesion area, increasing \( \lambda \) also increases cell volume. The slope of the area-volume curve also depends on \( S \): for the same \( \lambda \), an elongated cell has a smaller volume. The data shows that rounder cells \((S>0.5)\) are indeed larger than smaller cells \((S<0.5)\) for the same adhered area (Fig. 4-2c). The model can be implemented for arbitrary adhesion shapes, and the computed 3D cell shapes can be compared to reconstructed 3D shapes of cells obtained from confocal z-stack images (Fig. 4-2d).

In live cells, we expect the cortical tension and \( \lambda \) to vary spatially across the cell cortex. The spatial distribution of \( \lambda \) impacts cell volume. From our mathematical model, if \( \lambda \) is concentrated near the basal surface of the cell, then the cell volume is smaller. If \( \lambda \) is uniformly distributed in the apical cell surface, then the volume is larger. To obtain insights from data, we used immunofluorescence and imaged the distribution of phosphorylated myosin light chain (pMLC) using confocal z-stacks. The level of pMLC is a measure of active myosin assemblies in the cell, and is a direct measure of \( \sigma_a \). We also expect pMLC to reflect the level of \( \lambda \) since \( \Delta P \) is likely to be spatially uniform, and is governed by cell osmotic control. Fig. 4-3 shows the
measured vertical distribution of pMLC from confocal measurements for all stiffness conditions. On average, pMLC is more concentrated near the basal surface on stiffer substrates, and more uniformly distribution across the apical cell surface on softer substrates (Fig. 4-3b), in accordance with previous measurements (Han, et. al., 2012). This trend is reflected by the apical vs. basal pMLC ratio, \(\langle pMLC_{apical} \rangle / \langle pMLC_{basal} \rangle\), where \(\langle pMLC_{apical} \rangle\) is defined as mean intensity above the dotted line in Fig. 4-3c and \(\langle pMLC_{basal} \rangle\) is defined as the mean intensity below the dotted line (Fig. 4-3c). The dotted line separates the basal layer of the cell from the apical region, and is defined as the z-position 1μm above the the z-position displaying basal stress fibers. Cells distribute pMLC differently on different substrates, mostly due to integrin engagement and focal adhesion formation (Geiger, et. al., 2009). It is known that integrins and focal adhesions nucleate actomyosin bundles in stressfibers (Tojkander, et. al., 2012). Our mechanical model predicts that the cell volume generally increases with increasing \(\langle pMLC_{apical} \rangle / \langle pMLC_{basal} \rangle\) (Fig. 4-3d). This is because greater pMLC_{apical} corresponds to a more hemispherical cell with greater mean height. Indeed, we can fully explain all average cell volume data in all conditions across 3 different cell types by measuring the total level of pMLC (measured from epifluorescence) and the pMLC distribution (reported by the apical to basal ratio) (Fig. 4-3d). In order to connect measured pMLC intensities with \(\lambda\), a single fitting parameter is used for each cell type (Fig. 4-3d). Therefore, cortical tension and tension distribution can predict cell volume, based on the cortical force balance condition.

4.3.3 Cell tension, growth and connections to the Hippo signaling pathway

We have shown that cell cortical tension and the spatial distribution of pMLC can explain observed cell volume on different substrates. However, it is not clear how the cell regulates
growth and volume increase over the cell cycle and determine the cell volume at division. One possibility is that as cortical tension adjusts to increasing cell mass, the mechanical cue from increasing cortical tension can be a signal for regulating cell growth and division. YAP and its parologue TAZ are downstream effectors of the Hippo pathway, and have been shown to respond to stiffness of the substrate (Dupont, et. al., 2011). To examine the relationship between cell tension as measured by pMLC and YAP/TAZ, we performed quantitative immunofluorescence measurements and stained YAP/TAZ, pMLC and DNA for all three cell types in all conditions, and quantified single cell YAP/TAZ and pMLC levels using widefield epifluorescence (Fig. 4-4). The anti-body used stained for both YAP and TAZ, and therefore from hereon, YAP refers to both YAP and TAZ in this Chapter. Qualitatively, NuFFs and 3T3s show predominantly nuclear localization of YAP, in all conditions; however, the total amount of nuclear YAP, denoted as \( \text{YAP}_n \), did show a significant correlation with the average cell volume in all conditions (Fig. 4b). Note that the cell volume as a function of substrate stiffness shows opposing trends in NuFFs and 3T3s. Cells have largest volume on 12.6kPa for 3T3s, but smallest volume on 12.6kPa for NuFFs. Nevertheless, for both cell types, larger cell volume corresponds to higher levels of nuclear YAP. Both cell types show the largest cell volume and the highest level of nuclear YAP with aphidicolin treatment. Moreover, the total level of pMLC is correlated with total nuclear YAP, both at the individual cell level (Fig 4-4a-d), and also at the population average level across all conditions (Fig. 4-4). Here, 3T3s show a continuous rise in nuclear YAP level with increasing pMLC, but the nuclear YAP level saturates in NuFFs with increasing pMLC, suggesting that other factors may be at play to control nuclear YAP in NuFFs that is absent in the standard 3T3s.
From confocal images, it is possible to estimate the relative proportion of pMLC in the basal surface of the cell vs. the pMLC above the basal surface (apical surface). Since it is the apical surface of the cell that determines cell volume, we compute the total apical pMLC by summing apical intensities from Fig. 3b. We observe that the level of apical pMLC is correlated with nuclear YAP for all conditions, whereas basal pMLC is not correlated with nuclear YAP. These results directly implicate apical pMLC as a possible signal for nuclear translocation of YAP.

4.3.4 MSCs show bifurcated cell tension dependence

In fully differentiated cells, we observed that apical pMLC is correlated with the level of nuclear YAP. In conditions where cell volume is larger, the average nuclear YAP level is also higher. When we examine the same type of data for MSCs, these relationships no longer hold (Fig. 4-5). While on average the cell volume is correlated with nuclear YAP, nuclear YAP is no longer positively correlated with pMLC or apical pMLC (Fig. 4-5a). When we examine single cell data, we discover that depending on the stiffness of the substrate, the correlation between total nuclear YAP and pMLC bifurcates showing two distinct branches. As substrate stiffness increases, there appears to be more cells in the lower branch with lower nuclear YAP. The upper branch generally contains cells with lower nuclear to cytoplasm YAP intensity (N/C) ratio and higher overall YAP expression. The lower branch contains cells with higher N/C ratio, but lower overall YAP as well as lower nuclear YAP (Fig. 4-5b,c). The relative proportion of cells in the upper branch increases with increasing stiffness, which is consistent with the results of Dupont et al. Interestingly, only a single branch is observed in the cell area vs. volume correlation (Fig. 4-2a). We hypothesize that the two branches in the nuclear YAP / pMLC correlation represent two
phenotypes of MSCs, although more than 99% of our MSC population positively stained for both stem markers: CD90 and CD105. Clearly, these cells are not distinguishable through the use of these common differentiation markers. Stem cells might be sensitive to their neighboring cell identity (Smith, et. al., 2015) and cell density; it is possible that cell phenotype is influenced by the local environment.

To check whether cell tension and YAP relationships still hold for the observed branches, we examined the nuclear YAP and pMLC correlation for the separate branches, while assuming their average cell volumes are similar. We only included cells that are distinctly in either branch, and exclude cells that have low nuclear YAP and low pMLC near the origin. The upper/lower branch is defined by cells with $\Sigma YAP_n$ larger/smaller than the plateau drawn in Fig. 4-5b. We find that for individual branches, the correlations between $\Sigma YAP_n$, cell volume, and apical pMLC are again preserved (Fig. 4-5d).

4.4 Discussion and Conclusions

Cell volume is a fundamental property of living cells, and understanding how cells control their growth and volume has implications in development and wound healing as well as a variety of diseases. By performing quantitative immunofluorescence and single cell volume measurements, we discovered that cell volume depends on cell adhesion area and substrate stiffness. This dependence may be explained by how cells balance forces at the cell surface. At an upstream level, cells can sense mechanical force changes in the cell membrane through tension sensitive ion channels and the Rho pathway. When the cytoplasmic pressure, $\Box P$, increases (e.g. from import of organic molecules and ions to make more proteins) the cell also increases water content and increasingly activates RhoA and myosin contraction as more
proteins are synthesized through the cell cycle. As a result of this regulatory system, as the cell grows, more active myosin is developed in the cortex. The spatial distribution of myosin depends on additional factors such as integrin engagement and substrate stiffness; but the overall active myosin content must increase with increasing cell size. We find that the level of apical myosin, or myosin not engaged with integrin adhesions and stressfibers, is directly related to the amount of nuclear YAP level, which also explains why β-integrin influences YAP nuclear localization (Elosegui-Artola, et al., 2016).

If YAP plays a role in cell cycle and growth regulation, then the level of myosin can potentially influence YAP phosphorylation and allow the cell to sense its own size. Indeed, our data is suggestive of a size checkpoint between G1-S that is determined by cell tension. Fig. 4-6 shows the same nuclear YAP and pMLC correlation, but now labeled by cell DNA content. Nuclear YAP level rises with increasing pMLC at different rates on different stiffness substrates, but the maximal YAPn is reached at the same level of pMLC (Fig. 4-6). However, there is diversity in this behavior. For 3T3s, nuclear YAP seems to continue to increase in S/G2 together with pMLC and the rate of YAPn increase depends on the substrate. In NuFFs, YAPn still increases with pMLC in G1 and stops rising at the same level of pMLC, but there are some cells in G1 with large pMLC at the level of the YAPn plateau. The plateau value varies with substrate stiffness. These G1 cells with high pMLC are likely very large in volume. For MSCs in the lower branch, the behavior is similar to NuFFs, showing stiffness dependent YAPn plateau. MSCs in the upper branch are entirely different. They have high YAP expression, but no obvious checkpoint based on tension between G1 and S or distinguishing YAP levels between G1 and S.

In addition to the proposed mechanism of a tension-based cell cycle checkpoint, we find that cell volume in different conditions can be explained quantitatively from a theoretical model.
of 3D cell shape. We also discover that synchronization using serum starvation and aphidicolin has opposite effects on cell volume. The heterogeneous distribution of cell volume can be understood by considering the distribution of cells through the cell cycle. The cell cycle distribution is not uniform, but concentrated near younger cells. Since expression levels of many proteins depend on cell cycle, this result suggests that cell cycle averaged expression level changes would depend heavily on the relative duration of each cell cycle phase. Any perturbations that influence the cell cycle would indirectly influence expressions of many types of proteins. The YAP and the Hippo pathways have been proposed to influence cell cycle (Shen and Stanger, 2015). Quantitative single cell measurement would reveal how mechanical tension and the Hippo pathway can regulate cell growth and proliferation in a variety of conditions.
Figure 4-1: Cell volume is heterogeneous and depends on substrate stiffness. (a) A diagram of the microfluidic channel used for fluorescence exclusion cell volume measurements. The channel height is $h_1 + h_2 = 15\,\mu m$. The fluorescence signal is directly proportional to $h_2$, and the total integrated fluorescence signal after background subtraction gives the cell volume. Images of 3T3, NuFF and MSC cells in the microfluidic device showing DIC and fluorescent channels. The DIC channel is used to trace the 2D cell adhesion boundary and compute adhesion area and shape factor, $S$. The scale bar corresponds to 10um. (b) Histograms of cell volumes on 3kPa, 12.6kPa and glass substrates for 3T3, NuFF and MSCs. The wide distribution reflects intrinsic variation in cell size as well as effects due to cell cycle variation. The distributions skew to the left, reflecting that there are more young than old cells. (c) The average cell volumes for 3kPa, 12.6kPa, glass (GPA), serum starvation and aphidicolin treatment. At 12.6kPa, 3T3s and MSCs are larger, while NuFFs are smaller. Serum starvation generally decreases cell volume while aphidicolin treatment generally increases cell volume. The average cell adhesion area and adhesion shape are also shown. The shape factor is defined as $S = \frac{4\pi \text{Adhered Area}}{\text{perimeter}^2}$. MSCs show the largest adhered area at 12.6kPa, but for NuFFs and 3T3s, largest adhesion area occurs on glass substrates. (Scale Bar = 10 microns; all error bars represent standard error. Statistical significance: *** $p<10^{-6}$; ** $p<0.001$; * $p<0.01$; n.s.: $p>0.05$)
Figure 4-2: Cell volume in relation to cell adhesion area and cell shape. (a) Cell volume versus cell adhesion area for 3T3s, MSCs and NuFFs on different substrates. Each point is a single cell, color coded by the shape factor, S. In all cases, area is correlated with volume, but the data is heterogeneous. Moreover, the slope of the correlation depends on the substrate stiffness. For the same area, more circular cells have a larger volume. Variations in cell shape and levels of contractility contribute to the observed variation. (b) Cartoon of an adhered cell. The volume is defined by the apical surface (specified at all points by $R$, the vector between the center of the adhered area and the surface, and $\theta$, the angle made by the vector $R$ and the adhesion plane). Due to pressure difference across the membrane $\Delta P$, the cell uses active myosin contraction, $\sigma_a$, in the apical surface to balance the pressure difference. The mean curvature, $H$, is related to the apical surface shape $R$ (see SM for more details) and $h$ is the cortical thickness. (c) Model predictions of the cell volume as a function of total apical myosin and adhesion area. Model predicts that the cell volume increases with increasing adhesion area and total active myosin contraction; however, the slope depends on the adhesion shape. For the same $\sigma_a$, more circular cells are larger in size. This is consistent with data in (a). (d) Representative 3D cell shapes reconstructed from confocal z-stack images (blue) are compared with model cell shapes (red) computed for the same adhesion shape.
Figure 4-3: Total level and spatial distribution of pMLC are predictors of cell volume. (a) Immunofluorescence widefield images of pMLC for 3T3, NUFF and MSCs used for the quantification of total pMLC in each cell. Confocal z-stack images are also taken at 1μm z-steps to measure the relative amount of pMLC at each z-position. For stiffer substrates and relatively flat cells, there is typically higher concentration of pMLC near the basal surface. For rounder cells, the apical pMLC distribution is more uniform. (b) The average total pMLC (ΣpMLC) and relative ratio of apical vs. basal pMLC (\(<\text{PMLC}_{\text{apical}}>/\text{PMLC}_{\text{basal}}\)) on different substrates. The relative levels of pMLC are also plotted as a function of z-position for all three cell lines. We observe that the distribution of pMLC varies with substrate stiffness as well as cell type. (c) The spatial distribution (along the z-axis) of mean pMLC intensity of three cell lines for different ECM stiffness. In general, mean pMLC intensity is higher at the cell basal area; but as the ECM becomes softer, the difference between apical and basal pMLC decreases. The dotted line marks the approximate division between basal and apical (defined as 1μm above the z-position displaying basal stress fibers). (d) Computed cell volume as a function of total pMLC and the relative pMLC distribution. For each cell type the volume is scaled with respect to the cell volume on glass, and a single fitting parameter is used to relate total pMLC with integrated $\lambda, \int \frac{\sigma_{\text{adh}}}{\Delta P} dA$ (SM). The model predictions for volume across all stiffness are explicitly compared. (Scale Bar = 10 microns. All error bars represent standard error. Statistical significance: *** p<10^{-6}; ** p<0.001; * p<0.05; n.s.: p>0.05)
Figure 4-4: Cell volume is correlated with nuclear YAP/TAZ level in 3T3s and NuFFs. (a) Immunofluorescence widefield images with YAP in green and DNA in blue. The DNA channel is used to mask the nuclear region. The total nuclear YAP ($\sum YAP_N$) is obtained from epifluorescence images for different stiffness. (b) The total average nuclear YAP is plotted versus the average measured cell volume, average total pMLC level, and apical and basal pMLC levels. The individual cell data is also plotted in panels below, and color coded by the nuclear YAP intensity / cytoplasmic YAP intensity ratio. At both the single cell and ensemble level, higher nuclear YAP is correlated with higher total pMLC. Higher
nuclear YAP is also correlated with larger cell volume and higher apical pMLC, even though NuFFs and 3T3s display opposing trends as functions of substrate stiffness. Nuclear YAP is not correlated with basal pMLC. For NuFFs, nuclear YAP seems to plateau at large ΣpMLC, suggesting that nuclear YAP level reaches a maximum even as pMLC level is increasing. This suggests that there is another signal limiting nuclear YAP levels in NuFFs. Note in both 3T3s and NuFFs, the nuclear to cytoplasmic YAP concentration ratios are generally larger than 1. Visually, nearly all cells appear to have significant nuclear YAP. (Scale bar = 10 micrometer. All error bars represent standard error. Statistical significance: *** p<10^{-6}; ** p<0.001; * p<0.01; n.s.: p>0.05)

Figure 4-5: MSCs show bifurcated behavior in YAP nuclear localization and pMLC level. (a) Percentage of MSCs showing nuclear YAP localization. With increasing stiffness, more cells contain nuclear YAP, in agreement with Dupont et al. (b) The measured total amount of nuclear YAP, ΣYAP_N, decreases with increasing stiffness. Closer examination of single cell nuclear YAP and pMLC data shows a bifurcated behavior on different substrates. On stiffer substrates there are 2 branches. The upper branch has high overall YAP expression, but low nuclear to cytoplasmic (N/C) YAP intensity ratio. The lower branch has lower overall YAP expression, but high N/C. The proportion of the upper branch cells decreases with increasing stiffness. Thus, on softer substrates, it appears that most cells have lower N/C...
YAP ratio. On stiffer substrates, there are more cells with high nuclear N/C YAP ratio. (c) Representative images of MSCs with nuclear YAP localization and cytoplasmic YAP localization. (d) When the total nuclear YAP is plotted vs. volume, pMLC and apical pMLC, the positive correlation between nuclear YAP and these variables is recovered, similar to 3T3s and NuFFs. Cells in these separate branches are both positive for MSC markers CD90 and CD105 (Fig. S7). These results suggest that these are 2 branches which may not be distinguished by typical MSC differentiation markers. (All error bars represent standard error. Statistical significance: *** p<10^{-6}; ** p<0.001; * p<0.01; n.s.: p>0.05)”

**Figure 4-6. Nuclear YAP and pMLC relation suggests a late G1 checkpoint based on cell tension.**

(a) Two stiffness conditions with the greatest difference in average cell volume are selected for 3T3s, MSCs and NuFFs. The DNA histogram (left) is shown together with the total nuclear YAP vs. the total cell pMLC level (right). Cells are colored by their DNA content, with G1 cells identified as cells with DNA content below the dashed line in the DNA histogram (1.25 in the scaled DNA level). Cells beyond G1 have higher levels of nuclear YAP and pMLC. The rate of nuclear YAP and pMLC increase, however, varies by condition and cell type. (b) When cells in G1 in different conditions are compared, we observe that nuclear YAP rises with pMLC in G1 until a critical pMLC level, suggesting a checkpoint based on cell tension. For 3T3s, cells proceed to S after the critical level of pMLC and nuclear YAP continues to rise with pMLC. For NuFFs and the MSC lower-branch populations, cells in G1 can continue to increase in pMLC and cell size, but the nuclear YAP level plateaus after the critical level of pMLC. (c) G1-S transition checkpoint based on cell tension. Nuclear YAP increases with increasing pMLC, until a common critical tension level, at which cell transitions from G1 to S. If cells continue to grow in G1, nuclear YAP does not increase after the critical tension and plateaus. These cells are presumably arrested in G1.
Figure Appendix 1: Quantitative immunofluorescence analysis. (a) Examples of cell images in DIC and epifluorescence channels, showing the traced cell boundary (blue), inner (green) and outer (red) annulus, and dilated boundary for volume measurement (purple) (b) Examples of traced cell boundaries from immunofluorescence images. (All scale bars = 10 micrometer.)
CHAPTER 5: CONCLUSION AND FUTURE WORK

5.1 Review of Findings

This work reviews some of the basic mechanical concepts in cells, emphasizing continuum mechanics description of cytoskeletal networks. By combining mechanosensitive biochemical signal pathways, such as Rho-MLC signal pathway and osmotic pressure and hydrostatic pressure-driven flow of water and ion across the cell membrane, with force balance conditions at cell surface, we discuss how cells can maintain a homeostatic shape, size and relatively low membrane tension when the cells are subjected to sudden osmotic shock and mechanical forces. At long time scale, this force balance helps me to gain a quantitative mechanism of cell shape and volume control.

After proving form a theoretical point of view that, at time scale of less than hours, cells can quickly regulates its size and tension because tension-sensitive Rho-MLC signal pathway, which regulates active cortical contractile stress, and osmotic and hydrostatic pressure-controlled ion and water flow in Chapter 2, I, through collaborating with Professor Denis Wirtz’s lab, experimentally proved that mechanical tension directly affect Rho-MLC signal pathways, through Calcium current, which flows across the membrane mainly through mechanosensitive ion channels, such as TRPV4 channels, in Chapter 3.

In Chapter 4, I identified, through quantitative single-cell volume measurement and quantitative analysis of immune-staining results, that cortical tension as a main factor for cell shape and size adaptation throughout the cell cycle progression, which has been predicted from Chapter 2. In order to study more about the connection between active cortical tension and cell size homeostasis, I, with the help of my lab mates, quantified the nuclear localization of
YAP/TAZ, which is the downstream of mechanosensitive Hippo Pathway and has been identified of being able to bind with transcription factors in the nucleus by the biochemists. In Chapter 4, we ultimately find that cortical tension is closely related to YAP/TAZ nuclear location, and controls the cells as they leave G1 and entering S.

In conclusion, active and passive mechanical forces, such as active cortical tension and hydrostatic pressure play a crucial role in cell adaptation to variety of shape and size at short time scale, as well as in size-sensing mechanism during cell cycle progression.

5.2 Future Work: A Deeper Study in Cell Growth and Mechanosensitive Hippo Pathway

Throughout this dissertation, I studied the connection between mechanical forces, especially active cortical contraction cell size and shape homeostasis. However, what does cell size affect the actual cell growth, and how does YAP/TAZ nuclear location, which is closely connected to the active cortical tension, affect cell growth? To answer these questions, I, with the help of my lab mates, use the existing single cell volume measurement to tract down single cell overnight, to quantify its volume over time.

The cells we are using in this set of studies are HEK293 cells, with some knock out lines. With the help from the lab of Dr. Guan at University of California-San Diego, we get HEK293 with YAP Knockout and LATS 1/2 Knockout. These three cell lines have very different mean volume (Fig. 5-1), volume right before division (peak volume) and volume right after birth. This means, YAP Hippo pathway may have directly affected cell volume. In addition, the growth rates for these three cell lines are highly divergent, and the cell lines with most nuclear YAP (LATS 1/2 Knockout) has the highest growth rate and largest mean volume (Fig. 5-2).
Primary fix and stain results shows that cortical tension-controlled cell size homeostasis still holds for these three cell lines, and the checkpoint behavior described in Chapter 4 also exists. However, the amount of nuclear YAP/TAZ does seem to shift the cell population distribution in cell cycle, with most LATS 1/2 Knockout cells beyond G1. (Fig. 5-3)

Figure 5-1: Mean stationary volume, peak volume and volume right after division. Cells with most nuclear YAP/TAZ has the highest volume of all three measurements.
Figure 5-2: Growth rate of the five cell lines. Cells with largest amount of Nuclear YAP grow fastest. (Shorter doubling time)
Figure 5-3: Immunostaining result of Parental (293), YAPKO and LATS 1/2 KO lines. (a) Mean value of PMLC and nuclear YAP: The correlation between cortical tension and nuclear YAP still holds in these three cell lines. (b) Single cell data of DNA distribution and PMLC vs. nuclear YAP/TAZ. The nuclear YAP shifted the cell cycle distribution within the three cell lines. Even so, the G1 checkpoint behavior discussed in Chapter 4 (Fig. 4.6) still holds, as shown in (c).
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Curriculum Vitae

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Education:

Ph.D. Sept. 2012-Dec. 2017: Johns Hopkins University, Baltimore MD
Department of Mechanical Engineering
Advisor: Professor Sean X. Sun.

B.S. Sept. 2008-May. 2012: University of Pittsburgh, Pittsburgh, PA
Department of Civil and Environmental Engineering

Skills

Specialties and software skills

1. Continuum mechanics (Specialty: Linear and non-linear solid mechanics, Linear and nonlinear fluid dynamics, especially low Reynolds number fluid mechanics and interface mechanics)
2. Differential Geometry
3. Linear and non-linear dynamic systems
5. Finite element method (FEM) software: Comsol, Abaqus FEA & ANSYS
6. Graphing software: Adobe Illustrator and AutoCAD (From Undergraduate Experience)
7. LAMMPS: Molecular Dynamics Simulation (MD) (From Undergraduate Experience)

Lab experiences

8. Mammalian cell (both immortalised cell lines and primary cells) culturing
9. Epifluorescence and one/two photon confocal imaging (Certified user on Zeiss 710 and 800 fluorescence microscope)
10. Mammalian cell fixation and immunostaining (both on glass slide and in microfluidic channel)
11. Animal embryo dissection and live tissue culturing, fixation and imaging.

Awards

1. Dean’s Honor’s Student, University of Pittsburgh Engineering School (2008-2012)
2. Summer 2012: Mascaro Center for Sustainable Innovation Summer Research Award, University of Pittsburgh
3. 2010-2012 Swanson School of Engineering Research Award, University of Pittsburgh
Publications

Sole-first and co-first author publications


Other publications


Book chapters


Conference and presentations


### Teaching Experience

#### Graduate work at Johns Hopkins University

1. **Teaching Assistant**: Biomechanics of the Cell (EN.530.410) Spring 2015. 
   Instructor: Prof. Sean Sun
   **Class Description**: This upper undergraduate class introduces how to apply continuum mechanics in cell biology. Typically, it talks about how to use mathematical models to describe the role of proteins, membrane, and cytoskeleton.

#### Undergraduate work at University of Pittsburgh

2. **Teaching Assistant**: Introduction to Calculus I (Math 0220) Fall 2011

3. **Teaching Assistant**: Introduction to Algebra I (Math 0120) Fall 2010

4. **Teaching Assistant**: Introduction to Algebra I (Math 0120) Fall 2009

5. **Tutor**: Introduction to Calculus III (Math 0240) Spring 2012
   Math Assistance Center, University of Pittsburgh

6. **Tutor**: Introduction to Calculus II (Math 0230) Spring 2011
   Math Assistance Center, University of Pittsburgh