Force Mediated Lamin A/C Redistribution between Nuclear Membrane and Nuclear Interior Regulates YAP Mechanotransduction

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Abstract

Cells sense and interpret various mechanical signals including substrate stiffness, compression, and shear stress from their microenvironment. This mechanosensitivity plays an important role in cell fate decisions regarding differentiation, proliferation, and organ development. During mechanoadaptation, cells convert internal forces generated in response to mechanical cues into biochemical signals, leading to activation of specific signaling cascades. Incorrect responses to physical cues, however, likely contributes to diverse diseases including fibrosis, cancer progression, progeria or laminopathy as a result of gene disorders.

Yes associated protein (YAP), known as a mechanosensitive transcriptional regulator, shuttles between the nucleus and cytoplasm. YAP nuclear localization facilitates genes' activation regulating cell proliferation, tissue homeostasis, and cell differentiation via its interaction with transcription factors, including TEAD. YAP cytosolic localization, however, inhibits cell proliferation and osteogenic differentiation due to a lack of YAP-TEAD interactions. Defective YAP regulation leads to multiple diseases such as tumorigenesis, metastasis, and fibrosis due to uncontrollable cell proliferation mediated by activation of oncogenic transcription factors. The precise mechanical stimuli, including nuclear mechanics and different modes of nuclear deformation (e.g. compression and swelling), which regulate the dynamic localization of YAP in living cells remain unknown. Resolving this fundamental interaction is essential for understanding mechanobiology in physiology and pathology.

This thesis offers a comprehensive study of YAP mechanotransduction in living sparse cells by considering diverse mechanosensory stimuli including substrate stiffness, cell contractility, nuclear mechanics, and nuclear deformation. In contrast with previous findings derived from static fixed immunofluorescent approaches, I studied YAP mechanoregulation by transfecting NIH 3T3 cells with EGFP-YAP and tracking the dynamic movement of YAP on substrates with various stiffnesses. To my knowledge, I am the first to find that YAP is randomly localized in the nucleus and cytoplasm during cell movement and independent of substrate stiffness. I found that cell contractile work (strain energy) also varied during cell movement on different PDMS substrates, and that contractile work and YAP were tightly correlated temporally, leading to dynamic movement of YAP in living cells. To understand the mechanism driving contractile force's regulation of YAP translocation, I examined the role of nuclear deformation in YAP regulation. I

found that nuclear compression induced by contractile force transmitted via the LINC complex, which provides connection between actin cytoskeleton and nucleoskeleton, in turn regulated YAP localization. By modulating lamin A expression levels to change nuclear deformability, I also found that softer nuclei in lamin A siRNA cells required less contractile work to trigger YAP nuclear localization compared to the lamin A overexpressed cells with stiffer nuclei. I also applied different osmotic compressions to modulate nuclear volume and examine YAP localization. My results again showed that nuclear compression alone (mediated by either cell contractility or external hyper-osmotic shocks) is sufficient to regulate YAP nuclear translocation; however, in lamin A overexpressed cells, the discovery of unexpectedly higher YAP nuclear localization in larger nuclear volumes in addition to lower nuclear deformation due to stiffer nuclei compared to WT cells led me to suspect that lamin A might play a more crucial role in YAP mechanotransduction beyond nuclear stiffening.

To the best of my knowledge, I also show that not only lamin A total expression level, but also lamin A distribution between the nuclear membrane and the nucleoplasm is mechanosensitive to nuclear flattening, not to nuclear swelling. I found a conserved relationship between YAP nuclear transport and lamin A intensity in the nuclear membrane: cells with a high amount of lamin A accumulated in the nuclear membrane inhibited YAP nuclear transport. Nuclear compression mediated by either contractile force or external pressure triggered lamin A delocalization from the nuclear membrane to the nucleoplasm and subsequent YAP nuclear translocation. My results did not show significant changes in lamin A redistribution and YAP nuclear localization under nuclear membrane tension mediated by nuclear swelling, casting doubt on the hypothesis that membrane tension drives YAP nuclear localization. Instead, I then suggest that lamin A disassociation from the nuclear membrane due to local bending may be a key mechanism which enables YAP nuclear transport. These findings may provide better understanding of mechanotransduction and novel therapeutic perspectives for diseases associated with impaired nuclear mechanics and cellular dysfunction.

Résumé

Les cellules détectent et interprètent divers signaux mécaniques, y compris la rigidité du substrat, la compression et la contrainte de cisaillement provenant de leur microenvironnement. Cette mécanosensibilité joue un rôle important dans les décisions cellulaires relatives à la différenciation, la prolifération et le développement des organes. En réponse aux signaux mécaniques, les cellules convertissent les forces internes ou externes en signaux biochimiques qui entraînent l'activation de cascades de signalisation spécifiques et mènent à la mécanoadaptation des cellules. Des réponses incorrectes aux signaux mécaniques contribuent probablement à diverses maladies, notamment la fibrose, la progression du cancer, la progeria ou la laminopathie à la suite de troubles génétiques.

La protéine YAP (du nom anglais *Yes Associated Protein*), connue comme un régulateur transcriptionnel mécanosensible, fait la navette entre le noyau et le cytoplasme. La localisation nucléaire de YAP facilite l'activation des gènes régulant la prolifération cellulaire, l'homéostasie tissulaire et la différenciation cellulaire via son interaction avec des facteurs de transcription, y compris TEAD. La localisation cytosolique de YAP, cependant, inhibe la prolifération cellulaire et la différenciation ostéogénique en raison d'un manque d'interactions entre YAP et TEAD. Une régulation défectueuse de YAP entraîne de multiples maladies telles que la tumorigenèse, les métastases et la fibrose dues à une prolifération cellulaire incontrôlable médiée par l'activation de facteurs de transcription oncogéniques. Les stimuli mécaniques précis, y compris la mécanique nucléaire et les différents modes de déformation nucléaire (par exemple la compression et le gonflement), qui régulent la localisation dynamique de YAP dans les cellules vivantes restent inconnus. Résoudre ce mécanisme fondamental est essentiel pour comprendre la mécanobiologie de YAP en physiologie et en pathologie.

Cette thèse propose une étude complète de la mécanotransduction de YAP dans les cellules vivantes en considérant divers stimuli mécanosensoriels, notamment la rigidité du substrat, la contractilité cellulaire, la mécanique nucléaire et la déformation nucléaire. Contrairement aux découvertes précédentes dérivées d'approches immunofluorescentes statiques, j'ai étudié la mécanorégulation de YAP en transfectant des cellules NIH 3T3 avec EGFP-YAP et en suivant le mouvement dynamique de YAP sur des substrats de rigidités variées. À ma connaissance, je suis la première à découvrir que la protéine YAP est localisé de manière aléatoire dans le noyau et le

cytoplasme pendant le mouvement cellulaire, et ce indépendamment de la rigidité du substrat. J'ai trouvé que le travail contractile cellulaire (énergie de déformation) variait pendant le mouvement cellulaire sur différents substrats de PDMS (poly-di-methyl-siloxane). J'ai aussi démontré que le travail contractile cellulaire et la localisation de YAP sont étroitement corrélés dans le temps, conduisant à un mouvement dynamique de YAP dans les cellules vivantes. Pour comprendre le mécanisme qui régit la régulation de la translocation de YAP par la force contractile, j'ai examiné en détails les effets de la déformation nucléaire. J'ai trouvé que la compression nucléaire induite par la force contractile transmise via le complexe LINC, qui assure la connexion du cytosquelette d'actine et du nucléosquelette, à son tour régulait la localisation de YAP. En modulant les niveaux d'expression de la protéine lamine A pour modifier la rigidité nucléaire, j'ai également constaté que les noyaux plus mous dans les cellules siRNA de la lamine A nécessitaient moins de travail contractile pour déclencher la localisation nucléaire de YAP par rapport aux noyaux plus rigides observés dans les cellules où la lamine A est surexprimées. J'ai également appliqué différentes compressions osmotiques pour moduler le volume nucléaire et examiner la localisation YAP. Mes résultats ont de nouveau montrés que la compression nucléaire seule (médiée soit par la contractilité cellulaire soit par des chocs hyper-osmotiques externes) est suffisante pour réguler la translocation nucléaire de YAP. Cependant, la découverte, dans des cellules qui surexpriment la lamine A, d'une localisation nucléaire de YAP étonnamment plus élevée dans des volumes nucléaires plus importants en plus d'une déformation nucléaire plus faible due à des noyaux plus rigides que les cellules références m'ont amenés à soupçonner que la lamine A pourrait jouer un rôle crucial dans la mécanotransduction de YAP au-delà du raidissement nucléaire.

Je suis également la première, à ma connaissance, à montrer que non seulement le niveau d'expression total de la lamine A, mais aussi la distribution de la lamine A entre la membrane nucléaire et le nucléoplasme sont, mécanosensibles à l'aplatissement nucléaire, contrairement au gonflement nucléaire. J'ai trouvé une relation conservée entre le transport nucléaire de YAP et l'intensité de la lamine A dans la membrane nucléaire: le transport nucléaire de YAP est inhibé les cellules avec une grande quantité de lamine A présente dans la membrane nucléaire. La compression nucléaire médiée par une force contractile ou une pression externe a déclenché la délocalisation de la lamine A de la membrane nucléaire vers le nucléoplasme et la translocation nucléaire subséquente de YAP. Mes résultats n'ont pas montré de changements significatifs dans la redistribution de la lamine A et la localisation nucléaire de YAP sous une tension de la

membrane nucléaire médiée par un gonflement nucléaire. Ces résultats jetent un doute sur l'hypothèse selon laquelle la tension de membrane entraîne la localisation nucléaire de YAP. Au lieu de cela, je suggère que la délamination de la membrane nucléaire en raison de la flexion locale peut être un mécanisme clé qui permet le transport nucléaire de YAP. Ces découvertes pourraient offrir de nouvelles perspectives thérapeutiques pour les maladies associées à une mécanique nucléaire anormale ainsi qu'au dysfonctionnement cellulaire.

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Contribution of Authors

The present thesis consists of two manuscripts (Chapter 2 and Chapter 3). I am the first author of the manuscripts. The contributions of all authors to each manuscript are listed below.

Chapter 2. Lamin A redistribution mediated by nuclear deformation determines dynamic localization of Yes associated protein, Submitted to *Nature* in 2020, March 30

- Authors: Newsha Koushki, Ajinkya Ghagre, Luv Kishore Srivastava, Chris Sitaras, Haruka Yoshie, Clayton Molter, and Allen J. Ehrlicher
- Contributions: Conceptualization, A.J.E., N.K.; Methodology- development, L.K.S., H.Y.; Methodology- application, N.K.; Plasmid purifications and sequencing, A.G., C.S., L.K.S.; Investigation, N.K.; Writing- original draft, N.K.; Writing- review and editing, N.K., A.J.E., L.K.S., C.S., A.G., C.M.; Funding acquisition-A.J.E.; Resources, A.J.E; Supervision, A.J.E.

Chapter 3. Lamin A redistribution is only mechanosensitive to anisotropic nuclear compression and directly controls YAP mechanotransduction in mammalian cells, To be submitted to *Journal of cell science* in May 2020

- Authors: Newsha Koushki, Adele Khavari, Ajinkya Ghagre, Luv Kishore Srivastava, Allen Ehrlicher
- Contributions: Conceptualization, A.J.E., N.K.; Methodology- development, L.K.S.; Methodology- application, N.K.; Plasmid purifications and sequencing, A.G., L.K.S.; Investigation, N.K.; Writing- original draft, N.K.; Writing- review and editing, N.K., A.J.E., L.K.S., A.G.,; Funding acquisition-A.J.E.; Resources, A.J.E; Supervision, A.J.E.

Contribution to Original Knowledge

Chapter 2 introduces novel findings in YAP mechanotransduction. To my knowledge, this is the most comprehensive study of YAP mechanoregulation, considering diverse mechanical stimuli in order to identify the most direct parameter mediating YAP translocation. Contrary to previous findings, I showed that YAP localization is not dependent on substrate stiffness, but rather on nuclear compression from either cell contractile work or hyper-osmotic shocks. In Chapter 2, I discovered a novel relationship between YAP localization and lamin A distribution. Both YAP activity and lamin A total expression have been previously shown to depend on mechanical stimuli, cell cycle, and stem cell differentiation; however, no direct relationship between YAP and lamin A distribution have been reported. In this study, I showed that not only lamin A total expression level, but also lamin A distribution between the nuclear membrane and the nucleoplasm are mechanosensitive, and lamin A delocalization from the nuclear membrane is essential for YAP nuclear import.

Chapter 3 examines the details of nuclear deformation that lead to YAP translocation. There I show that lamin A redistribution between the nuclear membrane and nucleoplasm is only mechanosensitive to nuclear flattening, not to nuclear swelling. I showed that nuclear vertical deformation (Z), not lateral deformation (major axis in XY plane) is key in lamin A redistribution, and only nuclear membrane tension applied through nuclear flattening delocalizes lamin A from the nuclear membrane. Nuclear membrane tension applied by nuclear swelling did not mediate lamin A redistribution. After discovering which modes of nuclear deformation triggered lamin A delocalization, I again examined the effects of lamin A distribution under both nuclear flattening and swelling in YAP translocation. I showed that nuclear swelling did not mediate lamin A delocalization or YAP nuclear localization, but nuclear compression successfully delocalized lamin A from the nuclear membrane and increased YAP nuclear import. These results suggest that lamin A total expression level is mechanoresponsive, its distribution between the nuclear membrane and the nucleoplasm is sensitive to nuclear flattening, and it can play a crucial role in genes' transcription.

List of Abbreviations

AMOT	Angiomotin
CytoD	Cytochalasin D
DNK1/2	Dominant-negative EGFP-Nesprin1-KASH and EGFP Nesprin KASH plasmids
ECM	Extracellular Matrix
FAK	Focal adhesion kinase
FA	Focal adhesions
F-actin	Actin filament
HGPS	Hutchinson-Gilford Progeria Syndrome
LINC	Linker of Nucleoskeleton and Cytoskeleton
LAP	Lamin binding proteins
LAD	Lamina-associated domains
Lm	Normalized lamin A intensity in the nuclear membrane
mDia	Drosophila diaphanous
MLCP	Myosin-light-chain phosphatase
MLCK	Myosin light chain kinase
NPC	Nuclear pore complexes
PAA	Polyacrylamide
PDMS	Polydimethylsiloxane
PEG400	Polyethylene glycol 400Da
RMST	Root Mean Squared Traction
ROCK	RhoA- associated kinase

TAD	Transcription activation domain
TFM	Traction force microscopy
WT	Wild-Type
YAP	Yes associated protein
YR	YAP nuclear to cytosolic ratio

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Chapter 1: General Introduction

The cells in our body are under continuous dynamic modifications in our physiological environment. Cells' ability to sense and respond to different biochemical and mechanical stimuli enable them to adapt to variations in the surrounding environment and to maintain their proper biological functions. Dysfunction in cellular behavior, however, contributes to various diseases and pathologies[1]. Hence, investigation of cellular responses to different biochemical and mechanical signals is essential to better understand the mechanisms that allow cells to adapt to the modifications in their microenvironment. Cell chemical features and cellular responses to biochemical signals, including growth factors, hormones, and cytokines have long been recognized. Additionally, cells are able to sense both passive and active forces in our body and convert these mechanical cues to biological signals through a process called mechanotransduction. Multiple studies have been conducted to investigate how cells respond to mechanical cues, including extracellular matrix (ECM) rigidity, hydrostatic pressure, shear stress, and compression as well as how cells convert these biomechanical cues into biological functions through mechanotransduction[2,3]. However, the exact nature of how mechanosensing extends to biological functions has remained unclear and continues to be investigated. Here, I will briefly highlight some techniques to measure cellular response to the mechanical forces, and I will discuss multiple extra and intracellular components involved in cell mechanosensing and impact downstream signals that regulate cellular functions.

1.1 Cell mechanics

Quantitative investigations of how cells respond to mechanical stimuli can be performed using different methods including magnetic beads cytometry[4], traction force microscopy[5], microrheology[6,7] and atomic force microscopy[8], where calibrated mechanical forces are applied to the living cells, and cellular and nuclear deformation as well as alteration of mechanosensitive molecular shuttling can be measured simultaneously (Figure 1-1). In this work, I apply traction force microscopy to measure cellular forces exerted to the synthetic two-dimensional substrates with different stiffnesses. I also investigate how these cellular forces are translated into biological processes by examining force-induced alterations in nucleocytoplasmic shuttling of Yes associated protein (YAP) which acts as a transcriptional coactivator and regulates cell proliferation,

apoptosis, and differentiation. More detailed explanation of YAP biology and mechanotransduction will come later in this Chapter.

Cells possess both elastic characteristics under short time scale forces, and dissipative properties under constant long-time scale stresses; however, these mechanical characteristics vary under different actively applied stresses (e.g. shear flow, dynamic stretching, passive stresses such as ECM stiffness)[9]. Upon sensing the forces, cells need to form some contact proteins, including integrins which provide connection between the microenvironment and the cytoskeleton. Then, cells react to the forces by generating equal by opposite forces through formation of actin stress fibers and activation of myosin motors which create tension in actin cytoskeleton. This tension extends over the nucleus via the Linker of Nucleoskeleton and Cytoskeleton (LINC complex) which provides a link between cytoskeleton and the nucleus and plays an essential role in force transmission to the nucleus. Finally, the received forces activate transcriptional machinery and regulate expression of target genes which direct fundamental aspects of cellular functions and enable mechanoadaptation. In the following sections, I will introduce the important cytoskeletal and environmental parameters which are involved in force transition from the microenvironment to the nucleus.

1.1.1 Actin Cytoskeleton

Taking the vital role of mechanosensing in many biological processes, the cytoskeleton is a biopolymer network which plays a crucial role in sensing forces and regulating cell mechanics via four major components: 1) actin filaments (F-actin), 2) intermediate filaments, 3) microtubules, and 4) motor myosin and a wide range of filament crosslinkers regulatory proteins[10]. Among these components, actin stress fibers, actin bundles assembled by crosslinkers, and actin-myosin interactions play important roles in cell contractility and generating forces for various cellular functions, including cell adhesion, cell motility, and morphogenesis. The Rho family of GTPases is the main mechanosensitive regulator of actin dynamics and actin stress fiber formation by binding to GTP and activating Rho-associated coil-coil forming kinase (ROCK) and the mammalian homologue of Drosophila diaphanous (mDia) [11,12]. ROCK maintains actin stress fibers' integrity by phosphorylating LIM-kinase, which inactivates cofilin, known to prevent actin polymerization[13].

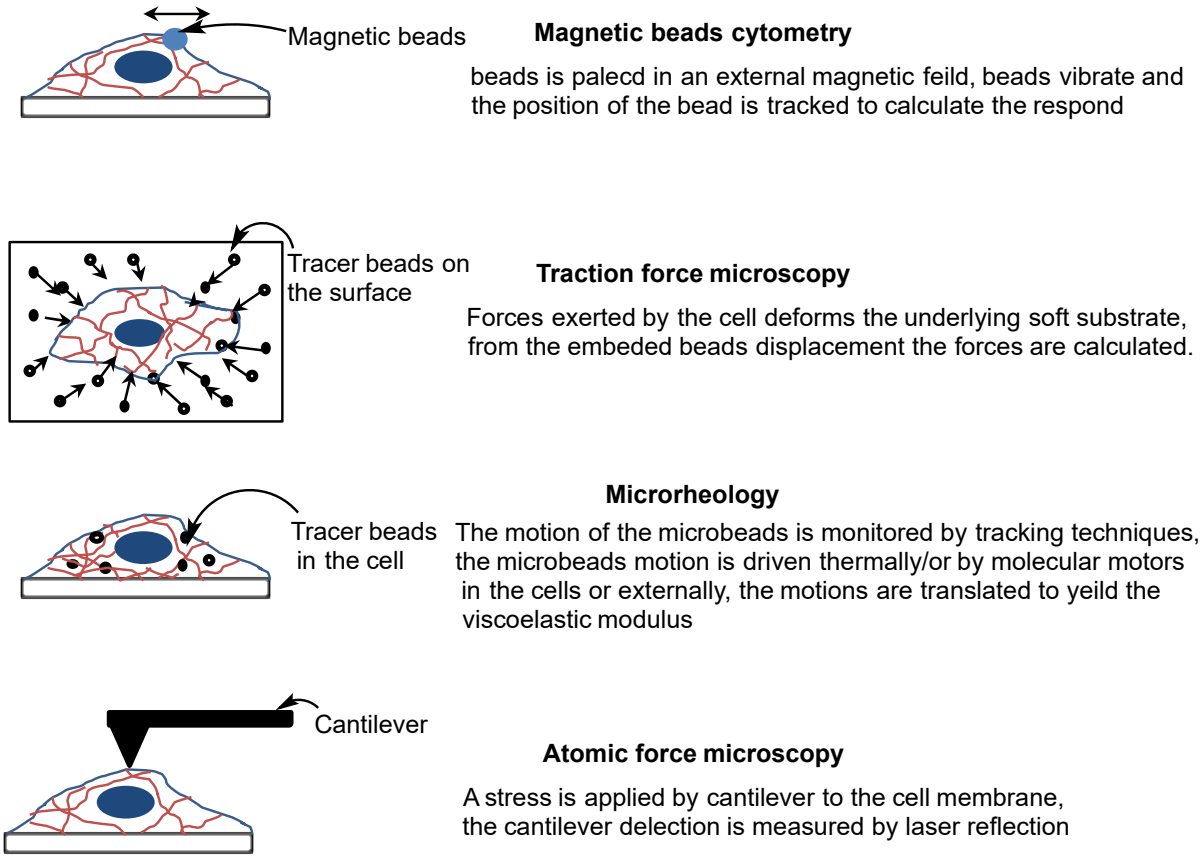

Figure 1-1 Schematic of different techniques to characterize mechanical properties of the cell. Inspired from Kasza et al, 2007, Current Opinion in Cell Biology, 101-107[10].

In this thesis, I am examining the effects of substrate stiffness on cell contractility and YAP mechanotransduction. I also study the effects of actin depolymerization and inhibition of myosin activity in cellular forces and YAP activity.

1.1.2 Myosin activity

Besides actin cytoskeleton, the actin-myosin interaction is an essential dynamic system for cell contractility. Myosin motors consists of two polypeptide chains that form two heads and a long tail. The head has a binding site for actin, which attaches to the actin fiber. The myosin's head also has a binding site for ATP where it enzymatically hydrolyzes ATP to ADP and releases energy. ATP binding allows myosin and actin to detach from each other followed by conversion of ATP into ADP and inorganic phosphate. The released energy from ATP hydrolysis changes the myosin head's angles into the cocked position. This phenomenon, along with conformational changes,

leads myosin's head to protrude off the actin surface and to interact with neighboring actin filaments[14,15]. This results in myosin sliding along the actin fiber and pulling it inward, leading to contraction[14]. In addition to actin polymerization, ROCK increases actomyosin contractility through phosphorylation of myosin-light-chain phosphatase (MLCP), which activates myosin motor proteins, and enables myosin-actin filaments interaction[16]. The local activity of motor myosin is further strengthened via actin binding proteins, including α-actinin, filamin, fascin, and spectrin, which covalently crosslink the F-actin network and directly impact the cellular forces and mechanical characteristics. Cell contractility and mechanics can also be affected by mechanical properties of the surrounding microenvironment, including ECM's rigidity.

1.1.3 ECM

Human tissues exhibit a wide range of elasticities from soft tissues such as the brain, with low mechanical resistance to stiff tissues such as bone and cartilage, which sustain high levels of stress[17]. ECM rigidity is known to effectively regulate actin cytoskeleton reorganization, cellular contractility, cell motility, and differentiation[18,19]. Cells are able to mechanically adapt themselves to various organ stiffnesses during homeostasis and organ development through the formation of dynamic adaptor proteins, known as molecular clutches, between the ECM, cell membrane, cytoskeleton, and nucleus[20]. Proteins in the molecular clutch are potentially involved in force transmission from the microenvironment to the actin cytoskeleton. After cell adhesion to the ECM, force transmission can be triggered in three different ways: 1) interaction between the ECM and integrins[21], 2) adapter proteins, including α -actinin[22], filamin[23], and talin[20], which directly connect integrins to the actin cytoskeleton, and 3) indirect interaction between integrins and actin cytoskeleton, triggered by vinculin[20] and focal adhesion kinase (FAK) (Figure 1-2). Giving the crucial role of ECM stiffness on cellular function, in this thesis, I fabricate Poly dimethyl siloxane (PDMS) with different stiffnesses to create a synthetic mechanical environment and to examine the role of microenvironment's rigidity on cellular response. Below I will describe some important proteins in the molecular clutch that are involved in force transmission from the microenvironment to the cytoskeleton, and the nucleus.

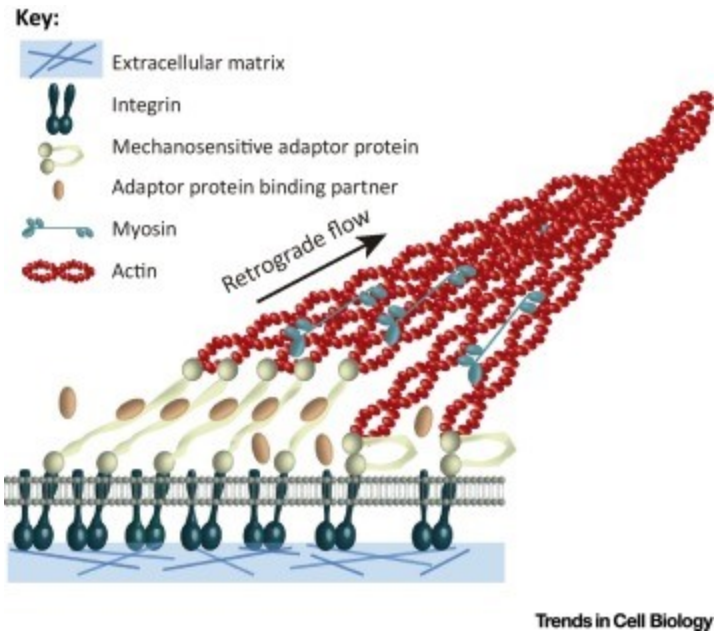

Figure 1-2 Schematic of continuous connection between the ECM, integrins, adaptor proteins, and actin filaments forming the molecular clutch. Reprinted from Trends in Cell Biology 28, Alberto Elosegui-Artola, Xavier Trepat, Pere Roca-Cusachs, "*Control of Mechanotransduction by Molecular Clutch Dynamics*", P356-367, Copyright (2018), with permission from Elsevier[20].

1.2 Integrated molecular clutch

In this section, I will explain some important parameters involved in force detection and force transmission from the microenvironment to the cytoskeleton and the nucleus.

1.2.1 Integrins

Cellular membranes, which directly contact the extracellular environment through integrins, ion channels, and G protein coupled receptors are the primary site of force transmission to the cell. Cells perceive the mechanical properties of ECM by applying force and pushing against the bottom of the matrix. Received mechanical cues then convert into intracellular signals, which direct fundamental aspects of cellular functions. The translation of ECM rigidity into biological signals starts from transmembrane receptors known as integrins. Integrins are heterodimers consisting of α− and β−subunits, and act as an interface between the intracellular and extracellular environments[18,24]. After cell attachment to ECM, integrins initiate the assembly of adaptor proteins, including FAK, vinculin, paxillin, and talin to form a link between a cell and ECM[3,18]. Integrin's cytosolic domain interacts with actin cytoskeleton via protein adaptors forming focal

adhesions (FA), while the extracellular domain links to ECM. FAs function as mechanosensors, which generate forces depending on matrix stiffness by pulling ECM. Stiff ECM increases the number of focal adhesions and traction forces that cells apply to the ECM compared to the soft matrixes[3,18,25].

1.2.2 Focal adhesions as mediator of mechanosensation

The term cellular mechanosensors has been applied to the majority of mechanosensitive proteins and molecules in cells which show changes in response to mechanical forces. The nature of these mechanoresponsive changes can be variations in intracellular shuttling, development of novel interaction sites, protein unfolding, or protein conformational changes[3]. In this thesis, mechanoresponsive changes are examined in terms of variations in intracellular shuttling of YAP protein, and redistribution of nuclear components, including lamin A under different mechanical environments. All these responses can be involved in force transmission from the extracellular environment to the nucleus where mechanosensitive genes are eventually activated. Some examples of force-induced molecular events are conformational changes in integrins[26], variations in ion channel activity[27], or force-induced kinase activity[28]. The best-known example of a mechanoresponsive adaptor protein is talin, which is an actin-integrin adaptor protein. Talin is known to unfold above a certain matrix rigidity threshold, and to expose its binding sites to vinculin[20,29], which in turn induces FAs growth and actin filaments reorganization[29,30]. Below the rigidity threshold, however, adhesion growth and stress fiber formation are abrogated as a result of low force transmission to talin, resulting in less tension in the cytoskeleton [29]. Focal adhesions' growth as a result of force-induced talin unfolding and recruitment of other adapter proteins is associated with actin stress fibers' formation and myosin motors' activities. This propagates mechanical stimuli across the actin cytoskeleton from the extracellular environment to the nucleus via the Linker of Nucleoskeleton and Cytoskeleton (LINC) complex.

1.2.3 LINC complex

Similar to integrins, which connect the cytoskeleton to the ECM, the LINC complex mechanically couples the actin cytoskeleton to the nucleus, and allows for mechanical force transduction to the nucleus, which is crucial for biological responses[31] (Figure 1-3). LINC disruption, however, is associated with the pathogenesis of Emery-Dreifuss dystrophy in humans[32]. The LINC complex is a protein complex composed of two protein domains, SUN and nesprin-1/2, which are associated

with both inner and outer nuclear membranes (Figure 1-3). SUN domain proteins span the inner nuclear membrane and are associated with both nuclear lamina[33] and chromatin[34], which facilitate force transmission from the cytoskeleton to the nucleoskeleton. In perinuclear space, between inner and outer nuclear membrane, SUN domain proteins interact with the nesprins' C-terminal KASH domain to form the LINC complex. N terminal domains of nesprins 1/2 also cross the outer nuclear membrane and bind to actin filaments directly or indirectly via dynein and kinesin motors. N-terminal motif of nesprin 3 interact with intermediate filaments through plectin and nesprin 4 and indirectly bind to microtubules (Figure 1-3)[35,36]. LINC complex perturbation causes nuclear distortion, chromatin dynamics[34], and actin derangement[33]. In this thesis, I transfect the cells with dominant negative KASH1/2 fused to GFP which inhibits LINC formation by binding to SUN proteins and saturating available binding sites in the nuclear envelop. Then I examine the role of the LINC complex in force transmission to the nucleus and YAP activation. Previous studies reported the importance of highly aligned perinuclear actin fibers localized over the apical surface of the nucleus in contractile force transmission via LINC complex [37,38].

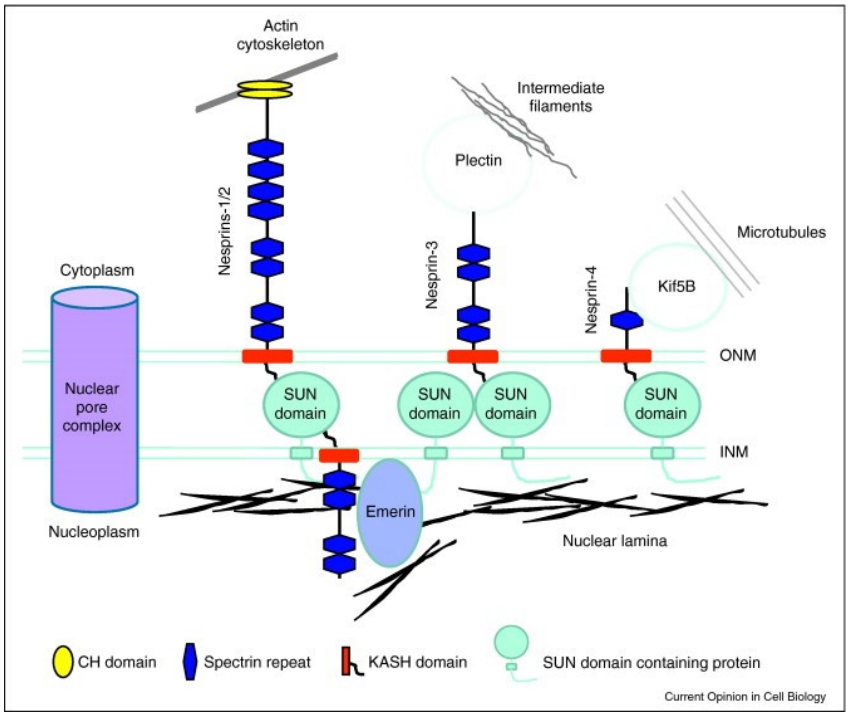

Figure 1-3 Schematic of LINC complex structure and their interactions with cytoskeletal components. Reprinted from Current Opinion Cell Biology 23, Jason A Mellad, Derek T Warren, Catherine M Shanahan, "*Nesprins LINC the nucleus and cytoskeleton*" , 47–54, Copyright (2011), with permission from Elsevier[39].

1.2.4 Actin perinuclear fibers

In addition to basal actin stress fibers, adherent cells have a highly aligned subset of actin fibers called the perinuclear actin cap, which are localized over the apical surface of the nucleus with the same curvature[37,38] (Figure 1-4). These fibers are connected to the nuclear envelope via the LINC complex and regulate nuclear shape by tuning intracellular tension. They also play a crucial role in force-mediated nuclear deformation, which directs chromatin organization, gene expression, and, ultimately, cellular functions[40,41]. Contractile actin perinuclear fibers compress the nucleus and squeeze it laterally, resulting in a thin disk-like shape of the nucleus by anchoring the focal adhesions (Figure 1-4). Actin perinuclear fibers have been shown to be mediated by cellular tension and inhibition of actomyosin contractility using Rho kinase inhibitor Y-27632 or myosin light chain kinase (MLCK) inhibitor ML-7, decreases the number of actin caps over the top of the nucleus[38]. Moreover, depletion of nesprin 1/2 results in an increase in the nuclear height. similar to inhibiting actomyosin contractility, suggesting that the compression induced by the tensed perinuclear actin cap is rapidly reduced upon LINC complex disruption, thereby severely impairing nucleo-cytoskeletal force transmission[38].

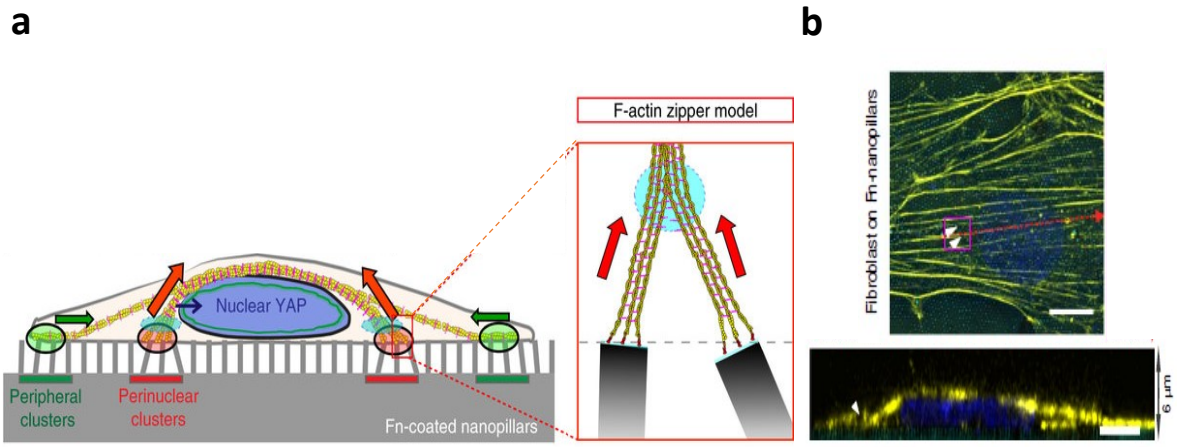

Figure 1-4 Actin perinuclear fibers and their connections with the nucleus. a) Schematic of actin perinuclear fibers spanned over the nuclease and connect to LINC complex, b) Top image is z-projection of actin fibers (yellow) and the nucleus (blue), Scale bar is 10 μ m. Bottom image is the XY image along dashed red line in the top image. Scale bar is 1 μ m. Reprinted by permission from Nature/Springer, Nature Cell Biology, "*Nanopillar force measurements reveal actin-cap-mediated YAP mechanotransduction"*, Jau-Ye Shiu et al, 262–271, COPYRIGHT (2018)[38].

In the previous sections, I explained the crucial role of various mechanosensitive proteins in force transduction to the nucleus. Below, I will discuss how these forces are ultimately transduced to the biological responses through mechanotransduction.

1.3 Nuclear mechanotransduction

The nucleus as the hallmark of eukaryotic evolution where gene expression and genome replication happen is considered the hub of mechanotransduction. The question of whether force-induced nuclear deformation directly regulates gene expressions has received increasing attention from researchers [42-44]. In this section, I will explain some of the important mechanoresponsive nuclear components, and the potential molecular mechanisms regulating nuclear mechanotransduction.

1.3.1 Nuclear structure

The nucleus is the largest and stiffest organelle in the cell and can be divided into the interior nuclear which contains chromatin, and the surrounding nuclear envelope, consisting of the outer and inner nuclear membranes[43,45]. Underlying the inner nuclear membrane are lamina, which are filamentous network proteins classified into A-type lamins, B-type lamins, and lamin binding proteins (LAP)[33,46]. Two isomers of A-type lamins, which tether the nucleoskeleton to the cytoskeleton via LINC complex are lamin A and C, encoded by LMNA genes[35]. Nuclear mechanosensing covers a wide range of states: force-induced nuclear membrane tension, changes in protein conformation, transcription factor localization, and chromatin reorganization, which remained incompletely understood.

1.3.2 Nuclear deformability dictates the mechanosensitive response to the physical inputs

Understanding how the nucleus responds to different mechanical forces is crucial to uncovering mechanotransduction and force-mediated signaling cascades that regulate gene expressions and differentiation. As the perceived forces eventually reach the nucleus, in this thesis, I hypothesize that nuclear deformability may play an important role in both force transduction to the nuclear component and in the corresponding gene expressions. Nuclear deformability impacts cellular processes, metabolism, and cell migration. Nuclear stiffness, the main obstacle for cell migration across narrow constrictions, varies between different cell types, and decreases during stem cell differentiation[47-49]. Nuclear lamins and tightly packed chromosomes are the two main mechanical components that affect nuclear deformability induced by applied forces[47]. In this work, I modulate lamin A expression levels to manipulate nuclear deformability and to examine how nuclear

deformability impacts the required force for YAP nuclear translocation. Very compliant nuclei are associated with lamin A siRNA cells, whereas lamin A overexpressed cells have stiffer nuclei.

Lamin A/C is the main architectural protein in the nucleus contributing to nuclear mechanics[50] and mechanotransduction[47,51,52]. It is composed of two major components of lamina and is an intermediate filament meshwork with a tripartite structure containing α-helical domain, amino-terminal and carboxy-terminal domains[53,54]. While B-type lamin is expressed in all the cells, A-type lamin is only expressed in differentiated cells, and its expression levels are different from various tissues[17,55]. Lamin A mutations and decreases in its expression levels increase the likelihood of nuclear envelope rupture and genome instability during cell migration across physically constrained spaces[47,56]. In addition, lamin A/C deficiency can cause multiple diseases, including Emery-Dreifuss muscular dystrophy, dilated cardiomyopathy, and Hutchinson-Gilford progeria syndrome as a result of defective nuclear mechanotransduction and impaired mechanically activated gene transcription[56-61].

Lamin A/C can mediate mechanical force transmission from the cytoskeleton to the nucleus through the LINC complex by binding to SUN domain proteins at the inner nuclear membrane, which are connected to the actin stress fibers, microtubules, and intermediate filaments via KASH domain proteins at the outer nuclear membrane[62]. Force-induced conformational changes in lamin A/C impact lamin interactions with chromatin via LAPs and ultimately alter genes' transcription[63-65]. Disruption of the LINC complex, however, can interfere with force transmission to the nucleus, leading to genetic disorders and defective mechanotransduction[54].

Force transmission to the nucleus has been shown to impact lamin A/C expression levels through force-induced conformational changes, which can promote or hinder molecular interaction with signaling molecules through exposure of functional cryptic sites or shielding of others[66,67]. Buxboim et al. demonstrated that cells cultured on the soft substrates with wrinkled and non-stressed nuclei exhibited low total lamin A/C levels due to destabilizing of the lamin A/C coiled-coil dimers, thus favoring phosphorylation at Ser22, Ser390, Ser404, and Thr424 by constitutive kinases, including Cdks, PKC, and Akt[17,66]. Lamin A/C phosphorylation triggers disassociation from lamina into the nucleoplasm, and ultimate degradation in the nucleoplasm, resulting in a decrease in total lamin A/C expression[17,66] (Figure 1-5). However, stiff substrates deriving cellular tension and nuclear flattening induce stretching and conformational changes in lamin coiled-coil

dimers, inhibiting phosphorylation by hindering access of kinases[17,66] (Figure 1-5). In this work, I examine the role of force-induced nuclear deformation in lamin A distribution between the nuclear inner membrane and nucleoplasm rather than lamin A/C total expression.

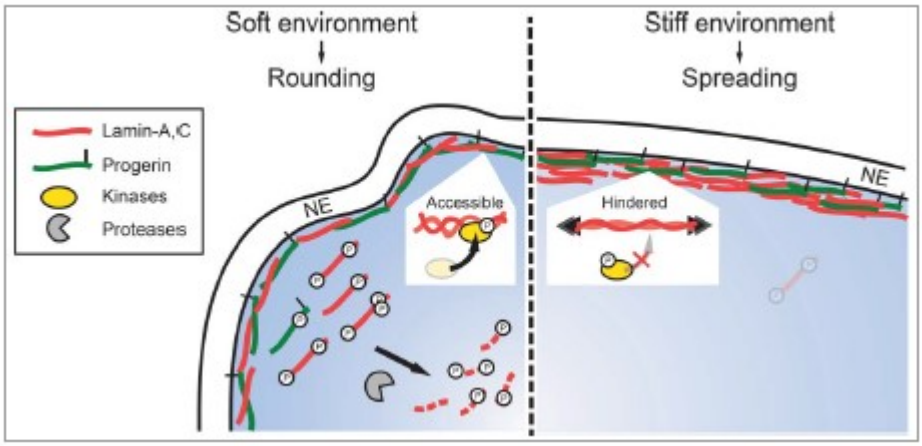

Figure 1-5 Nuclear mechanotransduction. Left side is the spherical relaxed nucleus on the soft substrate with phosphorylated and solubilized lamin A/C in the nucleoplasm. Right side is the highly stressed nucleus on the stiff substrate which inhibits lamin phosphorylation by hindering the access to the kinases. Reprinted from Nucleus, Sangkyun Cho , Amal Abbas, et al, "*Progerin phosphorylation in interphase is lower and less mechanosensitive than lamin-A,C in iPS-derived mesenchymal stem cells*", 9, 235-250, Copyright (2018), Open Access[68].

In addition to lamin A's crucial role as an important mediator of mechanical force transmission to the nucleus, studies have shown involvement of lamin A/C in mechanoactivation of cascades and transcription factors related to MSC differentiation[17,52,69]. Lamin A/C mechanosensitivity to substrate stiffness can induce adipogenic or osteogenic differentiation[17,52]. Lamin A deficiency mediates adipogenic differentiation, while lamin A overexpression has been found to enhance stiff-substrate promoted osteogenic differentiation[17,52]. Some studies also proposed the role of lamin A/C on nuclear pores' distributions and densities. Below, I will explain nuclear pore complex and the potential role of lamin A/C on nuclear pore distributions.

1.3.3 Nuclear pore complex (NPC)

NPCs are elaborate gateways anchored to the nuclear envelope, which connect the nucleoplasm and the cytoplasm, and direct selective transport of macromolecules between the cytoplasm and

the nucleus[70,71]. These immobile structures have cylindrical shapes containing multiple proteins called nucleoporins with a diameter of 120 nm and a mass range between 60-125 MDa, depending on species types. NPC has 8 cytoplasmic filaments protruding into the cytoplasm, and 8 nuclear filaments, which are connected via a ring, and form a basket shape structure. The inner lumen of NPC consists of a disorganized meshwork of FG-nups with phenylalanine–glycine repeats. Protein transportation through the nuclear pore is facilitated with repulsive interaction between protein and FG repeats mediating protein unfolding.

1.3.4 Role of lamin A/C in the nuclear pore distribution

Among the nuclear peripheral proteins, lamins have been shown to directly bind to nucleoporins, anchoring nuclear pore complexes in the nuclear envelope[72]. Lamins are crucial to avoid NPCs' aggregation whereas lamin depletion has been shown to cause uneven distribution of NPCs in the nuclear envelope[73]. Maeshima et al. demonstrated that NPCs dynamically increase during the cell cycle with low NPC density and a large number of nuclear pore-free islands (devoid of NPCs) in most G1 phase nuclei, whereas these pore-free islands gradually disperse during the cell cycle's progression to S, and NPCs concomitantly increase[74]. They also demonstrated that nuclear pore-free islands were drastically enriched with some nuclear membrane proteins, predominantly lamin A/C and emerin, but not lamin B. Lamin A/C depletion led to the disappearance of those pore-free islands, whereas lamin A/C overexpression facilitated formation of pore-free islands, suggesting that lamin A/C plays an essential role in nuclear pore distributions. Given the involvement of lamin A/C in the nuclear pore distributions and densities and considering the fact that pore-free islands are enriched with lamin A/C, in this thesis, I examine the role of lamin A distribution in the nuclear membrane on YAP protein shuttling between the nucleus and the cytoplasm. I study whether the lamin A distribution in the nucleus is mechanosensitive, and whether highly localized lamin A in the nuclear membrane affects protein translocation through the nuclear pores.

To understand how mechanical forces are transduced to gene transcription and biological processes via mechanotransduction, in this thesis, I investigate YAP mechanoregulation. Among different transcriptional factors involved in mechanotransduction, YAP has emerged as a key mechanosensitive transcriptional factor in diverse organisms and human development which fundamentally regulates cellular function through activation of target genes relevant to cell

proliferation, self-renewal, apoptosis, and differentiation. In the following sections, I will discuss YAP structure, YAP biology, and proposed molecular mechanisms regulating YAP activity.

1.4 Yes associated protein (YAP)

As previously mentioned, the perceived mechanical forces from the microenvironment propagate through the focal adhesions and the actin cytoskeleton, and the forces are eventually transmitted to the nucleus via the LINC complex. Nuclear deformation then can trigger activation of mechanosensitive transcription factors through force-mediated conformational changes in the nuclear compartments, including lamins and chromatin. These conformational changes determine the ease of transcriptional availability of chromatin, which regulates different target gene expressions[69,75]. However, how exactly nuclear deformations stimulate transcription activation remains unclear. In this work, to examine how mechanical signals and nuclear deformation are translated to biochemical response, I study the role of different mechanical forces on YAP activity. YAP as one of the crucial transcriptional coactivators plays an essential role in translating physical inputs into biological responses by activating transcription of some target genes relevant to tissue homeostasis, organ growth, stem cell differentiation, and cell proliferation[76,77]. I also investigate the regulatory mechanism which independently directs YAP translocation in the isolated living cells.

YAP is a 65 kDa mechanoresponsive protein, and appears in both the cytoplasm, where it is inactive, and in the nucleus, where it regulates gene transcription through interacting with TEADs[77]. YAP activity regulates the transcription of genes relevant to cell cycle control, leading to cell proliferation[78]. Thus, YAP controls organ size *in vivo* upon cell contact inhibition and *in vitro* by balancing cell proliferation and apoptosis. In addition, inactive YAP induces adipogenic differentiation, while active YAP in the nucleus mediates osteogenic differentiation of mesenchymal stem cells[79-81]. Dysregulation in YAP activity, however, can lead to tumorigenesis and cancer progression as a result of nonstop cell proliferation and organ overgrowth[77,82]. Therefore, understanding YAP's regulatory mechanism is central to therapeutic targets in cancer and regenerative medicine.

1.4.1 YAP biology

YAP itself does not have its own DNA binding motifs, but in the nucleus, it interacts with a host of transcription factors to induce expression of genes promoting cell growth and differentiation. At its carboxy-terminal half, YAP contains a transcription activation domain (TAD); at its amino-terminal half, however, YAP contains one or two WW domains, which trigger its interactions with proteins possessing PPxY motifs (Figure 1-6). Two of the predominant proteins which bind to YAP's WW domains are LATS1/2 and angiomotin (AMOT), which regulate YAP localization. In the nucleus, YAP activates and interacts with transcription factors, including RUNX, SMAD, and most importantly, TEAD family transcription factors that bind to DNA, resulting in specific gene expressions[83]. Some of YAP's important target genes include CTGF, Cyclin D1, AREG, and myogenic transcription factor Myf5[78].

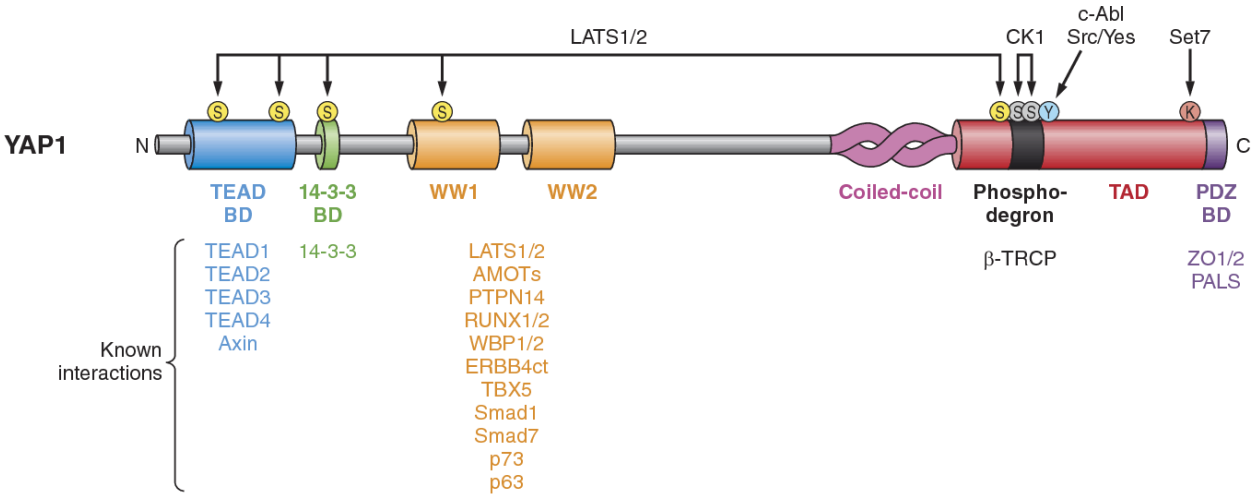

Figure 1-6 Schematic of YAP's multiple domains and its interactions with wide range of proteins and transcription factors. Reprinted from Physiological Reviews, Stefano Piccolo, Sirio Dupont, Michelangelo Cordenonsi, "*The Biology of YAP/TAZ: Hippo Signaling and Beyond*", 94, 1287–1312, Copyright (2014), with permission from The American Physiological Society[77].

1.4.2 Role of Hippo Pathway in YAP regulation

In Drosophila, the Hippo signaling pathway is one of the major canonical upstream pathways, and it is the first regulator of YAP localization[76]. At the core of the Hippo cascade are MST1/2 and LATS1/2 (Figure 1-7). MST1/2 interacts with its regulatory protein SAV1/WW45, and acts as an

enzyme to phosphorylate and activate LATS1/2 kinases[77]. The activated LATS1/2, in turn, phosphorylates YAP at five different serine/threonine residues (the most important ones being S127 and S381), located in HXRXXS consensus motifs[84]. Phosphorylation of YAP by LATS1/2 leads to nuclear exclusion and YAP inactivity as a result of YAP binding to 14-3-3 proteins, followed by YAP's cytoplasmic retention, and consequent degradation. Mutations of these serine residues, however, make YAP insensitive to phosphorylation and inhibition by Hippo signaling.

YAP activity also has been shown to be regulated by cell density in the context of contact inhibition of proliferation-induced cell growth arrest [85]. The Hippo cascade is inactive when cells grow in low density, leading to YAP nuclear localization, whereas high cell density activates the Hippo signaling cascade, leading to LATS1/2-mediated YAP phosphorylation and consequent YAP cytoplasmic localization[85].

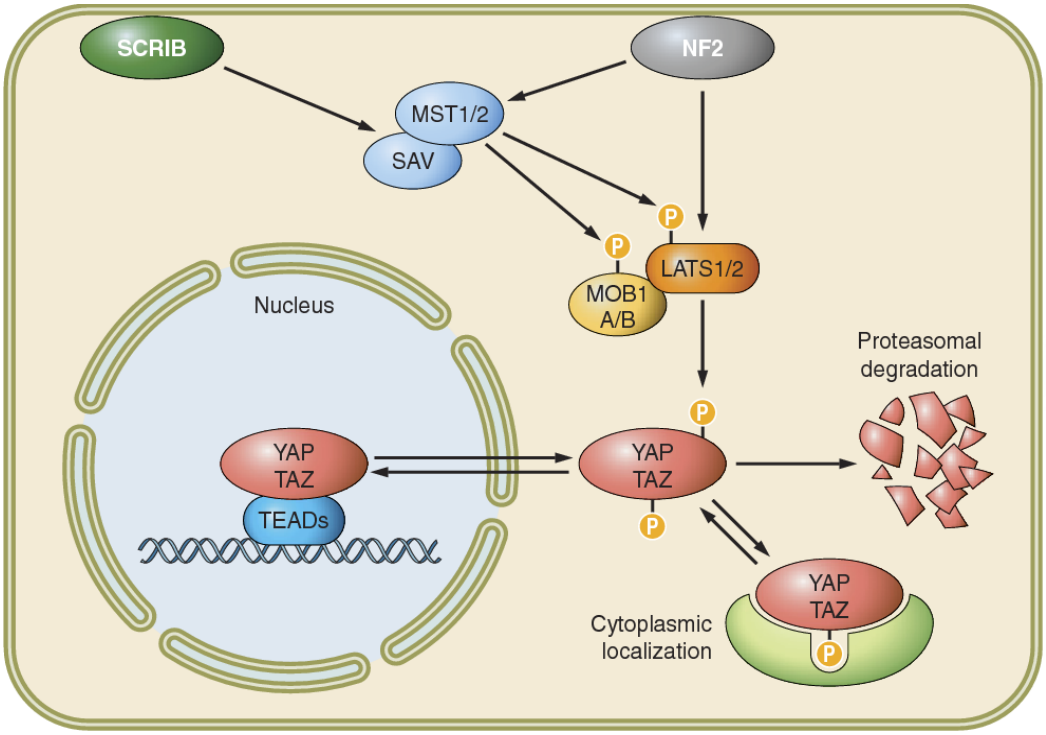

Figure 1-7 A model of YAP regulation mechanism through the hippo signaling pathway in mammalian cells. Reprinted from Physiological Reviews, Stefano Piccolo, Sirio Dupont, Michelangelo Cordenonsi, "*The Biology of YAP/TAZ: Hippo Signaling and Beyond"*, 94, 1287–1312, Copyright (2014) with permission from The American Physiological Society [77].

1.5 YAP mechanotransduction

Evidence indicates that YAP mechanosensitivity to mechanical cues emanating from ECM rigidity, cell geometry, dynamic stretching, and shear flow is independent of the Hippo pathway [79] (Figure 1-13). YAP, in turn, transduces these mechanical cues into the biological response specific to each cell type and mechanical stimulus[86].

Many studies have shown that YAP activity is regulated by ECM stiffness and substrate rigidity. Soft substrates (E<1 kPa) induce YAP cytoplasmic localization and inhibit YAP activity, whereas stiff substrates (E>5 kPa) impose YAP nuclear localization and upregulation of genes, including CTGF and ANKRD1, which are specific to YAP activation[79]. As ECM stiffness induces different degrees of cell spreading area, it has been shown that cell spreading area itself is sufficient to direct YAP activity[79]. Cells with large spreading (>3000 $(\mu m)^2$) area always show predominant YAP nuclear localization; however, small cells, with area less than 500 $(\mu m)^2$, show cytoplasmic YAP localization[79]. In addition, Cui et al. demonstrated that cyclic stretching of soft substrates induces YAP reactivation as a result of actin stress fibers' formation and increased cell contractility, confirming YAP's mechanosensitivity[87]. Regardless of previous studies which have been done in immunostained cells to determine YAP localization, in this work, I examine the effects of physical cues, including cell spreading area, substrate stiffness, and external compression on dynamic YAP in the living sparse cells.

1.5.1 Role of LATS in Mechanotransduction

YAP activity mediated by mechanical stimuli is regulated by a Hippo-independent mechanism. Previous studies demonstrated that LATS1/2 depletion could not rescue YAP activity in cells cultured on soft substrates[79], or cells treated with actin polymerization inhibitor[88]. In addition, LATS1/2-induced YAP phosphorylation was not increased after seeding the cells on soft substrates or inhibiting actin polymerization, confirming that the Hippo cascade is not the main mediator of YAP activity, and YAP mechanoregulation is independent from the Hippo pathway. However, the exact mechanism that would consistently explain YAP mechanosensation remains unknown. Below, I will explain some important proposed mechanisms regulating YAP activity in a Hippo-independent way.

1.5.2 YAP responds to cytoskeletal tension

Rho and actin filaments in a tensional state are required to elevate YAP activity, which can be assayed through the expression of endogenous target genes[79,88]. Stiff substrates entail the activation of GTPase Rho, which regulates actin stress fibers formation and actomyosin activity; cells spreading on the stiff substrates exert pulling forces against the substrates, leading to YAP nuclear localization. YAP activity mediated by cytoskeletal tension can be blunted by myosin light chain kinase (MLCK), non-muscle myosin type II, or Rho-associated protein kinase (ROCK) inhibitors. The treated cells with cytoplasmic YAP then function as if they are on the soft substrates[79,86]. Soft substrates inhibit actin polymerization and myosin contractility, leading to YAP cytoplasmic localization[79,88]. These confirm the importance of actin integrity and myosin contractility in YAP activation. However, the exact molecular mechanism by which actin contractility regulates YAP nuclear translocation remains unclear.

1.5.3 Actin-nucleus connection is essential for YAP mechanosensation

Driscoll et al. demonstrated that actomyosin generated force needs to transfer to the nucleus through the LINC complex to upregulate YAP nuclear localization[89]. Under applied external tensile stress to the cells cultured on the substrates, they showed that inhibition of ROCK or actin polymerization using Y27632 or CytoD, which disrupt connection between actin and the nucleus, significantly reduced force-mediated nuclear deformation; however, inhibition of MLCK using ML7, which decreases cell contractility, remarkably increased nuclear deformation under applied external tensile stress due to the maintenance of the LINC complex [89]. Investigating YAP regulation under dynamic stretching, they observed YAP nuclear localization induced by dynamic stretching; however, inhibition of actin stress fiber polymerization and ROCK abolished the force transferred to the nucleus and YAP activation. A decrease in cytoskeletal tension using ML7, however, did not inhibit YAP nuclear translocation mediated by dynamic stretching due to the potential nuclear deformation through the LINC complex[89]. These results suggest the key roles of the LINC complex and force transmission to the nucleus in YAP nuclear localization. In this work, I disrupt the LINC complex to examine how disruption of the LINC complex affects cell contractility-mediated nuclear deformation and YAP activation.

1.5.4 Nuclear deformation alone is sufficient to regulate YAP activity

The presence of the LINC complex which provides a connection between the nucleus and the cell cytoskeleton is crucial for force transmission to the nucleus and, consequently, nuclear structural deformation[56,89,90] and chromatin rearrangement[75], which regulate cell biological responses. Many studies have pointed towards the notion that transcriptional activities are associated with chromatin reorganization at the nuclear periphery via interaction between NPCs, which are connected with both the cytoskeleton and DNA as well as euchromatin[75,91]; however, the exact regulatory mechanism has remained unsolved.

Another way in which mechanical cues can trigger gene expression as a result of nuclear inner membrane reorganization is by impacting the accessibility of chromatin to transcription activators. Lamin A at the inner nuclear membrane has been shown to interact with heterochromatin, which is transcriptionally inactive, via long genomic regions termed lamina-associated domains (LADs); however, transcriptionally active euchromatin mostly settles at the center of the nucleus[92]. A-type lamins also have been shown to exist in the nuclear interior where they are highly dynamic compared to those associated with peripheral lamina in the inner nuclear membrane[65]. Nucleoplasmic lamins have been shown to play an important role in gene expression and chromatin regulation via their binding to both heterochromatin and euchromatins' subdomains[65]; however, the ways in which these nucleoplasmic lamins respond to different mechanical cues as well as how they regulate transcription activation and gene expression remain poorly understood. It is known that transcription factors can be mechanically activated and interact with the nucleoskeleton; hence, alterations in nucleoskeleton organization can lead to defects in transcription pathways and consequently cause multiple diseases[56,93,94]. Recent studies in YAP mechanosensation have reported that nuclear deformation itself plays a crucial role in YAP mechanoregulation[90]. It has been reported that either contractile or hyper-osmotic forces flatten the nucleus and open the nuclear pores, which enhance the rate of YAP nuclear import, while not impacting the YAP export rate (Figure 1-8). Moreover, it has been demonstrated that nuclear membrane tension also increases curvature of the lateral part of the nuclear membrane, leading to exposure of the inner surface of the nuclear pore to the cytoplasm and promoting translocation of mechanosensitive proteins to the nucleus[90] (Figure 1-8a). Although the proposed model provides new insight into how YAP transports to the nucleus, it does not fully explain why only tension

mediated by nuclear flattening, and not by nuclear swelling facilitates YAP nuclear import as well as why an increase in nuclear pore size does not impact the YAP export rate. Moreover, the proposed mechanism does not explain how increased nuclear curvature affects nuclear lamina and chromatin reorganization, which ultimately impact transcription activation and gene expression.

The essential role of both lamin A and YAP in cell fate decisions may lead one to question whether lamin A has crosstalk with YAP regulation in a substrate-dependent manner. Previous studies have reported that YAP nuclear localization mediated by substrate stiffness is blunted in LMNA muted cells[95]. Yet, in cancer cells with low lamin A expression levels, nuclear aggregation of YAP has been observed[96-98]. In addition, in mesenchymal stem cells, lamin A upregulated by a stiff substrate triggers YAP nuclear translocation, mediating osteogenic differentiation. It has also been shown that LMNA overexpression on stiff substrates leads to a decrease in YAP nuclear localization[17,52]. Findings thus far suggest a non-monotonic relationship between YAP and lamin A expression levels. In this thesis, I study the role of both lamin A expression levels and lamin A distributions in the nuclear inner membrane on YAP activation. I investigate whether applied contractile or external forces to the nucleus impact lamin A distribution in the nucleus and how lamin A distribution can be related to YAP nuclear translocation.

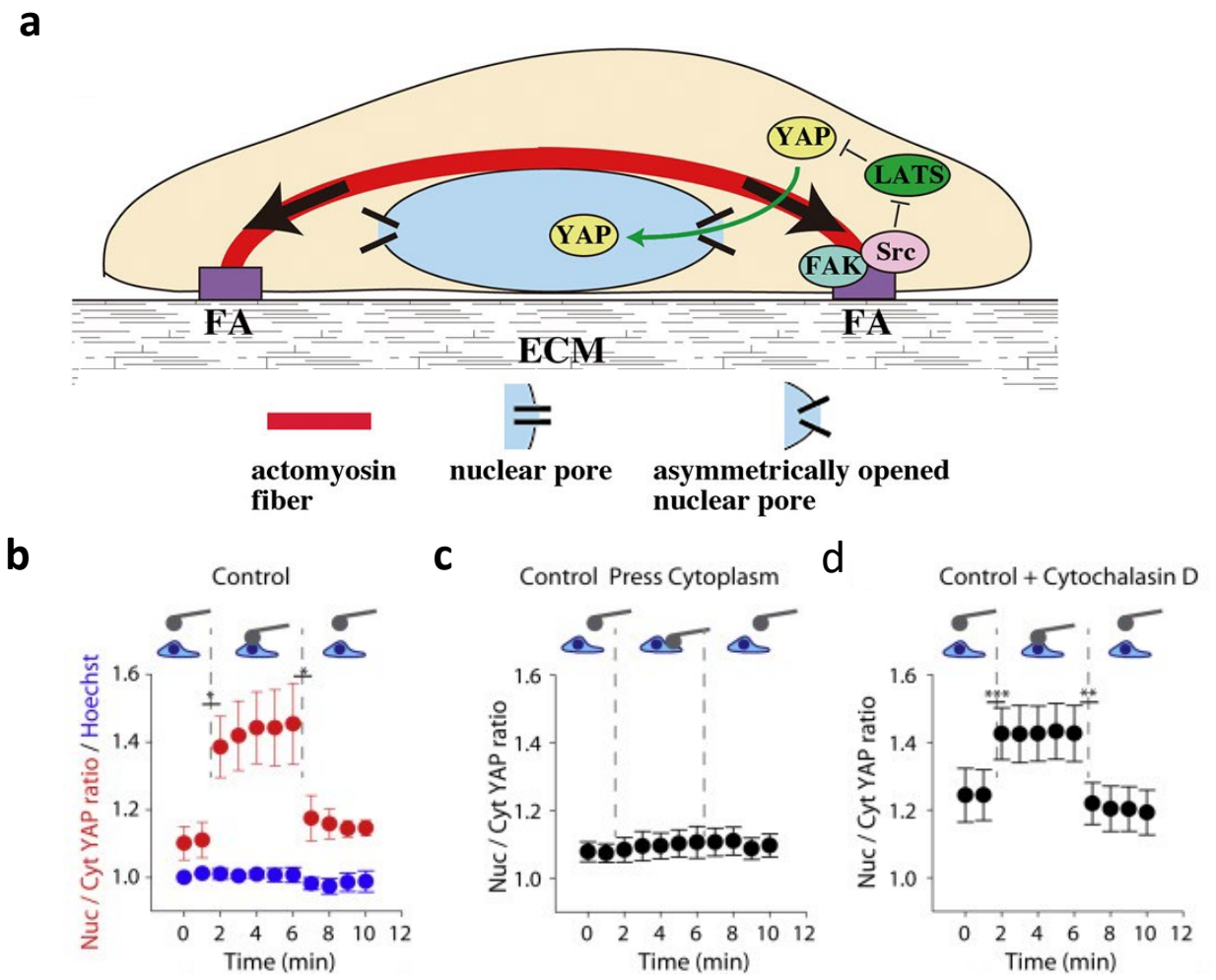

Figure 1-8 Nuclear deformation is sufficient to trigger YAP nuclear localization. a) Schematic of YAP nuclear localization mediated by actomyosin contractile force deforming the nucleus and increasing curvature of lateral part of nuclear membrane. Reprinted from Dobrokhotov et al., 2018, Clin Trans Med, 7:23, Copyright (2018), Springer Nature[99], Open Access, b) Quantification of YAP nuclear to cytoplasm ratio (red) and Hoechst nuclear average intensity (blue) during sequential force application to the nucleus using AFM: no force (1 min), 1.5 nN force (5 min), and no force (4 min). c) Same experiment as in (b) but applying the force on the cytoplasm rather than the nucleus, d) Same experiment as in (b) but for the CytoD treated cells. Reprinted from Elosegui-Artola et al., Cell 171, "*Force Triggers YAP Nuclear Entry by Regulating Transport across Nuclear Pores*", 1397–1410, Copyright (2007) with permission from Elsevier[90].

Preface to Chapter 2

In Chapter 2, I studied the effects of different physical parameters, including substrate stiffness, cell spreading, cell contractility, nuclear deformation, nuclear deformability, and lamin A distribution in YAP localization in the living sparse NIH 3T3 cells. Contrary to previous studies, I found that YAP dynamically translocated between the nucleus and cytoplasm independent of substrate rigidity and cell spreading during cell movement. I showed that cell contractile work (strain energy) which also fluctuated during cell movement on the specific substrate, accurately directed YAP localization through nuclear compression and independent of substrate stiffness. My results revealed that nuclear compression mediated by either contractile work or hyper-osmotic pressure was sufficient to effectively induce YAP nuclear import. Moreover, I found that nuclear deformability which determines the ability to resist applied force effectively impacted the required force to induce YAP nuclear localization. My results revealed that in the cells with stiffen nuclei mediated by lamin A overexpression, YAP nuclear localization happened in the lower deformation compared to the WT cells with softer nuclei, suggesting that nuclear deformation alone might not be the most robust, independent regulator of YAP. This led me to explore the effects of lamin A in YAP mechanotransduction beyond nuclear stiffening. I observed not only nuclear deformation-mediated YAP nuclear transport, but also that it impacted lamin A distribution between the nuclear membrane and the nucleoplasm. Force transfer to the nucleus-mediated lamin A delocalization from the nuclear membrane to the nucleoplasm. Lamin A delocalization also was consistently correlated with YAP nuclear localization independent of nuclear mechanics and any other experimental conditions.

Chapter 2: Lamin A redistribution mediated by nuclear deformation determines dynamic localization of YAP

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2.1 Summary

YAP is a key mechanotransduction protein with essential roles in diverse physiological processes. Dysregulation in YAP activity is associated with multiple diseases such as atherosclerosis, fibrosis, and cancer progression. Here we examine the physical stimuli that regulate dynamic YAP translocation to the nucleus. Through a combination of biophysical studies, we demonstrate that YAP localization is insensitive to cell substrate stiffness, but strongly determined by cellular contractile work, which in turn deforms the nucleus. We show that nuclear deformation from LINC-mediated cytoskeletal contractility or extracellular osmotic forces triggers YAP nuclear localization. By modulating the expression of lamin A and nuclear stiffness, we illustrate that nuclear rigidity modulates deformation-mediated YAP nuclear localization. Finally, we show that nuclear deformation causes relocalization of lamin A from the nuclear membrane to the nucleoplasm, and this is essential in allowing YAP to enter the nucleus. These results reveal key physical nuclear deformation mechanics that drive YAP nuclear import.

Keywords: YAP, nucleus, contractile work, TFM, substrate stiffness, cytoskeleton, LINC, deformation, mechanotransduction, mechanosensation

2.2 Introduction

Sensing and correctly responding to mechanical signals are essential aspects of biology. There are diverse mechanisms enabling cells to sense various mechanical stimuli, including ECM rigidity [87,88,100], dynamic stretching [87], cytoskeletal strain [101,102] and compression [103]. Many mechanosensory mechanisms regulate transcription factors, which in turn dictate fundamental

aspects of cellular function, homeostasis, and tumorigenesis [79,89,104]. Yes Associated Protein (YAP) is a crucial transcription factor that mediates the interplay between cellular mechanics and signaling cascades underlying gene expression, cell proliferation, differentiation fate decisions, and organ development [77,79,105,106]. Thus, the spatio-temporal localization of YAP provides critical information about the regulatory state of the cell. Increased YAP activity can cause abnormal and uncontrollable cell proliferation and invasiveness, leading to multiple diseases, including fibrosis and diverse cancers as a result of activating genes associated with oncogenic transcription factors [82,107,108].

Microenvironment mechanics appears to influence YAP localization, with several studies reporting that YAP nuclear localization and activity tends to increase with increasing substrate stiffness [29,79,109]. This has led to the hypothesis that substrate stiffness directly influences YAP localization and its resulting effects such as proliferation and differentiation [79,110,111]. However, the abrogation of these effects by cytoskeletal disruption demonstrates that YAP activity is more directly related to cytoskeletal processes and contractility rather than to substrate mechanics [79,84,109]. Elosegui-Artola et al. demonstrated that disrupting the actin-LINC complex (Linker of Nucleoskeleton and Cytoskeleton) attenuates YAP activity's correlation with substrate stiffness, further suggesting a role of cytoskeletal contractility in YAP activity [90]. However, how contractile forces vary during cell movement, and how they actually determine dynamic movement of YAP in real-time remains unclear, but they are hypothesized to be related to nuclear mechanosensing. Recent work which demonstrates that direct application of force to the nucleus is sufficient to regulate YAP activity reinforces the idea that nuclear deformation is a key mechanism of YAP regulation [90]. These findings suggest that nuclear deformability and nuclear deformation have essential roles in cells correctly responding to cues from their mechanical environment.

Among the proteins underlying the inner nuclear membrane, lamin A is known as one of the crucial intermediate filament proteins that confers physical support of the nucleus and modulates nuclear stiffness [47,56,62,112]. Mutations in lamin A are associated with impaired nuclear mechanotransduction resulting in a variety of human diseases [56,57,60]. Lamin A expression level also modulates nuclear rigidity; suppression of lamin A softens the nuclei and increases nuclear deformability [56,113,114], whereas lamin A overexpression increases nucleus' stiffness [114,115]. Lamin A expression levels can be affected by cell division [116] and stem cell differentiation [117]. Previous

studies have also illustrated an interplay between lamin A expression and substrate stiffness [17,118]. Soft substrates where the cells have limited spread area, promote lamin A turnover, phosphorylation and subsequent lamin A degradation. Whereas stiff substrates stabilize lamin A as a result of larger forces being transduced to the nucleus, leading to nuclear tension and conformational change in lamins which prohibits access of kinases [17,47,119]. The similar impacts of mechanical cues on lamin A expression levels and YAP activation, and the correlation of these proteins with stem cell differentiation and cell division suggest a relationship between lamin A, nuclear mechanics, and YAP mechanosensing, however, no such link has been established.

2.3 Results

2.3.1 YAP nuclear localization is dynamic and independent of cell spread area and substrate stiffness

To examine the dynamics of YAP localization, we transfected living NIH 3T3 cells with EGFP-YAP (pEGFP-C3-hYAP1, Addgene, plasmid #17843) and with EBFP2-Nucleus-7 (nuclear localization signal, Addgene, plasmid #55249) to visualize the nucleus. Similar to previous studies, our principal metric for YAP activity is the ratio of fluorescence of EGFP-YAP in the nucleus to EGFP-YAP in the cytoplasm and is henceforth referred to as the YAP Ratio (YR) (Figures 1A-1C, Figure S1A). To examine how cellular interactions with the substrate determine endogenous YAP and EGFP-YAP localization, we quantified the YR as cells spread on polydimethylsiloxane (PDMS) substrates with different Young's moduli (0.3 and 48 kPa) [120,121]. Both transfected and immunostained cells displayed a broad range of YRs on soft PDMS, stiff PDMS, and glass substrates without a stiffness trend (Figures 1A-1C, Figure S1A). We observed both nuclear (YR>1.5) and cytoplasmic (YR<1) localization of endogenous YAP and EGFP-YAP independent of substrate rigidity.

We also monitored the EGFP-YAP distribution during cell movement on PDMS substrates finding that EGFP-YAP localization is highly dynamic in time, with no stiffness correlation over time across diverse PDMS substrate moduli (0.3, 2, 5, 12, 18, 100 kPa) and fibronectin-coated glass (Figures 1D-1F, Figures S1B and S1C). The time-averaged YR on stiff substrates appeared identical to compliant substrates, suggesting that neither the magnitude of YAP localization nor the shuttling frequency is set by substrate stiffness (Figures 1D-1F).

Previous cell substrate studies on polyacrylamide (PAA) have reported a positive correlation between the YR and substrate stiffnesses; YAP cytoplasmic localization was only observed in round cells with small spread area on soft PAA substrates with moduli less than 1 kPa [79,90,109], whereas 5 kPa was identified as a critical modulus for high YR [79,88,90]. Using PDMS substrates, we observed that the YR did not correlate with substrate stiffness (Figures 1F and 1G). One possible explanation may be ascribed to non-mechanical variations in PAA substrates absent on PDMS [122].

To examine the impact of PDMS or PAA on YAP localization, we prepared PAA gels with uniform polymer mass and tuned the substrates stiffness by varying the crosslinker concentration. Similar to previous studies, cells on very compliant PAA gel with a Young's modulus of 1 kPa were round with low spread area and low YAP activity (YR<1) (Figure 1G, Figures S1D, S1E and S1I). However, cells on very compliant PDMS with a Young's modulus of 300 Pa displayed a wide range of spread areas and random distributions of YAP between their nuclei and cytoplasms, similar to those on the stiff PDMS (Figures 1E-1G, Figures S1F-S1H). Cells on stiff PAA substrates with moduli of 5, 20, and 50 kPa displayed highly dynamic EGFP-YAP localization with diverse spread areas similar to those we observed on PDMS substrates (Figures 1D and 1G, Figures S1D, S1E, S1J and S1K). These results suggest that YAP nuclear localization may be reduced on very soft (E≤1kPa) PAA substrates due to a lack of cell spreading.

To examine the roles of substrate adhesion and cell spread area on YAP translocation, we also tracked YAP localization in round suspended cells (Figure 1H). Here, during initial cell attachment, we consistently measured low YRs in round cells with small spread area (Figures 1H and 1I). As soon as those suspended cells attached and spread on the 5 kPa PDMS substrate, YAP distributed randomly between the nucleus and cytoplasm with no clear correlation with cell spreading (Figures 1H and 1I). Our results suggest that only in the case of rounded exceptionally small cell areas (<~1000 μm^2) is YAP consistently in the cytoplasm.

The apparent random spatio-temporal localization of YAP on both PAA and PDMS substrates, coupled with an absence of correlation between YAP localization and substrate stiffness or cell spreading implies that another mechanism may impact YAP localization. Actin stress fibers and intracellular forces may more directly regulate YAP localization [38,90,109]. We thus suspected that

dynamic changes in contractile stress may lead to dynamic and correlated changes in YAP localization.

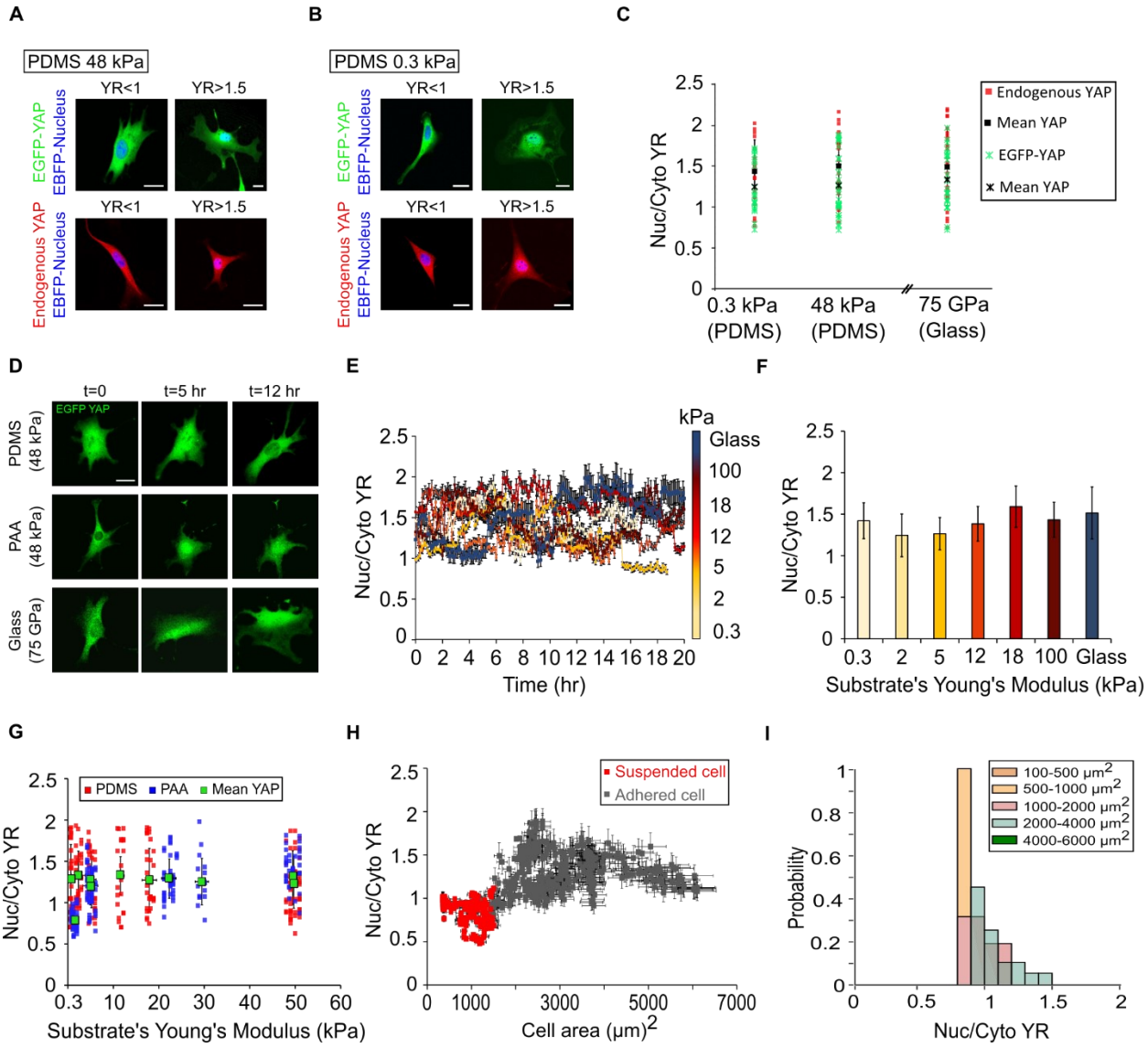

Figure 1. YAP localization is dynamic overtime and independent of substrate stiffness or cell spread area. A) Example image of EGFP-YAP and endogenous YAP merged with EBFP-NLS nucleus on stiff PDMS and, B) soft PDMS, C) YR variation on soft, stiff PDMS, and glass for EGFP and endogenous YAP. Black squares and black stars are mean YR values for EGFP-YAP and endogenous YAP, respectively on each substrate (n>15 cells per each condition), D) Example of EGFP-YAP variation during cell movement on PDMS, PAA, and glass over time, E) Quantification of YR during cells movement on PDMS substrates with different stiffnesses and on the fibronectin-coated glass, F) Time average of YR in the same condition as in (E), G) YR variation for NIH 3T3 cells seeded on different PDMS (shades of red) and PAA (shades of blue) substrates with different stiffnesses (n>20 cells per each condition), H) Quantification of YR and

cell spreading area before (red) and during cells attachment (gray) on 5 kPa PDMS substrate (n>10 cells), I) YAP distribution based on cell spread area for an example cell before and during adhesion to PDMS with the same modulus. Scales bars are 20 μm. Error bars indicate standard deviation (SD).

2.3.2 YAP nuclear localization increases with cell contractility

We employed Traction Force Microscopy (TFM) [123] to track single-cell contractility in time on PDMS substrates with specified moduli, and compared these metrics with the dynamic YR; we found that as the contractile state of the cell changed in time, it was temporally correlated with the YR (Figures 2A-2E, Figures S2A-S2H). We then compared the instantaneous contractile states of single cells with their YRs over a broad range of substrate stiffnesses, finding that both traction stress and substrate bead displacement correlated with YRs of the corresponding cell (Figures 2F and 2G). These data separated into different correlated populations as a function of substrate stiffness. When we examined YR as a function of cell contractile work for different PDMS substrates, we found all data collapsed onto a single curve (Figure 2H), illustrating that cell contractile work appears to relate to YAP localization.

We then disrupted myosin activity and the actin cytoskeleton using 50 μM RhoA-associated protein kinase (ROCK) inhibitor (Y27632) and 1.5 μM cytochalasin D (CytoD), respectively, and monitored their effects on YAP localization (Figures S2I and S2J). Both pharmacological treatments inhibited cell contractility and suppressed YAP nuclear localization ~15 and ~40 minutes after CytoD and ROCK inhibitor treatments, respectively (Figures 2I and 2J, Figures S2I and S2J). Critically, these cytoskeletal poisons did not change the relationship between cell contractility and YR, and data from cytoskeleton-disrupted cells followed the same YAP-Strain Energy curve (Figure 2K), suggesting that contractile work predicts YAP localization across all probed cytoskeletal states.

As contractile work appears to determine YAP localization, we considered the cellular components which are mechanically impacted by cell contractile work, and may drive changes in the YR. Since the nucleus is at the heart of YAP activity and has been previously implicated in force-mediated YAP activity [38,43,89,90], we examined in detail how contractile work mechanically deforms the nucleus and relates to YAP nuclear localization.

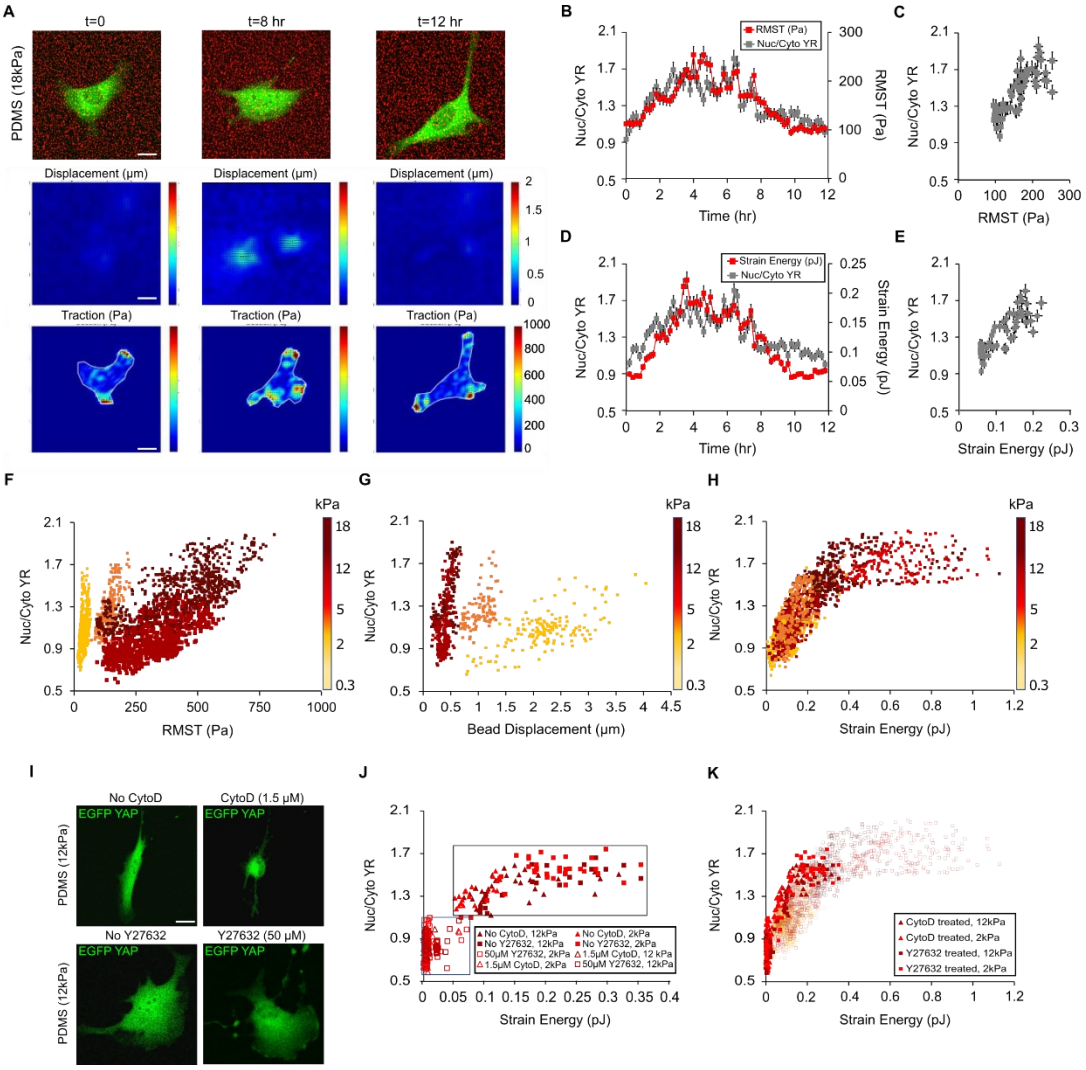

Figure 2. YAP localization is correlated with cell contractility. A) Representative traction stress and bead displacement maps of EGFP-YAP transfected NIH 3T3 cell during its movement on 18 kPa PDMS substrate, B) YR vs RMST over time for the same cell as in (A) with time interval of 12 minutes, C) Scatter plot of YR as a function of RMST, D) Quantification of YR vs Strain Energy for the same cell over time, E) Scatter plot of YR vs Strain Energy for the same cell, F) YR vs RMST for multiple cells seeded on PDMS substrates with different stiffnesses (n>20 cells per condition), G) YR vs bead displacement for the same cells as in (F), H) Scatter plot of YR as a function of Strain Energy for the same cells, I) Example of EGFP-YAP transfected cells before and after pharmacological treatments, J) Quantification of YR vs Strain Energy for multiple cells on PDMS substrates with different Young's moduli before (solid markers) and 30 minutes after (open markers) pharmacological treatments (n>10 cells per condition), K) All data of YR as a function of Strain Energy for nontreated (partially transparent data) and pharmacologically treated cells. Scale bars are 20 μm. Error bars indicate standard deviation (SD).

2.3.3 Cell contractility regulates YAP localization via nuclear deformation

To examine the interplay between contractility and nuclear deformation, we quantified the nuclear volume using confocal Z-stacks for cells with different contractility. We found a relationship between contractile work and nuclear volume (Figures 3A and 3B). One key connection between the cytoskeleton and nucleus is the LINC complex [62,89,90]. To examine the role of the LINC complex in transducing contractile work into nuclear compression, we disrupted the LINC complex by transfecting the cells with two dominant-negative EGFP-Nesprin1-KASH, EGFP-Nesprin2-KASH plasmids (DNK1/2), and iRFP-YAP at the same time (Figure 3A). Lombardi et al. showed that overexpression of DNK1/2 inhibits interaction between nesprins and SUN proteins, key components of the LINC complex, at the nuclear envelope by nonspecific binding to endogenous SUN proteins [124]. Our results demonstrated that suppressing the LINC complex in DNK1/2 transfected cells decreased the effect of cell contractility-induced nuclear compression (Figure 3B).

We then examined how the presence of LINC-mediated contractility influences YAP localization. Here we found that YRs were generally decreased in LINC disrupted cells relative to wild-type (WT) cells for any given cell contractility (Figure 3C, Figures S3A and S3B). This paralleled our observation of the effect of LINC complex disruption on nuclear compression, suggesting a connection between nuclear volume and YR. When we examined YRs as a function of nuclear volume, we found a complete overlap between WT and DNK1/2 transfected cell data, suggesting that nuclear volume may directly regulate YAP activity (Figure 3D). These findings suggest that acto-myosin contractility and LINC-mediated cytoskeletal-nuclear coupling thus contribute to nuclear compression, which appears to describe contractility-based YAP localization.

Returning to the possible influence of substrate stiffness on mechanotransduction mechanisms, we measured YRs as a function of nuclear volume on diverse PDMS substrates with different stiffnesses. Again, we found that cell contractility-driven nuclear compression is correlated with YAP nuclear localization in a substrate stiffness independent way, with similar trends being observed between WT and LINC disrupted cells (Figure 3E). We also observed no trend between nuclear volumes and substrate stiffness (Figure S3C).

Our results highlight the role of nuclear volume and deformation in mechanosensing, which is consistent with the idea that nuclear deformation is necessary to open nuclear pores, allowing YAP nuclear translocation [90]. We also demonstrated that inhibition of contractile force transfer to the nucleus decreases YAP nuclear import, suggesting that the amount of stress applied to the nucleus moderates its deformation, and consequently YAP activity. We then postulated that varying nuclear stiffness would also modulate contractile-dependent nuclear deformation and YAP localization. This means that the nucleus' stiffness is likely key in determining specific deformation-mediated mechanosensation to applied stresses.

2.3.4 Stiffer nuclei require more contractile work to trigger YAP nuclear localization

The interplay between lamin A expression level and nuclear stiffness led us to question whether changes in lamin A-mediated nuclear stiffness would result in changes in contractile work-mediated nuclear compression, and corresponding changes in YAP localization. We manipulated lamin A expression levels and measured corresponding nuclei deformation and nuclei stiffness under different osmotic compressions applied by different concentrations of 400 Da polyethylene glycol (PEG) (see Methods) [103] (Figures 3F and 3G, Figure S3D). To quantify total single-cell lamin A expression levels, we transfected the cells with GFP-lamin A chromobody [125]. We found that nuclear stiffness increases as a function of lamin A expression (Figure 3G, Figure S3D).

We then examined how contractile work deforms nuclei with different stiffnesses, and how this in turn regulates YAP localization. We found that in lamin A silenced cells with compliant nuclei (Figure 3G), nuclei were observed to be smaller due to contractility-induced nuclear compression (Figure 3I) with increased YRs (Figures 3H and 3J). However, in lamin A overexpressed cells, more contractile work was required to compress the nucleus and localize YAP to the nucleus (Figures 3H-3J, Figure S3E). Since lamin A expression determines the nuclear stiffness, this suggests that contractile work is not a direct regulator of YAP activity, and that nuclear volume may be a more robust independent variable. However, we note that the lamin A overexpressed nuclei deviate from the overall trend of YAP dependence on nuclear volume (Figure 3K).

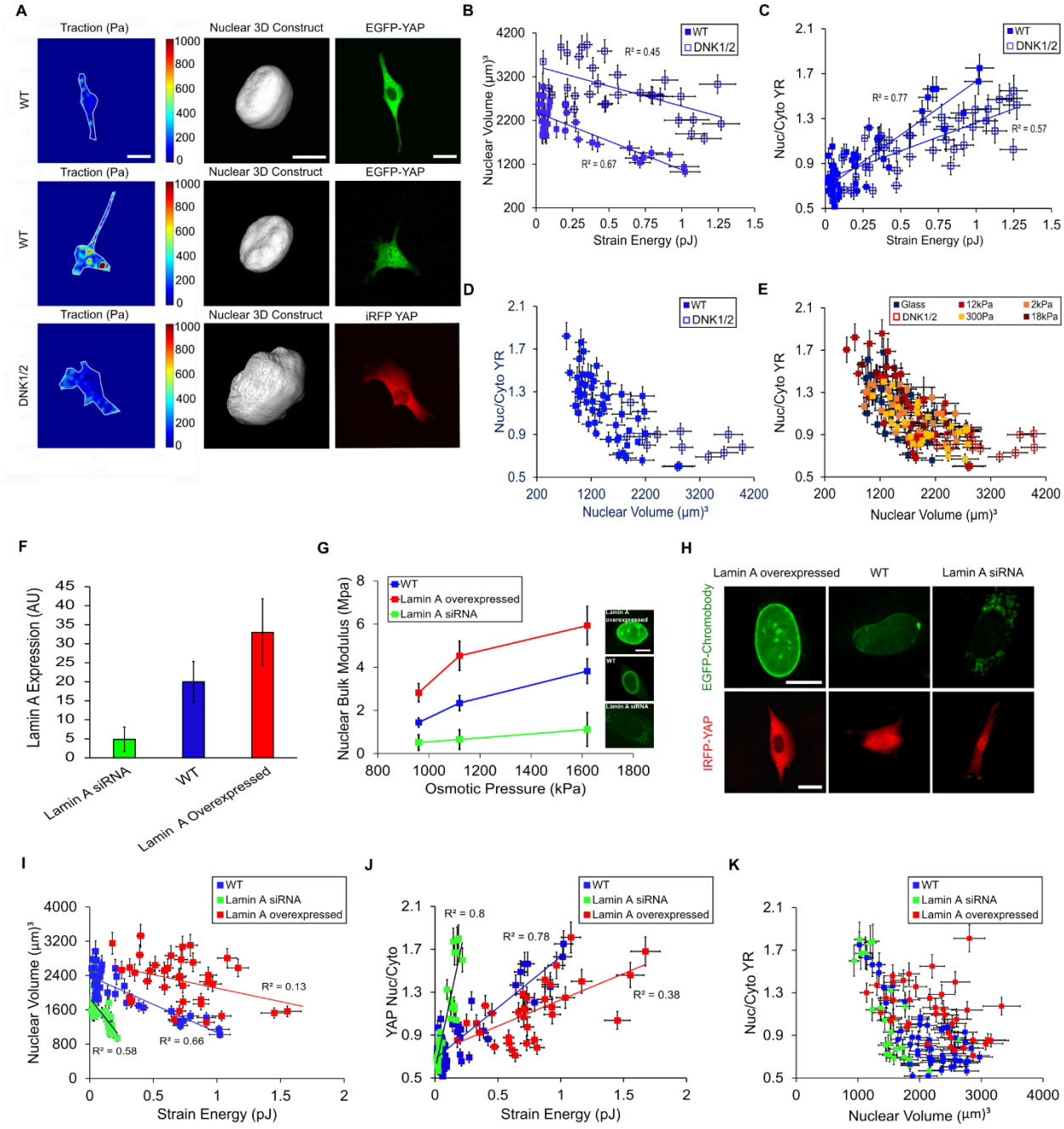

Figure 3. Strain energy-mediated nuclear deformation and YAP activity is directed by LINC complex and nuclear mechanics. A) Representative nuclear volumes and EGFP-YAP distributions in WT and DNK1/2 overexpressed cells with diverse contractility states on 12 kPa PDMS substrate, B) Quantification of nuclear volume vs Strain Energy for WT and DNK1/2 cells seeded on 12 kPa PDMS (n>10 cells per each condition), C) Quantification of YR vs Strain Energy for the same cells as in (B), D) YR as a function of nuclear volume for the same cells, E) Correlation between YR and nuclear volume for WT and DNK1/2 overexpressed cells seeded on PDMS substrates (n>10 cells per each condition), F) Quantification of lamin A expression level for lamin A siRNA, lamin A overexpressed and WT cells (n>20 cells per each condition), G)

Quantification of nuclear bulk moduli under different osmotic pressures applied to lamin A siRNA, lamin A overexpressed and WT cells (n>20 cells per each condition), H) Examples of GFP-chromobody and iRFP-YAP in different cells with different lamin A expression level, I) Quantification of nuclear volume vs Strain Energy for cells with different lamin A expression levels (n>15 cells per each condition), J) YR vs Strain Energy for the same cells as in (I), K) YR vs nuclear volume for the same cells. Scale bars are 20 μm for cells and 10 μm for nuclei. Error bars indicate standard deviation (SD).

2.3.5 YAP translocation correlates with nuclear deformation independent of contractile work, actin filaments and LINC complex

Having observed a correlation between YAP localization and contractility-modulated nuclear volume under the diverse conditions of cytoskeletal poisons, LINC suppression, and varied lamin A expression, we then postulated that modifying nuclear volume through *any* mechanism may be necessary and sufficient for YAP nuclear localization. To test this hypothesis, we applied external osmotic forces on the cells using different concentrations of PEG400, which has previously been shown to reversibly compress cells and nuclei [103,126]. We then measured the nuclear volumes and YRs for WT cells before and after applying different osmotic pressures, finding the same conserved relationship between nuclear volume and YAP localization (Figures 4A and 4B). Nuclear volume and YRs did not change under 960 kPa pressure, whereas increase in YR and decrease in nuclear volume were observed under 1.62 MPa (Figures 4A and 4B, Figures S4A and S4B). We then considered the role of substrate adhesion in YAP localization by applying osmotic compression to cells in suspension under 10% PEG, which in adhered cells consistently increased YRs.; in compressed suspended cells we did not measure increases in YRs, suggesting that substrate adhesion is an essential aspect of YAP localization in nuclear compression (Figures S4C and S4D).

Next, we examined the role of the actin cytoskeleton in adhered cells in osmotic pressure-mediated YAP activity by depolymerizing F-actin with CytoD. Here we again found the same nuclear volume and YR trend, demonstrating that actin cytoskeleton is not essential for externally compressive YAP mechanosensing (Figures 4C and 4D). Nuclear volumes increased and YRs decreased after depolymerizing actin filaments; however, 1.12 MPa osmotic pressure was sufficient to translocate YAP into the nucleus in the absence of actin (Figure 4D). We also revisited the role of the LINC complex in YAP nuclear localization under external pressure by blocking LINC complex via overexpression of DNK1/2. Compared to WT cells, a lower osmotic pressure

(1.12 MPa) was required to significantly deform the nucleus and activate YAP in LINC disrupted cells, demonstrating that external compressive forces deform the nucleus and activate YAP independent of the LINC complex, but that the LINC complex may serve as a mechanoprotective role in compression (Figures 4E and 4F). Critically, all YAP activity and nuclear volume data for WT, CytoD treated and DNK1/2 transfected cells appear as a single correlated distribution independent of experimental conditions, demonstrating an apparent connection between nuclear volume and YAP activity (Figure 4G).

While YAP activity appears related to nuclear volume, we questioned how relative nuclear deformation influences changes in YAP activity. To examine this, we measured the change in YAP ratios ($\Delta YR/YR_0$) as a function of the change of nuclear volume ($-\Delta V/V_0$) under different external pressures applied to WT, DNK1/2, and CytoD treated cells. For all conditions, the change in YR as a function of change in nuclear volume fell on a single curve, suggesting that nuclear deformation is related to YAP localization in a magnitude-dependent and conserved manner (Figure 4H). We also observed that the nuclei of DNK1/2 and CytoD treated cells deformed relatively more under same amount of pressure as compared with WT cells, further suggesting a potential mechanoprotective role for LINC and the actin cytoskeleton in the context of external compression (Figure 4H, Figure S4E). These results suggest that while acto-myosin contractile work is an essential regulator of YAP under typical cellular conditions, that nuclear deformation underlies YAP localization. This would suggest that nuclear deformability impacts required stress to trigger YAP nuclear transport.

2.3.6 Lamin A-mediated nuclear stiffness regulates the amount of stress required to trigger YAP nuclear transport

We next examined the role of nuclear stiffness modulated by lamin A expression level, finding that cells with increased lamin A expression required more applied stress to reach an equivalent YR. SiRNA lamin A cells required lower pressure (960 kPa) than WT or lamin A overexpressed cells to compress nuclei and localize YAP in the nucleus (Figures 4I and 4J), while 1.12 MPa was required to trigger YAP nuclear localization in WT and lamin A overexpressed cells (Figures 4K and 4L, Figures S4F and S4G) and increased further under 1.62 MPa osmotic pressure (Figures 4K and 4L, Figures S4H and S4I). These findings suggest that lamin A-mediated nuclear stiffness affects the amount of stress required to activate YAP.

These findings describe a preserved relationship between YAP and nuclear volume, observed under different pressures when modifying the stiffness of the nucleus via lamin A expression (Figure 4K). However, we noted that lamin A overexpression itself also appeared to impact YAP localization, with lamin A overexpressed cells yielding higher YAP ratios than those observed in similar volume in WT cells (Figures 4I and 4K, Figures S4F and S4H): lamin A overexpression also led to larger than expected changes in YAP ratios as a function of nuclear deformation (Figure 4L, Figures S4G and S4I). These data suggest that lamin A may play a role beyond that of modulating nuclear stiffness and may be directly related to YAP mechanosensing.

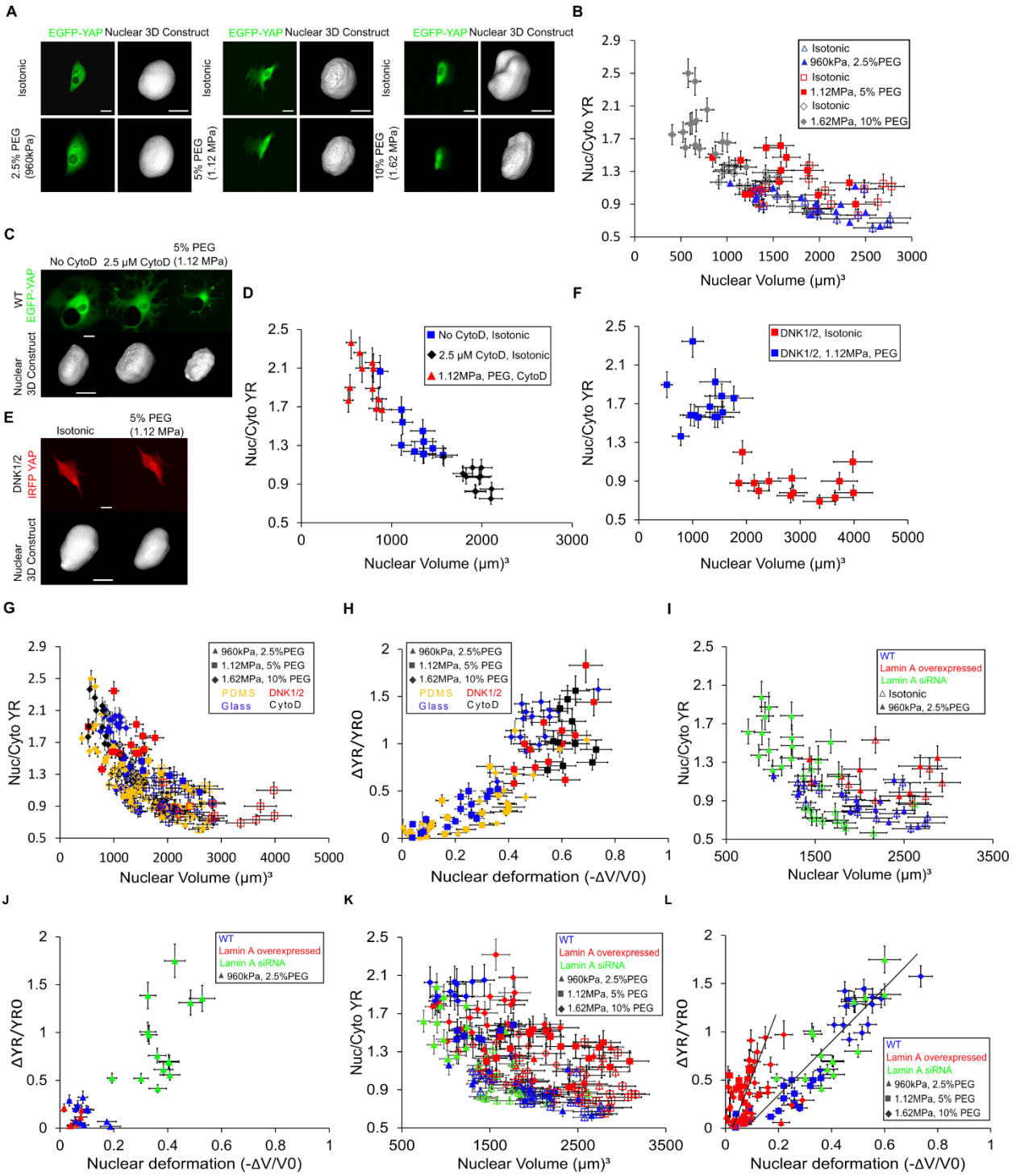

Figure 4. YAP translocation correlates with nuclear deformation independent of contractile work, actin filaments and LINC complex. A) Example of YAP localization and nuclear volume in EGFP-YAP transfected cells under different osmotic pressures, B) Quantification of YR vs nuclear volume in isotonic (open markers) and relevant hyperosmotic conditions (solid markers) (n>10 cells per each condition), C) Example of changes in nuclear volume and EGFP-YAP localization in CytoD treated cells after applying 5% PEG400 (1.12 MPa osmotic pressure), D)

YR vs nuclear volume in isotonic condition (blue squares), 30 minutes after adding 2.5 μM CytoD (black diamonds) and 20 minutes after adding 5% PEG400 (red triangle) (n>10 cells), E) Example of changes in iRFP-YAP localization and nuclear volume in GFPDNK1/2 transfected cells before and after adding 5% PEG400, F) Quantification of YR vs nuclear volume for GFP-DNK1/2 transfected cells before (red squares) and after (blue squares) adding 5% PEG400 (n>10 cells), G) All data of YR vs nuclear volumes for CytoD treated (black markers), DNK1/2 (red markers) transfected cells seeded on the glass and WT cells seeded on the glass (blue markers) and 300 Pa PDMS (yellow markers) under different hyperosmotic conditions. All open markers are representative of the cells in isotonic condition and solid markers are the same cells after osmotic compression (n>10 cells per each condition), H) Quantification of YR change ($\frac{\text{YR after adding PEG} - \text{initial YR}}{\text{initial YR}}$) as a function of nuclear volumetric deformation ($-\frac{\text{Nuclear volume after adding PEG} - \text{Initial nuclear volume}}{\text{Initial nuclear volume}}$) for the same values measured in (G), I) YR vs nuclear volume for WT, lamin A siRNA, and lamin A overexpressed cells before (open markers) and after applying 960 kPa osmotic pressure (solid markers) (n>10 cells per each condition), J) YR change vs nuclear deformation for the same condition as in (I), K) Quantification of YR vs nuclear volume for WT, lamin A siRNA, and lamin A overexpressed cells before (open markers) and after adding applying different osmotic pressures (solid markers) (n>10 cells per each condition), L) Quantification of YR change vs nuclear deformation for the same condition as in (K). Scale bars are 20 μm for the cells and 10 μm for the nuclei. Error bars indicate standard deviation (SD).

2.3.7 Lamin A redistributes from the nuclear membrane to nucleoplasm under nuclear deformation and directly regulates YAP localization

When we looked more closely at the data, we found that cells with larger contractility had more compressed nuclei with more YAP in the nucleus as previously established, however, we also noted that the lamin A distribution was impacted; cells with lower contractility had more lamin A in the nuclear membrane (nucleus 1 in Figures 5A, 5C and 5D), whereas cells with larger contractility had a more uniform distribution of lamin A in their nuclei and relatively less in the nuclear membrane (nucleus 2 in Figures 5B-5D). This apparent connection between lamin A localization and contractility prompted further TFM experiments; here we measured the normalized lamin A fluorescence in the nuclear membrane (Lm) (see method) as a function of cell contractile work, finding that strongly contractile cells have lower Lm values (Figure 5E), and smaller nuclear volumes (Figure S5A). Additionally, examining YAP localization, we found an inverse relationship between Lm and YR (Figure 5F). We postulated that if contractile forces change the nuclear volume, they may impact lamin A localization, which in turn might directly

regulate YAP localization. To test this idea, we quantified Lm as a function of varied nuclear volume by applying different osmotic compressions to WT, lamin A siRNA, and lamin A overexpressed cells. Similar to YAP activation, in lamin A siRNA cells lower force (960 kPa) was sufficient to redistribute lamin A from the nuclear membrane to the nucleoplasm (decrease in Lm) due to high nuclear deformability (Figure 5G). In WT and lamin A overexpressed cells, lamin A stayed intact in the nuclear membrane under the same pressure (Figure 5G), but higher force (1.12 MPa) triggered reduction of lamin A at the nuclear membrane (Lm) of WT and lamin A overexpressed cells (Figure S5C). Minimal Lm values were obtained under 1.62 MPa as a result of nuclear deformation (Figure S5G). These results were similar to force-mediated YAP activation in different cells with different nucleus' stiffnesses (Figures 4I and 4K, Figures S4F and S4H).

We found a conserved trend of decreased Lm as a function of decreasing nuclear volume (Figure 5H). However, we noted that the lamin A overexpressed cells deviate from the trend and display lower Lm values for a given volume than WT and lamin A siRNA cells. To further analyze if lamin A does indeed redistribute in response to nuclear deformation, we compared the change in Lm (ΔLm) with the amount of nuclear deformation under osmotic compression. We found a similarly conserved trend under 960 kPa pressure (Figure S5B), whereas under higher pressures we observed a deviation of the lamin A overexpressed cells from WT and lamin siRNA cells (Figure 5I, Figures S5D and S5H) that is reminiscent of our observations in nuclear deformation-mediated YAP activation (Figures 3K, 4K, 4L, and 5H). These deviations and similarity between nuclear deformation-induced lamin A redistribution and YAP activation cumulatively suggest that YAP is influenced by nuclear volume, but that the lamin A distribution may be a direct independent variable in regulating YAP.

To examine how the lamin A distribution regulates YAP, we plotted YR as a function of Lm, and we found a strong correlation under different osmotic conditions (Figures 5J, Figures S5E and 5SI). When we collated all data, we found a strikingly conserved relationship between lamin A localization and YR, independent of lamin A expression, osmotic pressure, or even nuclear volume (Figure 5K). In lamin A overexpressed and WT cells, under 960 kPa osmotic pressure Lm remains intact with negligible redistribution and YAP remains cytoplasmic (Figure 5M). However, under 1.62 MPa pressure membrane Lm is remarkably redistributed followed by YAP localization

(Figure 5N). These findings suggest that the lamin A distribution might be a central regulatory variable for YAP localization.

To further determine if lamin A redistribution is indeed driving YAP translocation, we quantified how changes in Lm due to nuclear compression impacts YAP activity, finding a strong correlation between lamin A redistribution (ΔLm) and YAP activity under diverse osmotic conditions (Figure 5L, Figures S5F and S5J). Collating all data of osmotic compression, we found a pronounced relationship between YR changes and lamin A redistribution for all examined experimental conditions (Figure 5O), suggesting that lamin A redistribution describes YAP nuclear localization independent of nuclear stiffness mediated by overall lamin A expression levels. These findings shed new light on the mechanosensing mechanism mediated by nuclear deformation and lamin A reorganization.

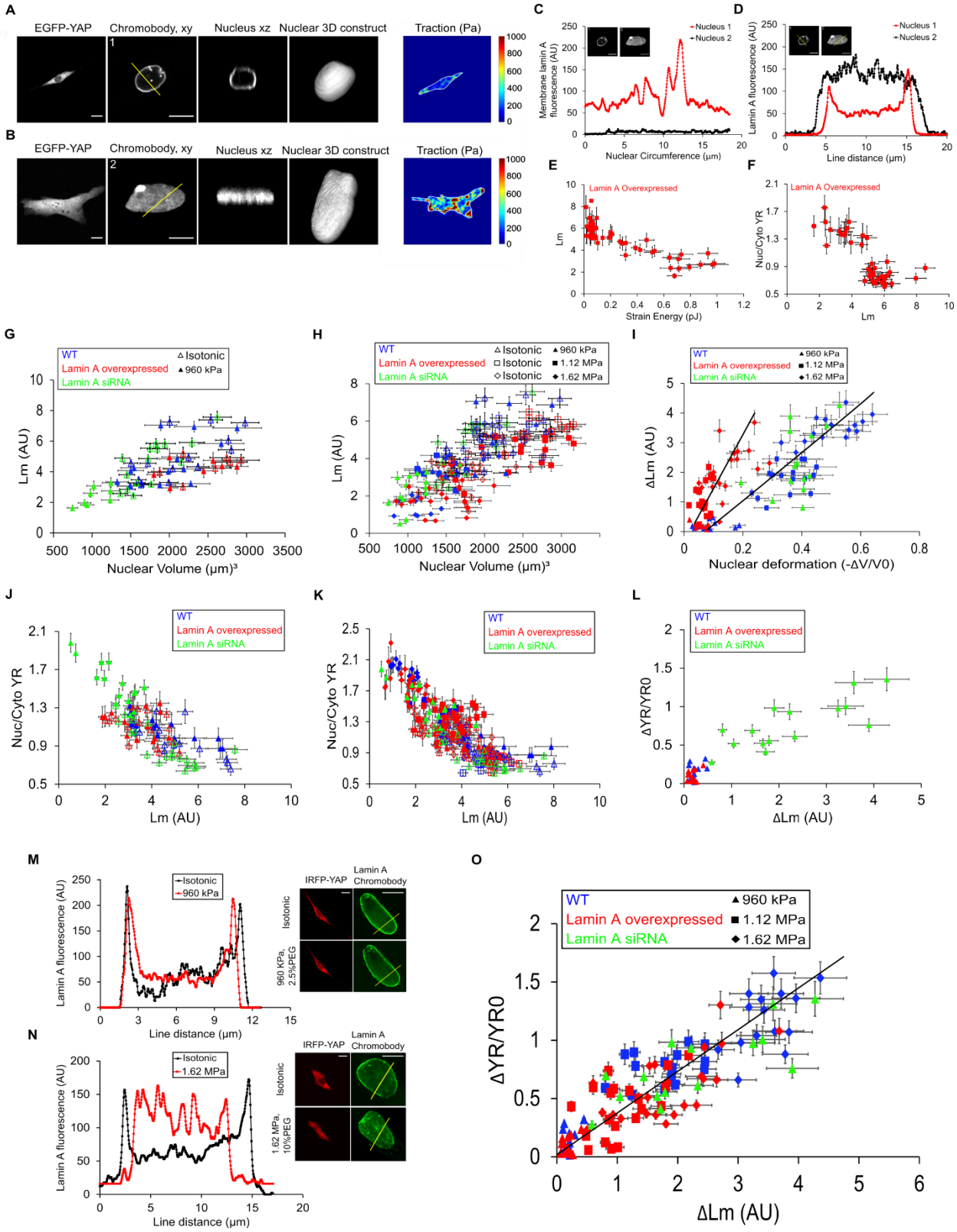

Figure 5. Lamin A redistributes from the nuclear membrane to nucleoplasm under deformation and directly regulates YAP localization. A) Example of YAP localization in low

contractile lamin A overexpressed cell with high nuclear volume and high lamin A localized in the nuclear membrane, B) Example of YAP nuclear localization in high contractile lamin A overexpressed cell with more flattened nucleus and even distribution of lamin A throughout the nucleus, C) Quantification of total lamin A fluorescence at the nuclear membrane for nucleus 1 (red line) and nucleus 2 (black line) shown in (A) and (B), D) Lamin A fluorescence along a chord crossing nucleus 1 and 2 in (A) and (B), E) Lm for lamin A overexpressed cells vs Strain Energy (n>15 cells), F) YR vs Lm for the same cells as in (E), G) Lm vs nuclear volume for WT, lamin A siRNA, and lamin A overexpressed cells before (open markers) and after (solid markers) applying 960 kPa pressure (n>10 cells per each condition), H) Collating all data of Lm vs nuclear volume for WT, lamin A siRNA and lamin A overexpressed cells under different experimental conditions (n>15 cells per each condition), I) Collating all data of Lm change ($\text{initial Lm} - \text{Lm after adding PEG}$) as a function of nuclear deformation for the same cells as in (H), J) YR vs Lm for WT, lamin A siRNA, and lamin A overexpressed cells before (open markers) and after applying 960 kPa pressure (solid markers) (n>10 cells per each condition), K) Collating all data of YR vs Lm before (open markers) and after (solid markers) adding different concentrations of PEG for the same cells as in (H) (n>10 cells per each condition), L) YR change vs Lm change for WT, lamin A overexpressed and, lamin A siRNA cells under 960 kPa pressure for the same cells as in (G) (n>10 cells), M) Representative of YAP localization and lamin A fluorescence along a chord crossing nucleus of an example cell before (black line) and after (red line) applying 960 kPa pressure, N) YAP localization and lamin A fluorescence along a chord crossing nucleus of another cell before (black line) and after (red line) applying 1.62 MPa pressure, O) Collating all data of YR changes vs Lm changes (ΔLm) for WT, lamin A siRNA, and lamin A overexpressed cells under same conditions as in (K) (n>15 cell per each condition). Scale bars show 10 μm for the nuclei and 20 μm for the cells. Error bars indicate standard deviation (SD).

2.4 Discussion

Diverse studies have examined how YAP activity is impacted by mechanical stimuli ranging from substrate stiffness to applied forces [79,88,90]. Here we demonstrated how contractility modulates nuclear deformation and revealed a direct relationship between YAP mechanosensation and lamin A redistribution. Furthermore, we showed that in contrast to previous studies [84,88,90,109], substrate stiffness does not determine YAP activity on PAA or PDMS surfaces. We showed that only in very small rounded cells is YAP principally cytoplasmic (Figure 1G), whereas in spread cells YAP is dynamically distributed between the cytoplasm and the nucleus, independent of substrate stiffness (Figures 1E and 1G). Examining cell contractility, we found that YAP activity appeared correlated with work regardless of substrate stiffness. Furthermore, we showed that contractility varies during cell movement, that YAP activity and contractility are highly correlated temporally,

leading to dynamic localization of YAP in single cells independent of substrate moduli (Figures 2B and 2D).

We measured that contractile work compresses the nucleus in a LINC complex-mediated way. This nuclear compression in turn regulates YAP localization, which is consistent with the idea of nuclear pore complex opening mediated by nuclear tension [90] (Figures 3B-3E). We also varied nuclear stiffness by modulating lamin A expression levels, which changes the amount of nuclear compression under applied physical forces. We found that for a given cell contractility, cells with stiffer nuclei had lower nuclear compression, larger nuclear volume, and lower YRs compared to those with softer nuclei (Figures 3I-3K). These findings suggest that nuclear deformation specifically rather than applied stress mediates YAP localization. Moreover, our results are consistent with previous reports of lamin A overexpression and nuclear stiffening decreasing YAP nuclear transport [17,90,115]. However, we observed unexpectedly higher YAP nuclear localization in larger nuclear volumes in lamin A overexpressed cells (Figures 4K and 4L), leading us to examine the interplay between YAP localization and the lamin A distribution.

We found that lamin A localization is mechanosensitive, and that either contractile work or osmotic force-induced nuclear deformation causes lamin A to delocalize from the nuclear membrane and to enter the nuclear interior. Recent studies also observed varied localization of A-type lamins with some at the nuclear periphery and some in the nuclear interior depending on cell cycle, differentiation and mechanical cues[17,66,127,128]; however, the pathways involved in nucleoplasmic lamin A regulation and the role of intranuclear lamin A in mechanosensing and transcription activation have remained open questions. While the molecular mechanisms behind lamin A disassociation from the nuclear membrane remains unclear, we speculate that this may be related to the local nuclear membrane curvature, and that under high bending curvature that lamin A may delaminate from the nuclear membrane (Figure 6). While nuclear membrane tension has been implicated as a mechanism for YAP regulation, previous work failed to stimulate YAP nuclear localization after applying hypoosmotic solutions that swell nuclei and place the nuclear membrane under tension [90]; this suggests that nuclear tension alone may not completely describe YAP nuclear localization. Our description of mechanosensitive lamin A redistribution under compression would potentially explain why YAP activation only occurs under nuclear flattening but not under nuclear swelling.

Quantifying this redistribution of lamin A from nuclear membrane to nuclear interior, we found that it matched the YAP redistribution in the cell, independent of all other experimental conditions (Figures 5K and 5O). The mechanistic role of lamin A in YAP transport remains unclear, however, it is likely that lamin A interacts with nuclear pore complexes (NPCs). Many studies have reported that lamin A plays a role in regulating the NPC distribution during the cell cycle, and does so in a differentiation dependent way [73,74]. Indeed, an inverse correlation between lamin A distribution and NPC density has been observed in the nuclear membrane [74], suggesting a potential relationship between lamin A localization and YAP translocation, which is consistent with our delamination perspective. Moreover, some transcriptionally active euchromatin regions are associated with nucleoplasmic lamin A [129,130], suggesting that a mechanosensitive redistribution of lamin A may directly impact gene expression and transcription activation.

Our study suggests that nuclear deformation-mediated lamin A reorganization may be a main non-Hippo-dependent YAP regulatory mechanism. This novel mechanism incorporates previously identified mechanical stimuli of YAP regulation, including substrate stiffness, cell contractility, nuclear deformation and nuclear mechanics. This is also consistent with previous reports of lamin A reduction being related to increased YAP activity in cancer progression [82,131,132]. We anticipate this link between nuclear stiffness, nuclear deformation, lamin A, and YAP activity will offer new insight and therapeutic strategies for other diverse diseases associated with modified nuclear mechanics and cellular dysfunction, including aging disorders [131], Emery-Dreifuss Muscular Dystrophy [57], and Hutchinson-Gilford progeria syndrome [60]; A clearer understanding of mechanical YAP regulation may also provide better strategies for directing stem cell engineering and homeostasis. Our proposed interplay between YAP, nuclear volume, and nuclear deformation are consistent with previous reports of cell compression [103,131,133] and high cell contractility [17,66] mediating osteogenic differentiation as a result of YAP activation.

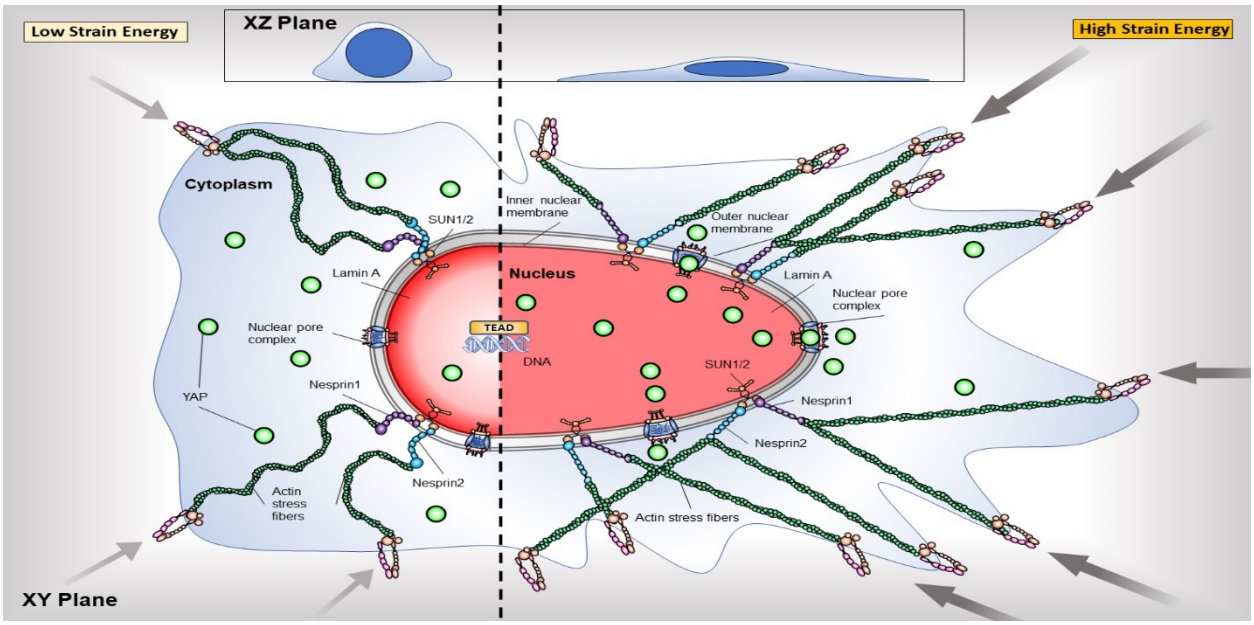

Figure 6. Proposed model regulating YAP nuclear localization. Right part of proposed model is representative of high tensional state of the nucleus with stretched nuclear pores and evenly distributed lamin A (red color) throughout the nucleus, inducing YAP nuclear localization. Left part of the proposed model is representative of lower tension state and less deformed nucleus with lamin A accumulated in the nuclear periphery (darker red) reducing YAP nuclear localization.

2.5 Supplementary materials

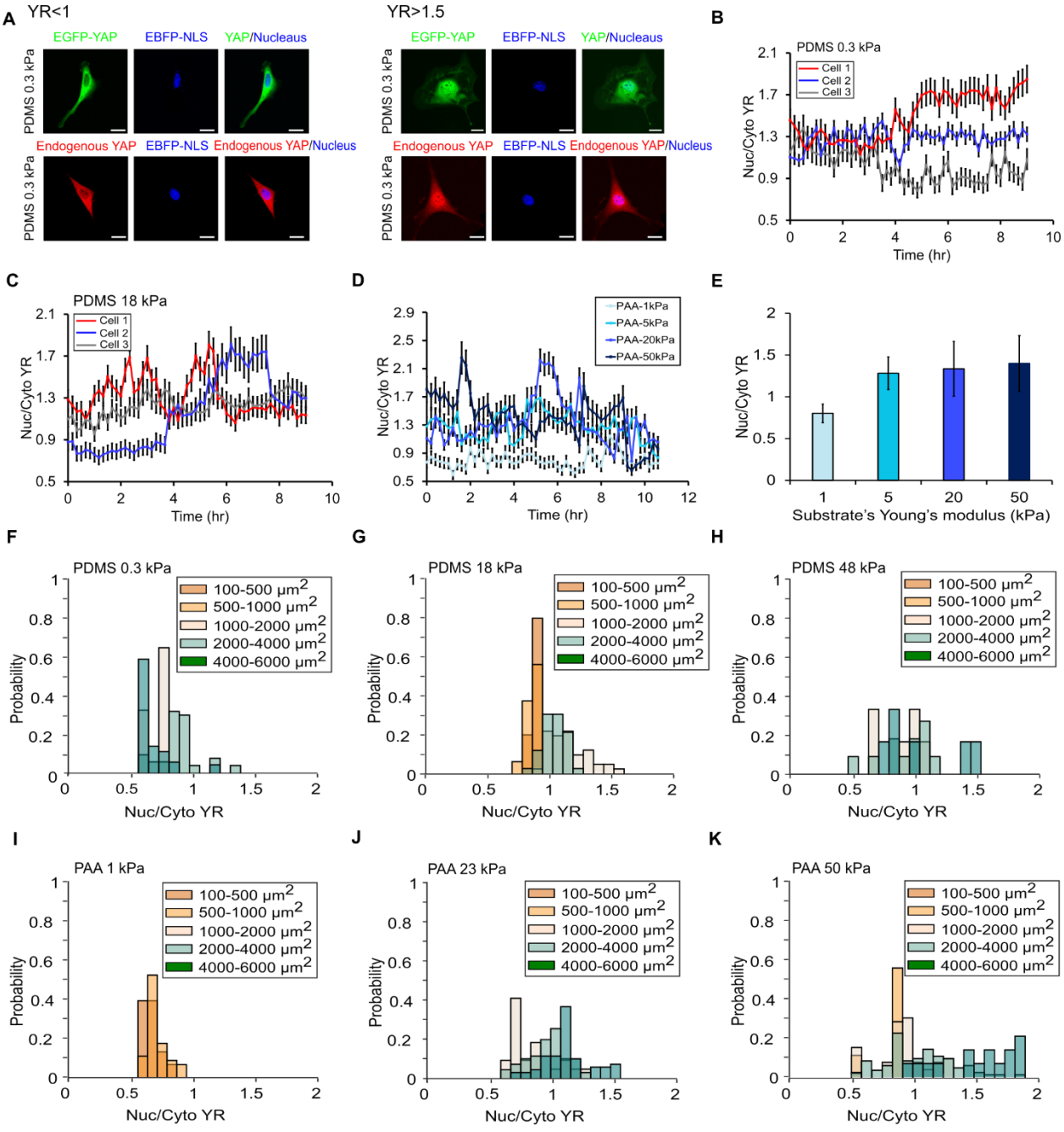

Figure S1. YAP localization is dynamic overtime and independent of substrate stiffness and cell spread area. A) Example image of different EGFP-YAP and endogenous YAP localization in NIH 3T3 cells labeled with EBFP-NLS nucleus on soft PDMS with Young's modulus of 0.3 kPa, B) Quantification of YAP localization variation overtime in 3 different EGP-YAP transfected cells moving on soft PDMS with Young's modulus of 0.3 kPa, and C) Stiff PDMS with Young's

modulus of 18 kPa, D) Quantification of YAP ratio variation in different EGFP-YAP transfected cells during cell movement on PAA substrates with various stiffnesses, E) Time average of YAP ratio on different PAA substrates for the same conditions as in (D), F) YAP ratio distribution based on cell spread area for different cells seeded on PDMS substrates with Young's modulus of 0.3 kPa, G) 18 kPa, and H) 48 kPa. Different colors are representative of different range of cell spread area (n>10 per each condition), I) YAP ratio distribution based on cell spread area for different cells seeded on PAA substrates with Young's modulus of 1 kPa, J) 23 kPa and K) 50 kPa. Different colors are representative of different range of cell spreading area (n>15 per each condition). Scale bars are 20 μm. Error bars indicate standard deviation (SD).

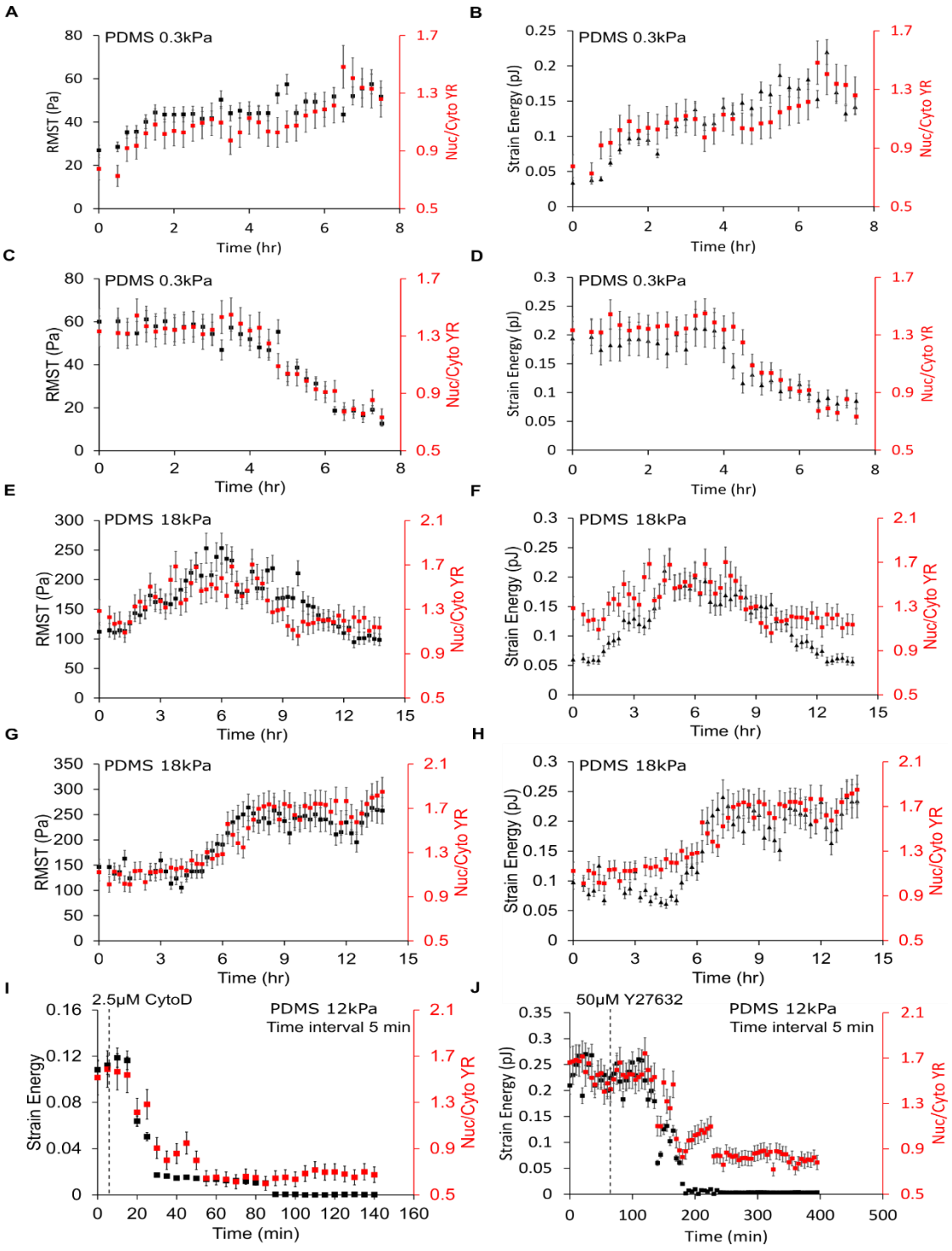

Figure S2. YR is correlated with cell contractility, best with Strain energy. A) Example of quantified RMST and EGFP-YAP ratio during cell movement on PDMS substrate with Young's modulus of 0.3 kPa, B) Quantification of Strain Energy and YAP ratio for the same cell in the same condition as in (A), C) Quantification of RMST and YR for a different cell seeded on PDMS with the same stiffness, D) Strain Energy and YR measured for the same cell in the same condition

as in (C), E) Example of tracking RMST and EGFP-YAP localization at the same time during cell movement on PDMS with stiffness of 18 kPa, F) Quantification of Strain Energy and YR for the same cell in the same condition as in (E), G) Another example of tight correlation between YAP ratio and RMST for a different cell seeded on PDMS with stiffness of 18 kPa, H) Strain Energy and YR measured for the same cell in the same condition as in (G), I) An example of YAP cytoplasmic localization as a result of losing contractility due to actin filament depolymerization about 15 minutes after CytoD treatment of EGFP-YAP transfected cells seeded on PDMS with Young's modulus of 12 kPa, J) An example of decrease in YAP ratio due to actomyosin inhibition about 50 minutes after adding ROCK inhibitor to the EGFP-YAP transfected cells seeded on PDMS with the same stiffness. Error bars indicate standard deviation (SD).

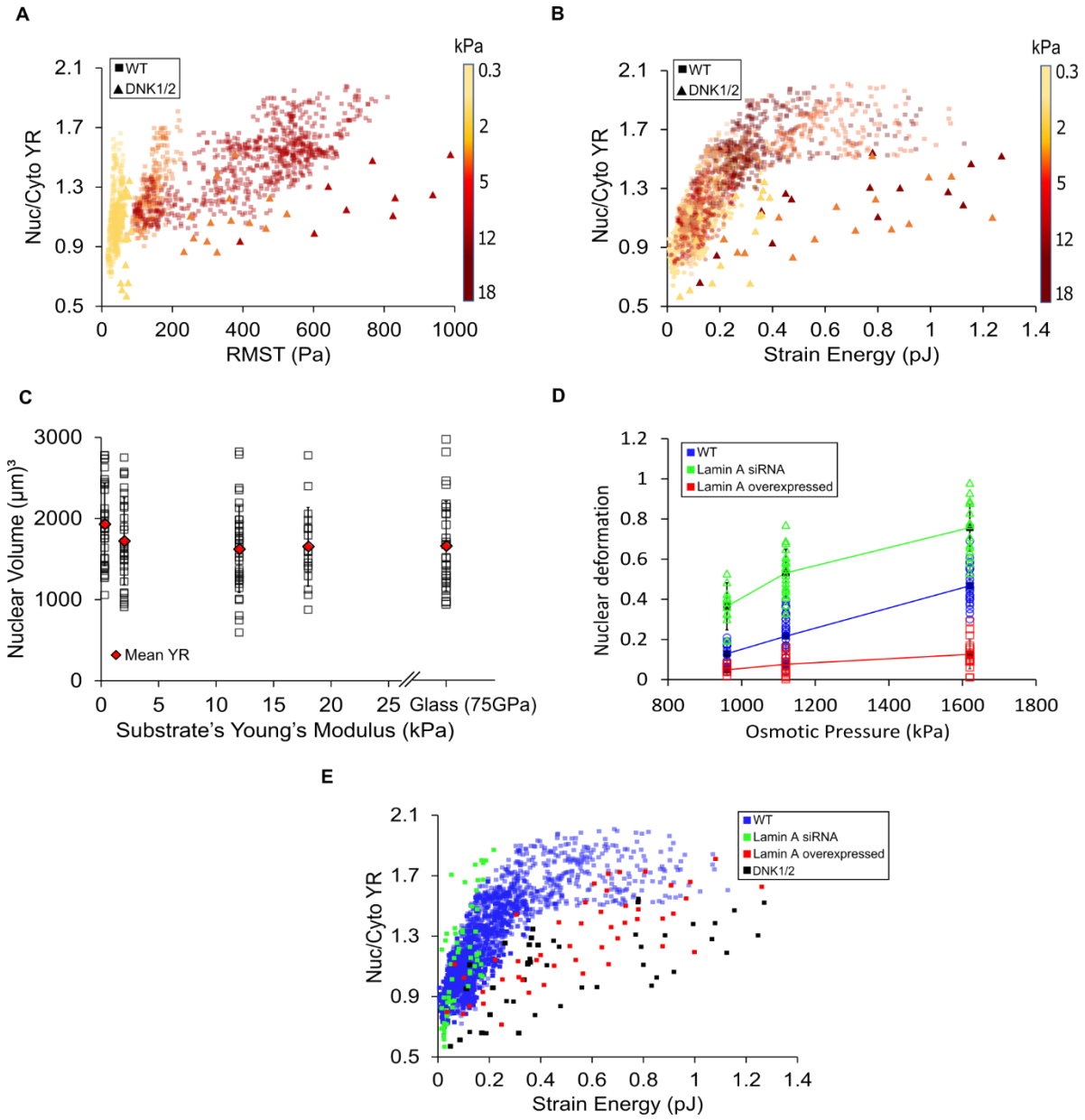

Figure S3. Strain energy-mediated nuclear deformation and YAP activity is directed by LINC complex and nuclear mechanics. A) Comparing RMST and YAP ratio for WT (squares) and DNK1/2 (triangles) cells seeded on PDMS substrates with different stiffnesses. Color bar is representative of substrate stiffness (n>15 per each condition), B) Quantification of YAP ratio and Strain Energy for the same cells in the same conditions as in (A), C) Quantification of nuclear volume of different cells seeded on divers PDMS substrates and glass (n>15 per each condition). Diamonds are representative of mean nuclear volume of the cells on each stiffness, D) Quantification of nuclear volumetric deformation (-ΔV/V0) under different osmotic pressures applied by PEG400 to the WT, siRNA lamin A and lamin A overexpressed cells, E) All the data of Strain Energy as a function of YR for WT (blue markers), lamin A siRNA (green markers),

lamin A overexpressed (red markers) and DNK1/2 cells (black markers) (n>15 per each condition). Error bars indicate standard deviation (SD).

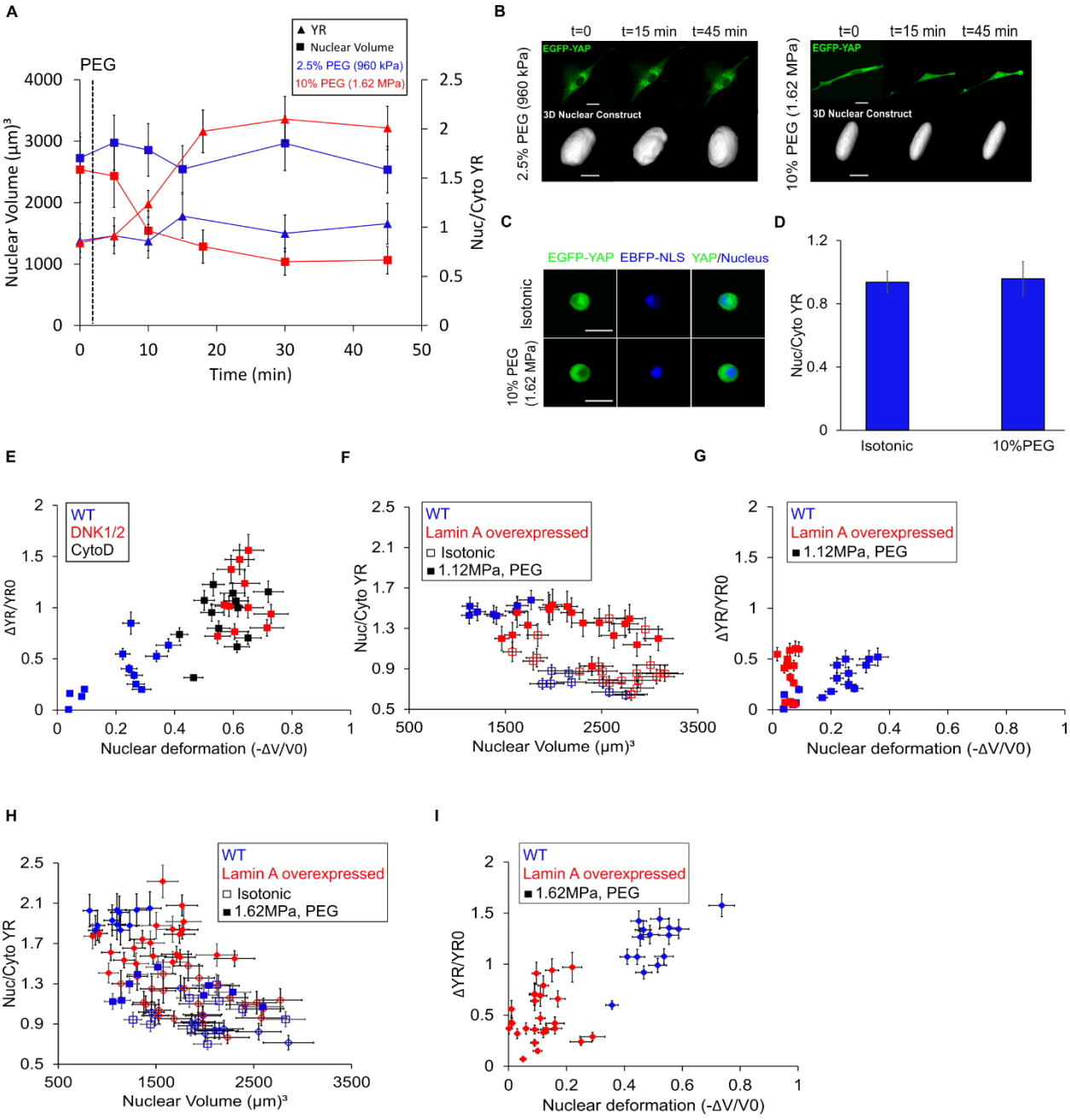

Figure S4. Nuclear deformability induced by lamin A expression level directly regulates required force to activate YAP. A) Example of tracking nuclear volume and EGFP-YAP localization overtime in 2 different cells before and after adding 2.5% (blue markers) and 10% PEG (red markers), B) Example image of changes in nuclear volume and EGFP-YAP localization over time under different hyperosmotic conditions (2.5% and 10% PEG). Scale bars show 20 μm

for cells and 10 μm for nuclei, C) Example image of changes in YAP nuclear localization in suspended cells transfected with EGFP-YAP. Scale bars show 20 μm, D) Quantification of mean YR values of different suspended cells before and after adding 10% PEG (n>15 cells per each condition), E) Quantification of differential YAP ratio as a function of nuclear deformation for WT (blue markers), DNK1/2 (red markers), and CytoD treated (black markers) cells under 5% PEG (1.12 MPa osmotic pressure) (n>10 cells per each condition, F) Quantification of YAP ratio as a function of nuclear volume for WT (blue markers) and lamin A overexpressed (red markers) cells before (open markers) and after (solid markers) adding 5% PEG (1.12 MPa osmotic pressure) (n>10 cell per each condition), G) Measuring differential YAP ratio as a function of nuclear deformation for the same cells in the same conditions as in (F), H) Quantification of YR as a function of nuclear volume for WT (blue markers) and lamin A overexpressed (red markers) cells before (open markers) and after (solid markers) adding 10% PEG (1.62 MPa osmotic pressure) (n>10 cells per each condition), I) Quantification of differential YAP ratio as a function nuclear volumetric deformation for the same cells in the same conditions as in (H). Error bars indicate standard deviation (SD).

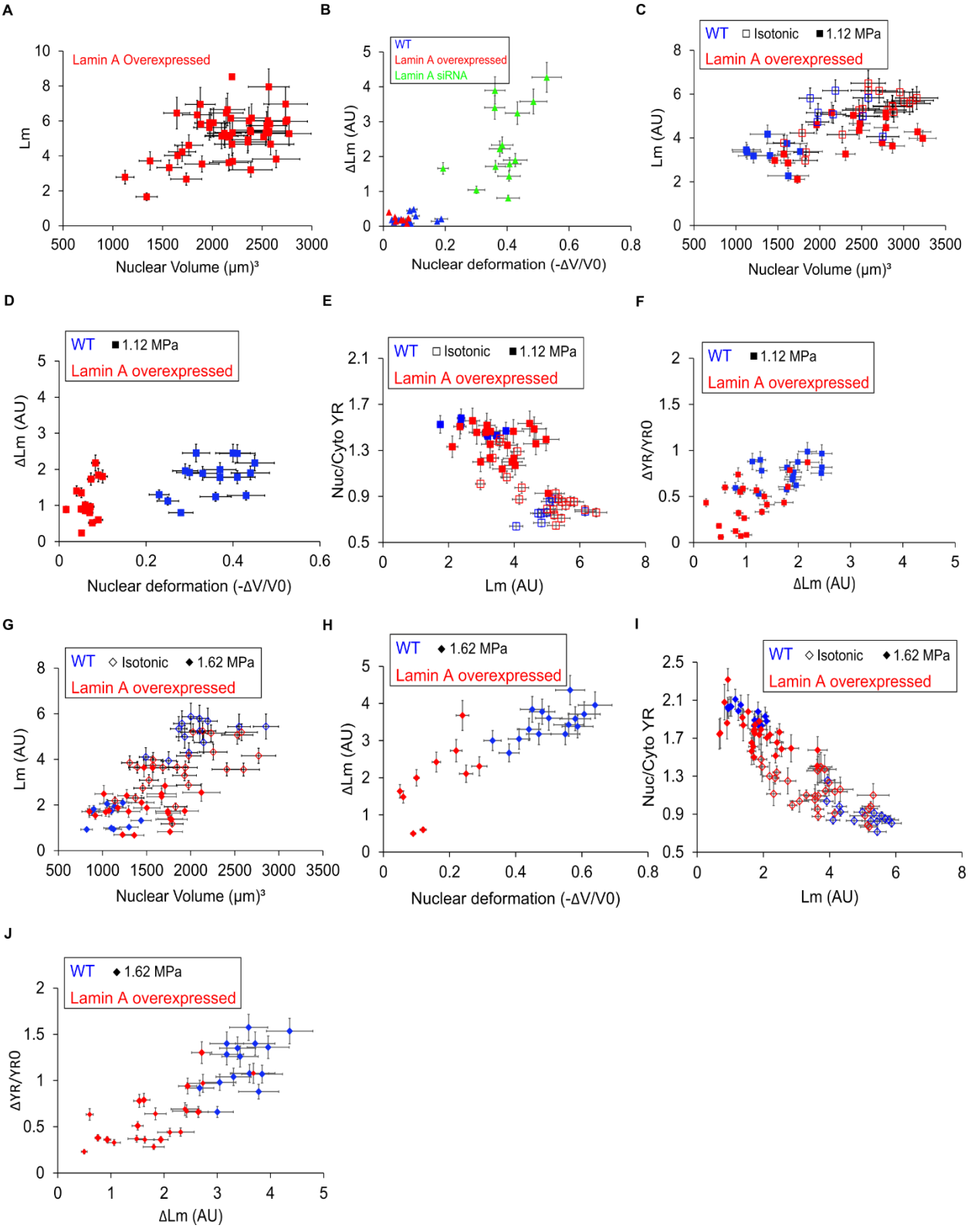

Figure S5. Lamin A redistributes from the nuclear membrane to nucleoplasm under deformation and directly regulates YAP localization. A) Quantification of nuclear membrane

lamin A as a function of nuclear volume for lamin A overexpressed cells (n>15 cells), B) Quantification of lamin A redistribution ($Initial\ Lm\ before\ PEG - Lm\ after\ adding\ PEG$) as a function of nuclear deformation for WT (blue), lamin A siRNA (green) and lamin A overexpressed (red) cells under 2.5% PEG (n>10 cells per each condition), C) Quantification of membrane lamin A vs nuclear volume for WT (blue markers) and lamin A overexpressed (red markers) cells before (open markers) and after applying 5% PEG (1.12 MPa osmotic pressure, solid markers) (n>10 per each condition), D) Quantification of change in membrane lamin A as a function of nuclear deformation for the same cells under the same condition as in (C), E) Quantification of YR as a function of Lm for the same cells before (open markers) and after (solid markers) adding 5% PEG, F) Quantification of change in YAP ratio as a function of change in membrane lamin A distribution for the same cells under the same pressure as in (C), G) Assessment of membrane lamin A vs nuclear volume for WT (blue markers) and lamin A overexpressed (red markers) cells before (open markers) and after applying 10% PEG (1.62 MPa osmotic pressure, solid markers) (n>15 per each condition), H) Quantification of lamin A redistribution as a function of nuclear deformation for the same cells under the same condition as in (G), I) Quantification of YR as a function of Lm for the same cells before (open markers) and after (solid markers) adding 10% PEG, J) Quantification of change in YAP ratio as a function of change in lamin A distribution for the same cells under the same pressure. Error bars indicate standard deviation (SD).

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2.7 Author contributions

Conceptualization, A.J.E., N.K.; Methodology- development, L.K.S., H.Y.; Methodology-application, N.K.; Plasmid purifications and sequencing, A.G., C.S., L.K.S.; Investigation, N.K.; Writing- original draft, N.K.; Writing- review and editing, N.K., A.J.E., L.K.S., C.S., A.G., C.M.; Funding acquisition-A.J.E.; Resources, A.J.E; Supervision, A.J.E.

2.8 Conflict of interest statement

The authors declare that no conflict of interests exists.

2.9 Materials and Methods

2.9.1 Fabrication of PDMS substrates

To determine the effects of substrate rigidity on dynamic localization of EGFP-YAP protein, polydimethylsiloxane (PDMS) substrates with different stiffnesses were prepared as described previously [120,121]. In brief, PDMS solutions were supplied by mixing same weight ratio of component A and B of commercial PDMS (NuSil® 8100, NuSil Silicone Technologies, Carpinteria, CA) with different concentrations of Sylgard 184 PDMS crosslinking agent (Dimethyl, methylhydrogen siloxane, which contains methyl terminated silicon hydride units) to obtain substrates with various stiffnesses (Table 1). Then, 170 µl of each solution was applied to the clean 24*24 mm glass coverslips and cured at 100 °C for two hours. For traction force microscopy, prepared PDMS substrates were coated with 1 µm thick layer of fiduciary particles using spin coater (Laurell Technologies, WS-650 Spin Processor) and incubated at 100 °C for an hour.

Additional crosslinker concentration (weight %)	Young's modulus (kPa)
0	0.3 ± 0.05
0.1	2 ± 0.06
0.2	5 ± 0.05
0.36	12 ± 0.71
0.45	18 ± 0.68
1.8	100 ± 2.8

Table 1. Young's moduli of different PDMS substrates containing different concentrations of Sylgard 184 crosslink agent.

2.9.2 Polyacrylamide Fabrication

Polyacrylamide (PAA) gels were prepared based on the previously described protocol [134]. PAA gel solutions were prepared with varying concentrations of acrylamide and bis-acrylamide mixed with ammonium per sulfate (APS), TEMED and fluorescent fiduciary beads for traction microscopy (Table 1). Acrylamide and bisacrylamide solutions were mixed and degassed for 15-20 minutes under fume hood. Further APS and TEMED were added to the gel solutions and mixed by pipetting. The final solution was added onto the hydrophobic glass slide (treated with RainX)

and a coverslip was placed gently on top of the gel drop. After polymerization, the gel sandwiches were placed inside MiliQ water bath and then the glass slides were gently removed.

(kPa)	Acrylamide %	BIS %	Type	Acrylamide (ul)	BIS (ul)	PBS (ul)	APS (ul)	TEMED (ul)	Beads (ul)
1.08	3	0.1	AFM	75	50	835	10	10	20
5.01	4	0.3	AFM	100	150	710	10	10	20
9.69	8	0.1	AFM	200	50	710	10	10	20
15.28	8	0.15	AFM	200	75	685	10	10	20
20.85	8	0.225	AFM	200	112.5	647.5	10	10	20
23.84	8	0.3	AFM	200	150	610	10	10	20
40.40	8	0.48	AFM	200	240	520	10	10	20

Table 2. Young's moduli of different PAA hydrogels containing different concentrations of acrylamide, Bis, APS and TEMED.

2.9.3 Surface modification

In order to covalently bind fibronectin to PDMS or PAA substrates, Sulfo-SANPAH (ThermoFisher Scientific) solution dissolved in 100 mM HEPES was added on top of the substrates and they were exposed to UV for 2 minutes. After UV activation, Sulfo-SANPAH solutions were removed and 5 µg/ml fibronectin (Sigma) solution diluted in PBS was added on to of the samples, followed by incubation in room temperature for 9-12 hours. Finally, fibronectin solutions were removed, and substrates were rinsed with PBS 3 times. After UV sterilization of coated substrates, trypsinized cells were seeded on top of the samples and they were allowed to adhere overnight.

2.9.4 Cell culture

NIH-3T3 *Mus musculus*, mouse cell line was obtained from ATCC and cultured in Dulbecco's modified Eagle medium (DMEM) (Wisent) supplemented with 10% fetal bovine serum (FBS) (Wisent) and 1% Penicillin-Streptomycin antibiotic (P/S) (Thermo Fisher). The cells were

incubated at 37 °C in 5% CO_2 environment and, they were allowed to grow on the substrates for 18 hours before imaging.

2.9.5 Transfection and Confocal microscopy of live cells

To quantitatively track EGFP-YAP mechanotransduction with time**,** we transiently transfected NIH-3T3 cells with 2 plasmids, pEGFP-yap-C3-hYAP1 (Addgene, plasmid #17843) [135] and EBFP2-Nucleus-7 (nuclear localization signal, Addgene, plasmid #55249), using GenJet transfection reagent (Signagen). 18-24 hours later, cells were seed on fibronectin-coated PDMS, PAA and glass substrates and after cell attachment, they were transferred to a lab-built heated stage perfused with 5% CO_2 and mounted on a confocal microscope (Leica TCS SP8 with a 10x objective). With this setup, we could image cells with transmission and fluorescence microscopy for extended periods, while maintaining a controlled culture environment.

In order to examine the effects of LINC complex on contractile and force-mediated nuclear deformation and YAP translocation, we transfected the cells with two dominant-negative GFP-Nesprin1-KASH and GFP-Nesprin2-KASH (DNK1/2) plasmids which were kindly provided by Dr. Catherin Shanahan's laboratory (King's College, London) [124]. Previously it has been shown that overexpression of dominant-negative Nesprin-KASH disrupts interaction between nesprins and SUN proteins at nuclear envelop by nonspecific binding to endogenous SUN proteins resulting in mislocalization of nuclear nesprins and disruption of LINC complex [90,124]. To specify YAP localization, at the same time we transfected the cells with iRFP-YAP which was a gift from Xavier Trepat (Institute for bioengineering of Catalonia (IBEC), Barcelona) and EBFP-Nucleus to avoid any crosstalk between GFP-DNK1/2 and YAP and 18 hours after transfection we seeded the cells on PDMS substrates coated with fluorescent beads followed by incubation at 37 °C for 12 hours. Finally, alive transfected cells seeded on PDMS traction substrates were imaged overtime using confocal Leica SP8 with 10x objective. To measure traction stress and strain energy at the same time, fluorescent beads coating PDMS and PAA substrates also were imaged along with EGFP-YAP transfected cells and EBFP-Nucleus.

To quantify YAP nuclear to cytoplasmic ratio, image segmentation was performed using MATLAB code for every time frame to measure the ratio of EGFP-YAP fluorescence inside the

nucleus to EGFP-YAP fluorescence in the cytoplasm during cell movement on PDMS, PAA and glass substrates.

2.9.6 Immunostaining

To compare endogenous YAP localization with EGFP-YAP, we fixed the cells with 4% paraformaldehyde for 15 min in room temperature and washed 3 times with PBS. We stained the nuclei with 0.5 µl/ml bisBenzimide H 33342 trihydrochloride (Sigma) and after 20 minutes, cells were washed with PBS. The cells were permeabilized with 0.1% Triton X-100 diluted in PBS for 10 minutes. To avoid any nonspecific hydrophobic binding, 2% bovine serum albumin (BSA) was added to the cells and incubated for 30 minutes in room temperature. After washing with PBS, we made solution of 10 µg/ml of YAP mouse monoclonal antibody (sc101199, Santa Cruz) and donkey Anti-Mouse IgG H&L (Alexa Fluor® 647) secondary antibody (ab150107, abcam) in BSC separately. The cells were separately incubated first with primary antibody and then with the secondary antibody for an hour in room temperature. Fluorescence images were acquired with a Leica SP8 confocal microscope and endogenous YAP ratio values were measured using image segmentation and quantifying the ratio of YAP intensity inside the nucleus to YAP intensity in cytoplasm, and then the values were compared with YR values obtained from EGFP-YAP transfected cell.

2.9.7 Cell spread area

To examine the effects of cell spread area, we started confocal imaging 10 minutes after seeding the EGFP-YAP and EBFP-Nucleus transfected cells on fibronectin-coated PDMS substrates and continued imaging for 18 hours using confocal Leica SP8 with low magnification (x10 objective). Then we quantified YAP ratio and cell projected area for every time frame acquired by confocal microscope. The projected cell area of the cells was determined with Fiji software.

2.9.8 Traction Force Microscopy

Active contractile stress in actin cytoskeleton were quantified using Traction Force Microscopy (TFM) as previously described [121]. In brief, EGFP-YAP transfected NIH 3T3 cells were cultured on fibronectin coated compliant PDMS substrates of known moduli and a thin PDMS layer of embedded fiduciary fluorescent particles was spin coated on the top surface. After 12 hours incubation at 37 °C, EGFP-YAP transfected cells and fluorescent particles were imaged simultaneously over time using Leica TCS SP8 confocal microscope with low magnification

(x10/NA 0.4 air objective) at a resolution of 0.28 μm/pixel. The reference images of the particles were acquired at the end of the experiment by detaching the cells from the substrate surface.
Cell-substrate traction stresses and strain energies were calculated for each acquired time frame as described previously [123]. Briefly, local displacements of the fiduciary particles were calculated by comparing the particle positions with cells on the substrate and reference particle positions without cells. From the particle displacement and known Young's moduli of the PDMS substrate, cellular contractile stresses and strain energies were calculated. Quantifying YAP ratio based of above EGFP-YAP and EBFP-Nucleus images for the relevant time frames, we could measure YAP localization for every contractile state of the cells on PDMS substrates with different stiffnesses.

2.9.9 Pharmacological treatments

To further quantify the effects of contractile stress and work (strain energy) on YAP subcellular translocation, we started imaging of the EGFP-YAP transfected cells and fluorescent particles on top of PDMS substrates with different stiffnesses before any treatment and then we added 1.5 μM Cytochalasin D (CytoD, ThermoFisher Scientific) to the cells which depolymerize actin filaments and decrease contractility. We then continued imaging for another 2 hours after adding CytoD followed by killing the cells at the end of the experiment for the reference images required for traction stress analysis. Quantifying YAP ratio and relevant traction stress as well as strain energy for each time frame before and after actin filaments depolymerization enable us to track changes in YAP localization in real time during losing cells' contractilities in real time. Moreover, we applied 50 μM ROCK inhibitor (Y27632, abcam) to nontreated EGFP-YAP transfected cells seeded on different PDMS substrates to investigate how inhibition of actomyosin activity affects dynamic movements of YAP in alive single cells. We continued imaging for 6 hours after ROCK inhibitor treatment and at the end of experiment the cells were detached for traction analysis.

2.9.10 Quantification and modulating of lamin A expression

To quantify the total lamin A present in the nuclei, all the cells were transfected with GFP tagged lamin A Chromobody (Chromtek), which facilitates real-time imaging of lamin A by labeling the total lamin A without interfering with its function and distribution in the nuclei [125,136]. As previously it has been verified that lamin A Chromobody is the accurate quantitative metric of lamin A expression and distribution [136], we transfected NIH 3T3 cells with GFP-Chromobody to quantify lamin A expression and distribution. To modulate lamin A expression in GFP-

Chromobody transfected NIH 3T3 cells, we overexpressed lamin A by transiently transfecting the intact cells with m-Cherry tagged plasmid DNA for lamin A, which was a gift from Michael Davison (Addgene, plasmid # 55068). To surpass lamin A expression, we transfected the cells with RFP tagged inducible shRNA construct for lamin A (Dharmacon) by adding 0.5 µg/ml doxycycline to WT cells transfected with GFP tagged lamin A chromobody [137], followed by incubation at 37 °C for 72 hours.

After modulating lamin A expression, cells were seeded on the fibronectin-coated glasses and after 24 hours incubation they were imaged using confocal Leica SP8 microscope with x63/1.4 NA oil immersion objective. Lamin A expression level in different cells was assessed by identifying a mask covering the whole nucleus and then quantifying total GFP-Chromobody's fluorescence in the nuclear mask using MATLAB code.

In order to quantify normalized lamin A intensity localized in the nuclear membrane (Lm), we identified two separate masks that covered only the nuclear membrane and the nuclear interior. The ratio of GFP-Chromobody's fluorescence in the specified nuclear membrane mask to the GFP-Chromobody's fluorescence in the nuclear interior mask was quantified and referred to as Lm.

To study the effects of lamin A expression and distribution on YAP localization, all the cells were transfected with iRFP YAP as well as EBFP- Nucleus at the same time when we were transfecting GFP-Chromobody and modulating lamin A expression. 18 hours after transfection and 72 hours after doxycycline treatment cells were seeded on fibronectin-coated glass and PDMS traction substrates, followed by 24 hours incubation at 37 °C and 5% CO_2 environment. To quantify YAP ratio, total lamin A expression, lamin A localized in the nuclear membrane and contractility, iRFP-YAP, EBFP-Nucleus, GFP-Chromobody and fluorescent beads were imaged by confocal microscope with 63X/1.4 NA oil immersion objective.

2.9.11 3-D volume measurement of nuclei

In order to measure nuclear volume and nuclear deformation, XYZ stacks of EBFP tagged nuclei with a z-step size of 0.5 µm were imaged using Leica SP8 confocal microscope with x63/1.4NA oil immersion objective lens. The 3D visualization and quantification were performed by Fiji software. To quantify nuclear volume, the stacks were thresholded based on the top and bottom of the nuclei and then the number of voxels of the threshold region was counted and multiplied by the size of each voxel using Fiji software. To investigate the role of cell contractility in nuclear

deformation and YAP localization in WT, lamin A overexpressed and lamin A siRNA cells, we employed z stacks imaging of the EBFP tagged nuclei of the single cells seeded on PDMS substrates coated with fluorescent particles at the same time when iRFP-YAP, EBFP-Nucleus, GFP-Chromobody and fluorescent particles underneath of each cell were imaged. To analyze traction stresses and strain energies of the cells, at the end of the experiment, cells were detached for the null force image. We repeated the same experiment for LINC disrupted cells and CytoD treated cells to investigate how LINC complex disruption and actin filament depolymerization affect contractile force-mediated nuclear deformation.

2.9.12 Osmotic compression

To examine how externally deformed nuclei regulated lamin A distribution and YAP activation, hyperosmotic pressure was applied using different concentrations of 400Da polyethylene glycol (PEG 400, Sigma) [103,126,136]. First, XYZ stacks of EBFP-Nucleus, iRFP-YAP and GFP-Chromobody of the cells seeded on the fibronectin-coated glasses and PDMS substrates were imaged using confocal microscope with x63/1.4 NA. Then, different concentrations of PEG400 were added to the cells and again *z* stacks of the nuclei as well as iRFP-YAP and GFP-Chromobody were imaged (Table 3). We then quantified nuclear volume, YAP ratio (YR) and normalized nuclear membrane lamin A (Lm) after adding different concentrations of PEG400 to the cells and compared them with initial values obtained in isotonic condition to investigate how applied force deforms nucleus, redistribute lamin A and activates YAP.

PEG concentration wt%	Osmotic pressure (MPa)
2.5	0.96
5	1.12
10	1.62

Table 3. Quantified osmotic pressures relevant to different concentrations of PEG400.

2.9.13 Quantification of nuclear bulk moduli

To determine how nuclear deformability influenced the required force to activate YAP, we modulated lamin A expression level as mentioned above and 18 hours after seeding the cells on fibronectin-coated glasses, we applied different hyperosmotic pressures using different

concentrations of PEG400. We acquired XYZ stacks of EBFP-Nucleus before and after adding PEG. We then quantified change in the nuclear volume when the cells with different lamin A expression were exposed to different hyperosmotic shocks and using the following equation,

$B = -\Delta P / (\Delta V / V_0)$

where B= bulk modulus, ΔP = osmotic pressure, ΔV= change in nuclear volume and V_0 = Initial nuclear volume, we calculated nuclear bulk modulus [126,136].

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Preface to Chapter 3

In Chapter 3, I studied lamin A mechanosensitivity under different modes of nuclear deformation emanated from contractility, hyper-osmotic, and hypo-osmotic shocks to understand which modes of nuclear deformation (lateral (nuclear major axis) and vertical (z) axis) regulates lamin A and to confirm its relation with YAP localization. I found that only nuclear flattening and nuclear vertical deformation mediate lamin A delocalization from the nuclear membrane and YAP nuclear transport; critically, I did not observe significant changes in lamin A and YAP distribution under nuclear swelling, suggesting that nuclear membrane tension does not universally explain YAP transport. These findings can help us to better understand cell mechanosensation from a physical perspective, and they can provide new insight into the therapeutic approaches for multiple diseases associated with defective mechanotransduction and impaired nuclear mechanics, including cancer and laminopathies.

Chapter 3: Nuclear swelling inhibits YAP nuclear entry by stabilizing lamin A/C in the nuclear inner membrane

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3.1 Abstract

Lamin A/C total expression levels change vastly during organ development, stem cell differentiation, and in response to microenvironment mechanics. Mutations in lamin A/C, however, lead to dysfunctional nuclear mechanics which are associated with various diseases, including cancer metastasis, muscular dystrophy, and HGPS. Nevertheless, how lamin A/C mechanics influence the mechanotransduction behind these diseases remains unclear. Here we show that lamin A/C nuclear mechanosensing and YAP mechanoregulation is only sensitive to nuclear compression, whereas nuclear swelling does not impact lamin A/C distribution nor YAP localization. We observed that only vertical nuclear compression induces lamin A/C delocalization from the nuclear membrane, which in turn influences YAP nuclear transport. Surprisingly, both YAP and lamin A/C are unaffected by nuclear swelling, suggesting that nuclear membrane tension does not directly regulate YAP localization. These insights into anisotropic nuclear compression in lamin A/C delocalization and YAP mechanosensation provide clarity into mechanical deformation of the nucleus in mechanotransduction and may offer new perspectives in therapeutic approaches that target lamin A/C, YAP translocation, and nuclear mechanics.

Keywords: Lamin A/C, mechanotransduction, nuclear mechanics, nuclear deformation, traction force microscopy, Yes associated protein

3.2 Introduction

There are diverse mechanisms allowing cells to sense passive and active mechanical inputs, including extracellular matrix (ECM) rigidity [138,139], shear stress [102], dynamic stretching [87], and compression [103]. Many of these mechanisms rely on the formation of dynamic molecular clutches between ECM, integrin, force generating actomyosin cytoskeleton and adaptor proteins [29,140,141].

Similar to the cell-ECM boundary, which is connected with integrins to sense external mechanical stimuli [100,142], the nuclear envelope is a mechanosensitive interface between the nucleoskeleton and cytoskeleton where intracellular forces are transmitted via LINC complex (Linker of Nucleoskeleton and Cytoskeleton) [69,143-146]. This continuous mechanical coupling between extracellular environment, cytoskeleton, and the nucleus enables cells to respond correctly to different mechanical stimuli.

The nucleus is the hub of mechanotransduction, and transcription activation has been directly connected with nuclear deformation [43,47,69,143]. Defects in nuclear mechanosensation is also linked to a vast number of diseases, including cancer progression [147-149], and cardiopathies [150]. One of the proposed nuclear mechanosensory mechanisms is deformation-mediated conformational changes in nuclear components, including lamins and chromatin, which effectively determines the activity of transcriptional machinery and impacts gene regulation [66,75,151,152].

A-type lamins form filamentous networks underlying the inner nuclear membrane and contribute to nuclear mechanics [62,112,153,154] and mechanotransduction [75,155-158]. Lamin A/C is found in both the inner nuclear membrane and in the nuclear interior, and it integrates extracellular mechanical cues and biological signals by activating downstream signal transduction pathways [52,65]. This is believed to occur through force-induced conformational changes in lamin A/C, which affect its interaction with transcription activators and chromatins [64,159,160]; however, how force-mediated lamin A/C conformational changes is associated with downstream signaling activation remains poorly understood.

Lamin A/C expression levels are highly variable, depending on cell type [161,162], stage of tissue development [116], and differentiation state [52,117]. Deficiencies or mutations in lamin A/C are referred to as laminopathies [163,164], and lead to various diseases, including muscular dystrophies [56,57], and Hutchinson-Gilford Progeria Syndrome (HGPS) [165,166]. Moreover, it has been shown that lamin A/C expression level is mechanosensitive, and exposing cells to diverse mechanical signals, such as ECM rigidities, impacts differential lamin A/C expression[17]. This is hypothesized to be a protective nuclear mechano-adaptation to buffer mechanical stimuli arising from intra or extracellular forces [17,55]. Previous studies identified phosphorylation of lamin A/C as a key mechanism in modulating lamin A/C disassembly, solubility, and degradation in nucleoplasm as a result of low cell contractility [17,66,118]. Cell contractile forces modulated by substrate rigidity can

deform the nucleus and modify conformational states of lamin A/C, which hinders the accessibility of kinases and leads to concomitantly less lamin A/C solubility and high lamin A/C levels; however, less tensioned nuclei on soft substrates favor lamin A/C phosphorylation, which triggers lamin A/C solubilization in the nucleoplasm and subsequent degradation [17,47,66,67,75]. The molecular mechanism by which lamin A/C mechanosensitivity mediates biological processes remains unclear.

Lamin A/C also seems to be critical in nuclear pore complexes' (NPCs) density and organization in a cell-cycle dependent way [74]. It has been reported that pore-free regions appear in the nuclear membrane in areas enriched with lamin A/C and emerins. Conversely, depletion of lamin A/C increased nuclear pore densities, suggesting a proscriptive role of lamin A/C in nuclear pore organization [74]. Considering the mechanoresponsive property of lamin A/C expression levels, the effect of peripheral lamin A/C in regulating nuclear pore densities, and the importance of nuclear pores in nucleocytoplasmic translocation of large molecules, we suspected that lamin A/C distribution itself in the nucleus may be mechanosensitive, and peripheral lamin A/C agglomeration may interfere with molecular translocation through nuclear pores by affecting nuclear pore densities.

Yes associated protein (YAP) is a crucial mechanosensitive transcription coactivator that translocates between the nucleus and cytoplasm in response to different mechanical stimuli [38,79,88,90]. YAP is only active in the nucleus where it interacts with transcription factors, including the TEAD family, promoting proliferation and osteogenic differentiation. Cytoplasmic YAP, however, remains inactive, leading to apoptosis and adipogenic differentiation [79-81,88,111]. Dysfunction in YAP activity is associated with diverse diseases including fibrosis, tumorigenesis and metastasis as a result of oncogenic activation [82,86,107]. We suspected that the distinct roles of A-type lamins in NPC distributions [73,74] combined with the importance of nuclear pore opening in YAP nuclear transport [90] would link nuclear lamin A/C distribution with YAP nuclear transport. Previously we have shown that nuclear-deformation delocalizes lamin A/C from the nuclear inner membrane and enables YAP entry into the nucleus [167]; cells with deformed nuclei and evenly distributed lamin A/C had more nuclear YAP, whereas cells with large nuclear volumes had highly accumulated peripheral lamin A/C and cytoplasmic YAP. However, it is still unclear if lamin A/C relocalization is dominated by isotropic, lateral, or vertical modes of deformation. Moreover, it is

unknown if nuclear compression or swelling elicit the same YAP localization and lamin A/C redistribution response.

3.3 Results

3.3.1 Only nuclear compression triggers lamin A/C delocalization from the nuclear membrane.

Although overall lamin A/C expression levels have been reported to vary with substrate rigidity [17,66], the spatio-temporal dynamics of lamins are relatively unknown. Previously we observed spatial correlations between lamin A/C expression and local nuclear stiffness [136], and redistribution of lamin A/C from the nuclear inner membrane to the nucleoplasm in response to the nuclear deformation [167]; however the modes of deformation that induce lamin A/C relocalization are unknown .

To measure the spatio-temporal dynamics of lamin A/C, we imaged NIH 3T3 cells expressing GFP- lamin A/C chromobody (Chromtek) with confocal microscopy (Leica SP8). We quantified the lamin A/C intensity in the nuclear membrane normalized by the lamin A/C intensity in the nuclear interior (Lm), and the total nuclear volume (See Methods). To quantify effects of cell contractility on lamin A/C distribution, we also employed traction force microscopy (TFM). Consistent with our previous findings, we measured a strong correlation between normalized lamin A/C distribution, contractile Strain Energy (R^2= 0.82), and nuclear volume (R^2=0.71) for the cells cultured on Polydimethylsiloxane (PDMS) substrates with a Young's modulus of 18kPa (Figures 1A, 1B, S1A, and S1B) [120,121]. However, we observed a poor correlation between the contractile work and lamin A/C as a function of nuclear area (R^2=0.01) (Figures 1C and S1C). These findings support the idea of lamin A/C distribution is sensitive to the nuclear compression mediated by contractile force but not the nuclear area [167].

Observing the effects of cell contractility-mediated nuclear compression on lamin A/C redistribution from the nuclear membrane to the nucleoplasm, we suspected that nuclear deformation by any mechanism may induce lamin A/C delocalization. To test this idea, we applied different positive hyper-osmotic and negative hypotonic pressures using different concentrations of polyethylene glycol 400Da (PEG400) and de-ionized water, respectively. We then compared nuclear volumes and normalized lamin A/C fluorescence in the nuclear membrane (Lm) under isotonic, positive, and negative pressures. We found only ~10% decrease in Lm when the cells

were under 2.5% PEG (960 kPa positive hyper-osmotic pressure) (Figures 1D, and 1H), whereas 5% PEG (1.12 MPa hyperosmotic pressure) triggered on average a 30% reduction in Lm (Figures 1E, and 1H). Maximal lamin A/C delocalization from the nuclear membrane was ~80% obtained under 10% PEG (1.62 MPa hyperosmotic pressure) (Figures 1F, and 1H). Surprisingly, cells and nuclear swelling under negative hypotonic pressure (66% de-ionized water, -0.56 MPa hypotonic pressure) did not impose significant changes in Lm in the cells (~ ±11%) (Figures 1G, and 1H).

To understand how nuclear deformation impacts lamin A/C localization, we then examined changes in normalized lamin A/C fluorescence in the nuclear membrane ($\Delta Lm=Lm_{initial}-Lm_{exposed}$) as a function of nuclear volumetric deformation ($-\Delta V/V_0$) by comparing Lm values and nuclear volumes under varied pressures (Figure 1I). We again observed a significant relationship between nuclear compression and lamin A/C redistribution ($R^2= 0.86$), whereas nuclear swelling did not induce significant lamin A/C redistribution ($R^2= 0.23$) (Figure 1H). The absence of lamin A/C reorganization in response to swelling suggested that nuclear compression more directly impacts lamin A/C distribution than nuclear stretch or volume.

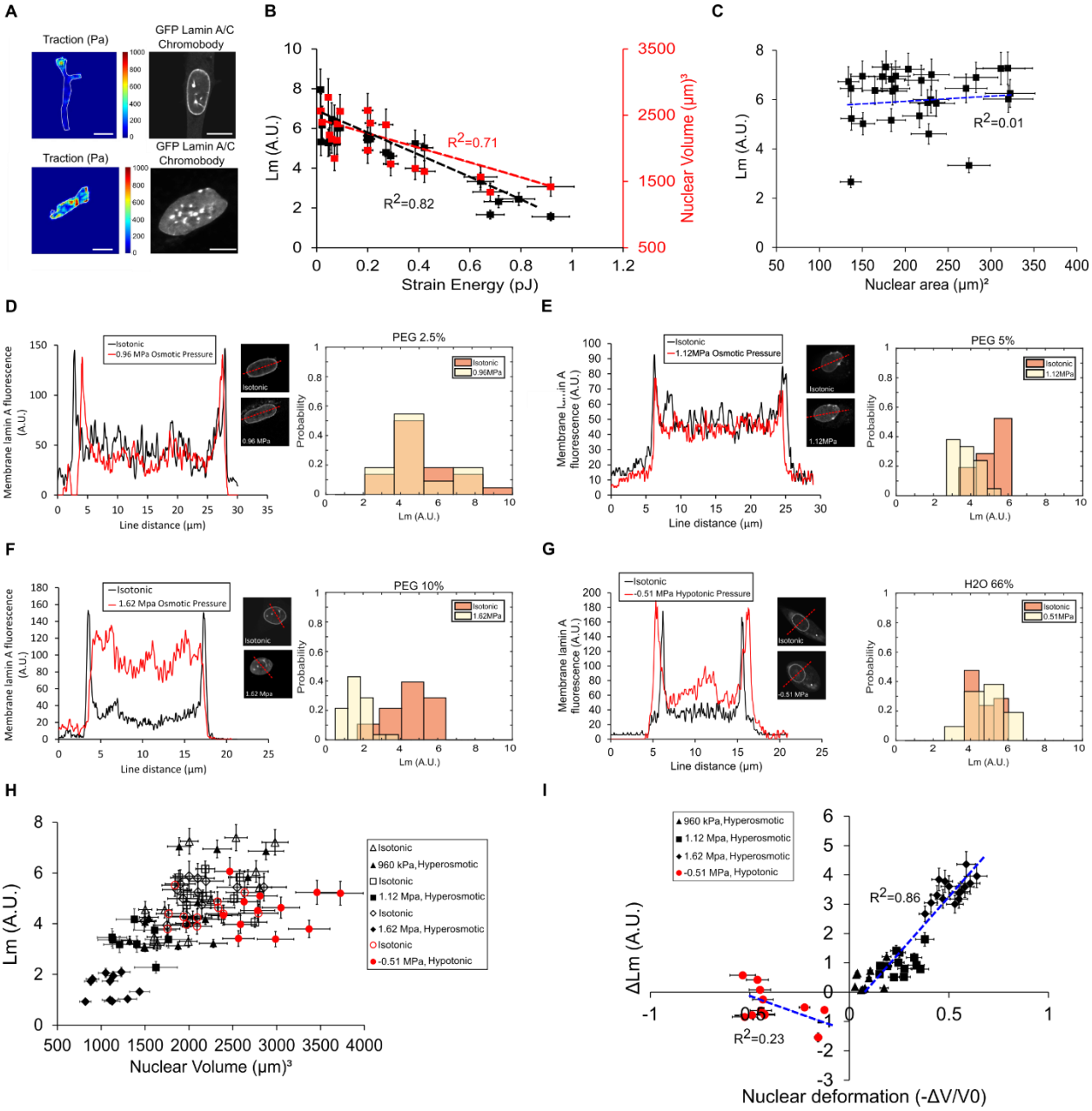

Figure 1. Lamin A/C relocalization from the nuclear membrane to the nucleoplasm occurs under nuclear compression but not under nuclear swelling. A) Representative images of two cells with different contractility and lamin A/C distributions; a weakly contractile cell with large nuclear volume had high lamin A/C density in the nuclear periphery, whereas a high contractility cell with a compressed nucleus had evenly distributed lamin A/C across the nucleus, B) Quantification of Lm and nuclear volume as a function of strain energy for cells on a 18 kPa PDMS substrate (n>10 cells), C) Quantification of Lm as a function of nuclear area for the same cells as in (C), D) Representative image of lamin A/C distribution, and quantified fluorescence intensity of lamin A/C along a chord crossing the nucleus of one example cell before and after applying 0.96 MPa osmotic pressure. Histograms are representative of lamin A/C distribution for different

cells before and after applying 0.96 MPa (n>10 cells), E) 1.12 MPa (n>10 cells), F) 1.62 MPa hyper-osmotic pressure (n>10 cells), and G) -0.51 MPa (66% de-ionized water) hypo-osmotic pressure (n>10 cells). H) Plot of normalized lamin A/C fluorescence in the nuclear membrane (Lm) as a function of nuclear volume under different hyperosmotic and hypotonic conditions (n>10 cells per each condition), I) Evaluation of the change in Lm (ΔLm) as a function of nuclear deformation under different pressures for the same cells as in (H). Scales bars are 10 μm for the nuclei and 20 μm for the cells. Error bars indicate standard deviation (SD).

3.3.2 Lamin A/C redistribution is highly sensitive to the nuclear vertical compression

To test which modes of nuclear deformation effectively impact lamin A/C redistribution, we first quantified nuclear length (major axis, X), width (minor axis, Y) in XY plane, and nuclear height (Z) for multiple cells in different contractile states. Our results revealed a relationship between cell contractility and the nuclear height (R^2=0.57) (Figure 2A), while cell contractility was only weakly correlated with the nuclear length (X) and width (Y) (R^2=0.11 and 0.002, respectively) (Figures S2A and S2B). These results suggested that contractility has minimal impact on the nuclear length and nuclear width. We then quantified the relationship between normalized lamin A/C fluorescence in the nuclear membrane (Lm) and the force-mediated nuclear X, Y, Z deformation under different cell contractility, hyper, and hypo-osmotic shocks.

Interestingly, our results showed that lamin A/C redistribution was isotropically sensitive to deformation. We observed that lamin A/C localization was more sensitive to the nuclear height (Z) (R^2=0.62) than the nuclear width (Y) (R^2=0.01) and length (X) (R^2=0.2) under different contractile works, positive, and negative pressures (Figures 2B, 2C, and S2C-S2F). To further test our hypothesis of lamin A/C redistribution under vertical and lateral nuclear deformation, we measured changes in normalized nuclear membrane lamin A/C fluorescence (ΔLm) as a function of the nuclear lateral (-ΔX, -ΔY) and vertical (-ΔZ) deformation. Again, we observed a weak correlation between lamin A/C redistribution and nuclear lateral deformation (R^2<0.2) (Figures 2D and 2E); whereas lamin A/C redistribution was highly correlated with nuclear vertical compression under positive hyper-osmotic compression (R^2=0.86) (Figure 2F). Nuclear vertical swelling, however, did not impose lamin A/C dislocation (R^2=0.03), suggesting that lamin A/C is only mechanosensitive to the nuclear flattening or vertical compression, but not to the nuclear swelling (Figure 2F).

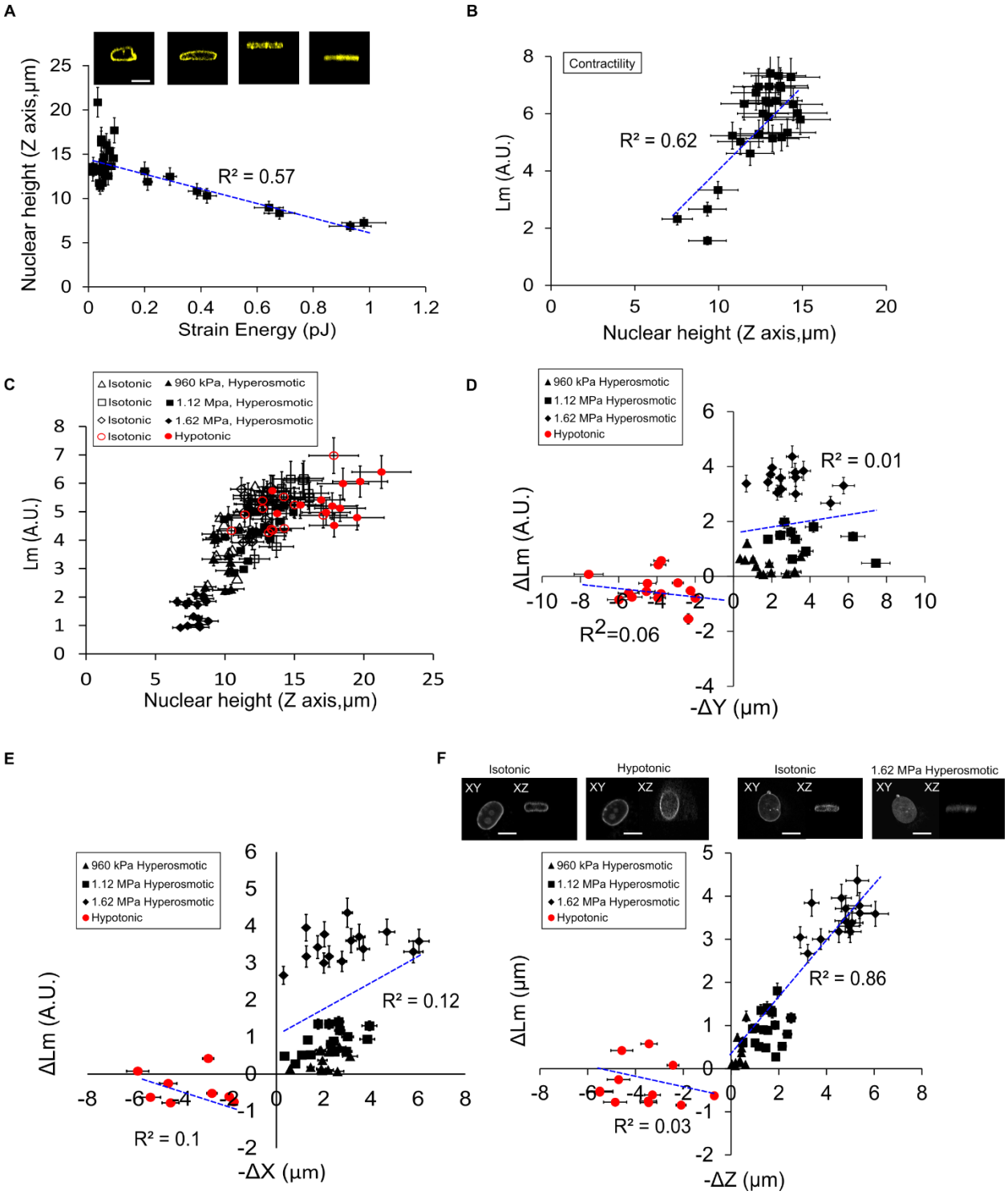

Figure 2. Lamin A/C redistribution is highly sensitive to the nuclear vertical compression. A) Quantification of the nuclear height (Z) as a function of cell strain energy (n>10 cells). Inset is representative of different nuclear heights of cells with diverse contractility, B) Quantification of Lm as a function of the nuclear height (Z) induced by contractility for the same cells as in (A), C) Quantification of Lm as a function of nuclear height when the cells were under different hyper-

osmotic (black markers) and hypotonic (red markers) deformations. Open markers are representative of cells in isotonic condition and solid markers are the same cells after applied pressures (n>10 cells per each condition), D) Evaluation of Lm redistribution (ΔLm) as a function of the nuclear width deformation (-ΔY), E) the nuclear length deformation (-ΔX), and F) the nuclear height deformation (-ΔZ) for the same cells under different pressures as in (C). Inset is representative of lamin A/C redistribution under different hyper-osmotic and hypotonic shocks for two example cells. Scale bars are 10 μm. Error bars indicate standard deviation (SD).

3.3.3 Lamin A/C redistribution through nuclear flattening impacts YAP localization

Previous studies have shown that YAP localization is sensitive to the nuclear flattening and nuclear pore opening mediated by intracellular or extracellular forces [90]; however, why only nuclear flattening but not nuclear swelling influences YAP nuclear localization remains unclear. Previously we identified the importance of lamin A/C delocalization in YAP translocation [167]. The apparent lamin A/C sensitivity to the nuclear flattening (Figure 2F) suggested that various modes of nuclear deformation impact YAP and lamin A/C redistribution differently.

To examine the relationship between modes of nuclear deformation and mechanotransduction, we transiently transfected NIH 3T3 cells with iRFP YAP and quantified YAP nuclear to cytoplasmic ratios (YR) (see methods) and the distribution of lamin A/C in different cells with varied contractility (Figures 3A-3D). Our results illustrated that strongly contractile cells with highly compressed nuclei displayed evenly distributed lamin A/C throughout their nuclei and predominantly had YAP localized in the nucleus; however, weaker contractile cells with larger nuclear volumes displayed denser lamin A/C in the nuclear membrane and had higher cytoplasmic YAP localization (Figures 3E and S3A). To resolve the roles of lateral and vertical nuclear dimensions, we quantified nuclear/cytoplasmic YAP ratios and the lamin A/C distribution in the nucleus as a function of nuclear X, Y, and Z axes. We found that both YAP and lamin A/C distributions were highly correlated with vertical nuclear compression (R^2=0.84, and 0.72, respectively), but only weakly related to the nuclear lateral compression (Figures 3F, S3B, and S3C). These findings suggest that nuclear deformation-mediated YAP nuclear localization and lamin A/C distribution is mostly due to nuclear vertical axis compression (Figures 3E and 3F).

To further test this idea, we applied different modes of external deformation (hyper-osmotic compression and hypotonic swelling) to the cells with cytoplasmic YAP and highly localized lamin A/C in the nuclear membrane. We then quantified both YAP and lamin A/C distributions as a

function of nuclear volume, and nuclear axes. Again, similar to lamin A/C, YAP localization was correlated with nuclear volume compression and nuclear height compression (R^2=0.54), but weakly correlated with nuclear lateral axis deformation (Figure 3G, and S3D-S3F). Our results did not show significant changes in YAP localization and lamin A/C distribution under 0.96 MPa hyper-osmotic compression (2.5% PEG400), whereas 1.12 MPa hyper-osmotic pressure triggered delamination from the nuclear membrane to the nucleoplasm (lower Lm values compared to those in isotonic condition) and YAP nuclear entry (Figure 3G). We measured minimal Lm values and maximal YAP ratios when the cells were under 1.62 MPa hyper-osmotic compression (Figure 3G); however, neither YAP nor lamin A/C distributions were influenced by nuclear swelling through hypo-osmotic pressure (Figure 3G).

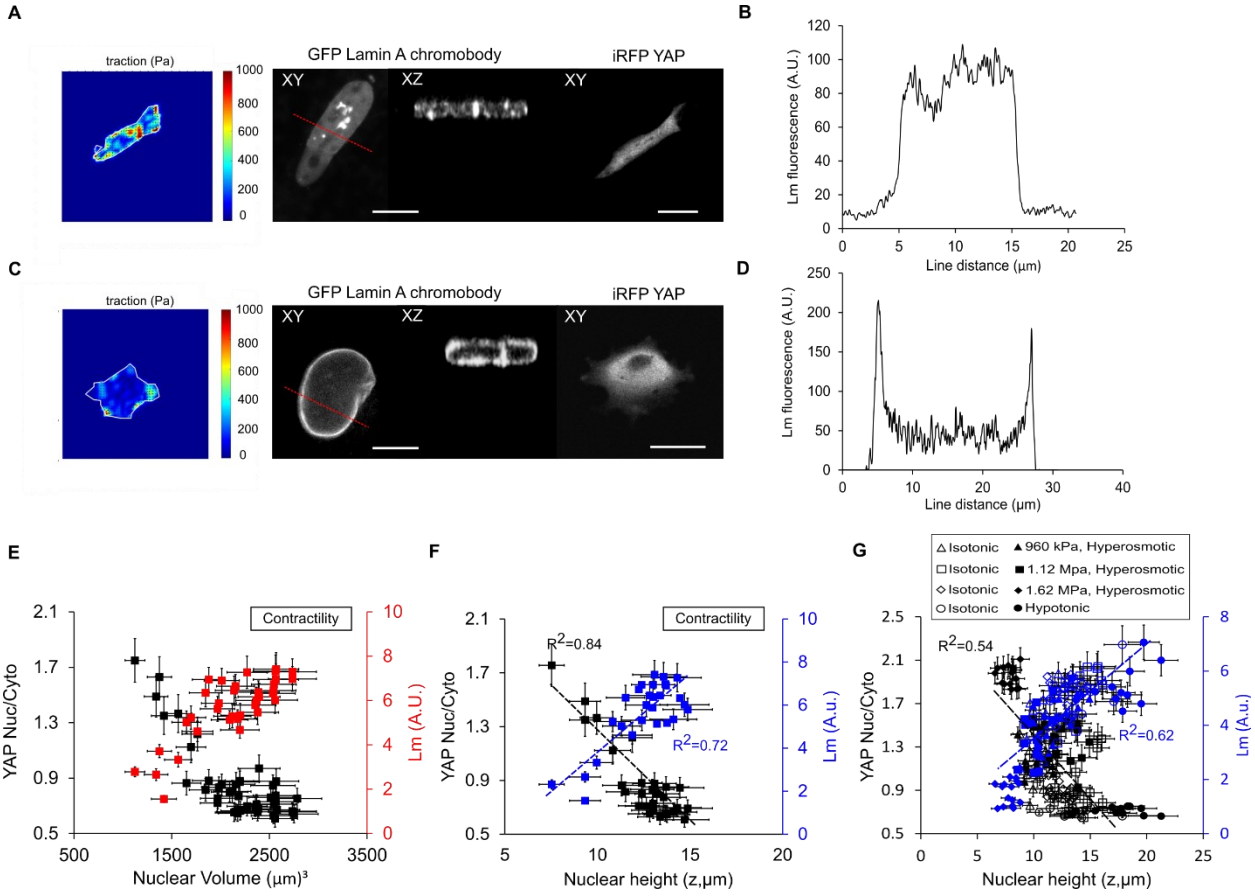

Figure 3. Lamin A/C delocalization through nuclear vertical compression mediates YAP nuclear localization. A) Representative images of high contractility cell's traction map, GFP lamin A/C chromobody transfected nucleus and iRFP-YAP transfected NIH 3T3 cell, B) Quantification of lamin A/C fluorescence along a chord across the nucleus in (A), C) Representative images of low contractility cell's traction map, GFP-lamin A/C chromobody

transfected nucleus and iRFP-YAP transfected cell, D) Quantification of lamin A/C fluorescence along a chord across the nucleus in (C), E) YAP nuclear to cytoplasmic ratio and normalized lamin A/C fluorescence in the nuclear membrane (Lm) as a function of nuclear volume for different cells seeded on PDMS substrate with Young's modulus of 18 kPa (n>10 cells), F) YAP nuclear/cytoplasmic ratio and Lm as a function of nuclear height (Z) for the same cells as in (E), G) YAP ratio and Lm as a function of nuclear height (Z)before (open markers) and after (solid marker) applying different hyper-osmotic and hypo-osmotic shocks (n>10 cells per each condition). Scale bars are 10 μm for the nuclei and 20 μm for the cells. Error bars indicate standard deviation (SD).

3.3.4 YAP translocation under hypotonic and hyper-osmotic shocks is directly controlled by lamin A/C localization

To resolve the role of nuclear flattening in regulating YAP localization, we quantified changes in the YAP ratio as a function of nuclear volumetric deformation and nuclear vertical (-ΔZ) deformation. Again, we observed the YAP distribution was highly correlated with nuclear volumetric (R^2=0.84) and vertical (R^2=0.82) compression when the cells were under hyper-osmotic shocks (Figures 4B-4D). These results were consistent with the idea of nuclear flattening and NPC opening being crucial determinants of YAP nuclear localization [90]. Hypo-osmotic swelling, however, had no clear effect on YAP ratios or Lm values (Figures 4A, 4C, and 4D); this is surprising, as hypo-osmotic pressure swells the nucleus, stretching the nuclear membrane and placing it under maximal tension. If tension drives NPC opening and YAP entry to the nucleus, we would expect hypo-osmotic pressure to increase YAP nuclear localization strongly, however, this was not observed.

To examine why YAP nuclear translocation happens under nuclear flattening, but not nuclear swelling (Figures 4C and 4D), we again considered the role of lamin A/C distribution in YAP nuclear entry under different modes of deformation. We quantified YAP and lamin A/C in the nuclear membrane when the cells under varying hyper-osmotic and hypo-osmotic pressures. Under all conditions, we found that the YAP distribution and lamin A/C localization were significantly correlated (R^2=0.8), suggesting that lamin A/C rather than nuclear deformation per se may uniquely determine YAP localization (Figure 4E). To further test this idea, we also quantified the change in YAP ratios as a function of change in Lm values in the nuclear membrane under different modes of deformation. Interestingly, we again found a strong correlation between YAP and lamin A/C distribution under both hyper and hypo-osmotic conditions (R^2=0.85) (Figure 4F). This

reveals that it is perhaps lamin A/C that is directly regulating YAP nuclear shuttling, and that membrane stretch and NPC activity may not uniquely describe YAP mechanotransduction.

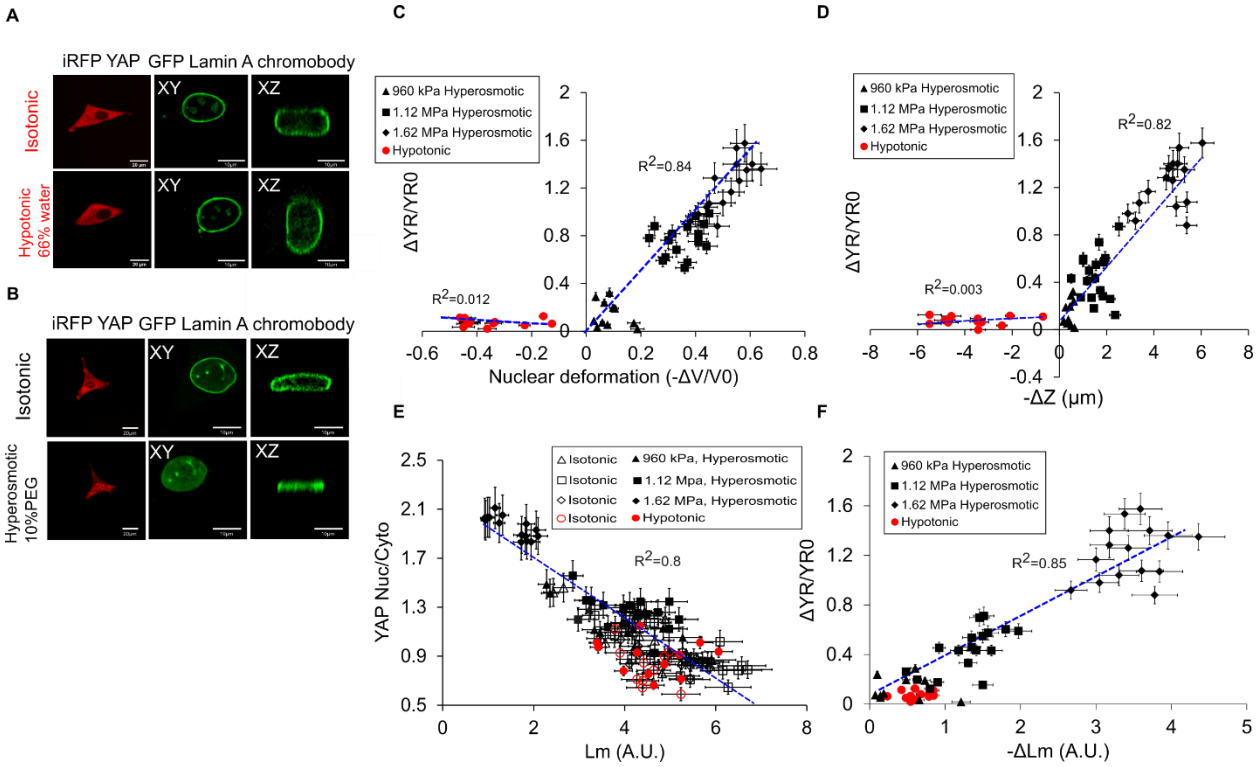

Figure 4. A) Representative images of iRFP-YAP and GFP-lamin A/C chromobody transfected cells under isotonic and hypo-osmotic condition, B) Representative images of iRFP-YAP and GFP-lamin A/C chromobody transfected cells under isotonic and hyper-osmotic condition, C) Quantification of differential YAP ratio as a function of nuclear volumetric deformation under different forces (n>10 cells per each condition), D) Quantification of changes in YAP ratio as a function of nuclear vertical deformation for the same cells as in (C), E) Analysis of YAP ratio as a function of Lm for the same cells as in (C) in the same conditions, F) Quantification of YAP redistribution as a function of changes in Lm for the same cells as in (E) under same conditions. Scale bars are 10 μm for the nucleus and 20 μm for the cells. Error bars indicate standard deviation (SD).

3.4 Discussion

Nuclear mechanosensing and conversion of forces into the biological responses involve: 1) cellular and nuclear components that are mechanically prone to change their allosteric accessibility to the signaling molecules, 2) force-induced translocation of transcriptional factors, and 3) chromosome conformational changes and reorganization[17,20,29,75]. Previous studies reported that lamin A/C levels are sensitive to the tensional state of the cytoskeleton, which can trigger nucleoplasmic

lamin A/C assembly to the lamina in the nuclear membrane[17,55,65]; however, highly relaxed nuclei can mediate phosphorylation of lamin A/C by exposing cryptic sites to kinases, leading to lamin A/C disassembly which facilitates ultimate degradation and decreased lamin A/C total level[17,55,65]. Here, we revealed that not only total lamin A/C levels, but lamin A/C distribution between the nuclear periphery and the nucleoplasm were impacted via force application to the nucleus. Our results reveal that only nuclear compression mediated by either contractile force or hyper-osmotic shocks led to physical delocalization of lamin A/C from the nuclear membrane to the nucleoplasm. However, lamin A/C localization was not impacted by nuclear swelling-induced isotropic tension. High contractility cells or cells under hyper-osmotic compression showed even distributions of lamin A/C across the nuclear interior; however, more relaxed cells with low contractility showed heterogeneous lamin A/C distribution with highly accumulated lamin A/C in the nuclear inner membrane. The mechanism behind the physical lamin A/C detachment from the nuclear membrane remains unknown, however, we speculate that it is related to the nuclear radius of curvature, where pronounced local bending induces delamination. To analyze which modes of nuclear deformation mediate lamin A/C redistribution, we measured nuclear length, width, and height deformation under different contractile forces, hyper-osmotic and hypo-osmotic shocks. We found that lamin A/C relocalization is only sensitive to the nuclear height, not to the nuclear lateral axes, and that nuclear flattening can trigger its delocalization from the nuclear membrane.

In addition to acting as a load bearing element and providing mechanical stability to the nucleus, lamin A/C's role in chromatin reorganization and transcription activation have gained attention during the last decade[62,75,159]. Our results elucidate the converse correlation between lamin A/C localization in the nuclear membrane and YAP nuclear translocation. Previously, nonmonotonic relationship between YAP nuclear localization and lamin A/C total expression levels have been reported; LMNA knockdown cells resulted in decreased YAP levels[95], while lamin A/C upregulation mediated by substrate stiffness led to increased YAP nuclear localization. Additionally, a lack of lamin A/C in cancer cells with soft nuclei leads to YAP nuclear agglomeration[97,168]; however, in MSCs overexpression of LMNA caused abrogation of YAP nuclear transport[17]. Here we suggest that lamin A/C distribution in the nuclear membrane rather than lamin A/C total expression level may obtain a more conserved relationship with YAP distribution in these cases. In our previous work, we showed that not lamin A/C expression level but lamin A/C redistribution between the nuclear membrane and the nucleoplasm is tightly

correlated with YAP localization[167]. Here we further analyzed YAP translocation under both nuclear flattening and nuclear swelling, finding that YAP localized in the nucleus only when the nuclear membrane tension was induced by nuclear flattening. These results are consistent with the previous finding that nuclear pore opening induced by force-mediated nuclear flattening regulates YAP localization[90]. However, nuclear tension induced by the nuclear swelling did not induce significant variation in YAP localization, suggesting that nuclear pore opening cannot be the only mediator of YAP regulation. Variations in YAP localization under different modes of nuclear deformation were similar to the lamin A/C distribution under different kinds of forces, resulting in a conserved correlation between YAP localization and lamin A/C intensity in the nuclear membrane under different experimental conditions.

Recent evidence indicates the essential role of lamin A/C in nuclear pore reorganization, and the importance of nuclear pore density in transcriptional activities[73,74,116,169,170] which may explain the role of lamin A/C distribution in YAP localization. Additionally, lamin A/C is implicated in DNA replication and chromatin reorganization. This can impact force-induced transcriptional availabilities on the chromatin, which may potentially mediate YAP nuclear transport[65,75,159].

Understanding lamin A/C mechanoregulation and its impact on transcription activations provides new insight into how mechanical stimuli effectively regulate cellular biological responses, including gene transcription, differentiation, and proliferation to mechanical inputs. Moreover, several studies have indicated the role of lamin A/C and YAP dysregulation, separately, in multiple diseases associated with defective nuclear mechanics, and impaired mechanotransduction, including cancer[82,86,168], aging[171], and muscular dystrophy[86,149,160]; however, no direct correlation between lamin A/C levels and YAP in pathology have been previously identified. Our results indicating the novel interplay between the nuclear vertical deformation, lamin A/C redistribution, and YAP localization might potentiate novel therapeutic approaches to multiple related diseases. Additionally, these findings will offer a better understanding of cell proliferation, stem cell engineering, cell migration, and apoptosis in which both YAP activity and lamin A/C play crucial roles.

3.5 Acknowledgements

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3.6 Contributions

Conceptualization, A.J.E., N.K.; Methodology- development, L.K.S.; Methodology- application, N.K.; Plasmid purifications and sequencing, A.G., L.K.S.; Investigation, N.K.; Writing- original draft, N.K.; Writing- review and editing, N.K., A.J.E., L.K.S., A.G.,; Funding acquisition-A.J.E.; Resources, A.J.E; Supervision, A.J.E.

3.7 Supplementary

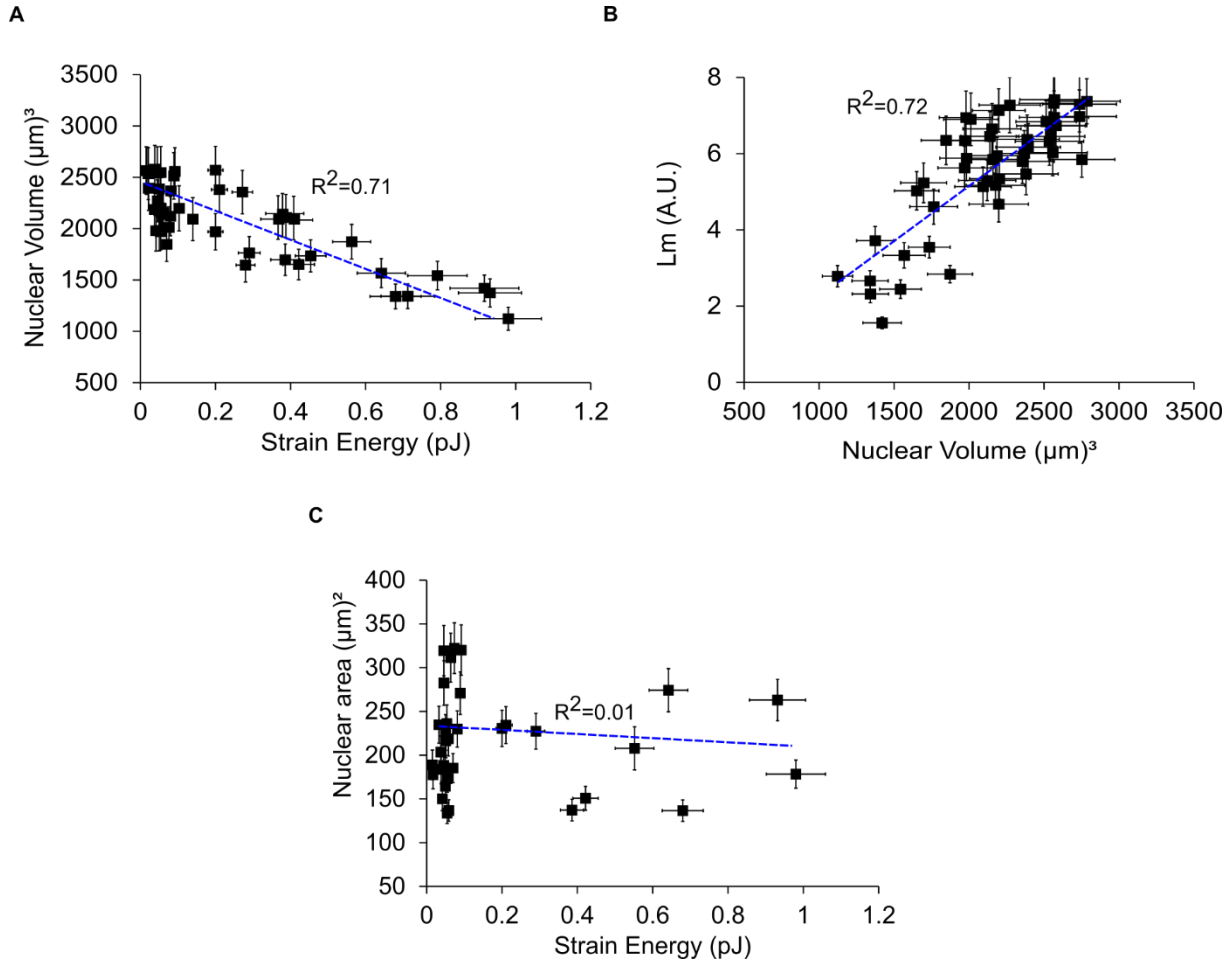

Figure S1. Lamin A/C distribution is regulated by contractile force transmission to the nucleus. A) Quantification of nuclear volume as a function of strain energy of the cells cultured on PDMS substrate with Young's modulus of 18 kPa (n>10 cells), B) Evaluation of Lm as a function of nuclear volume for the same cells as in (A), C) Quantification of nuclear area as a function of strain energy for the same cells as in (A). Error bars indicate standard deviation (SD).

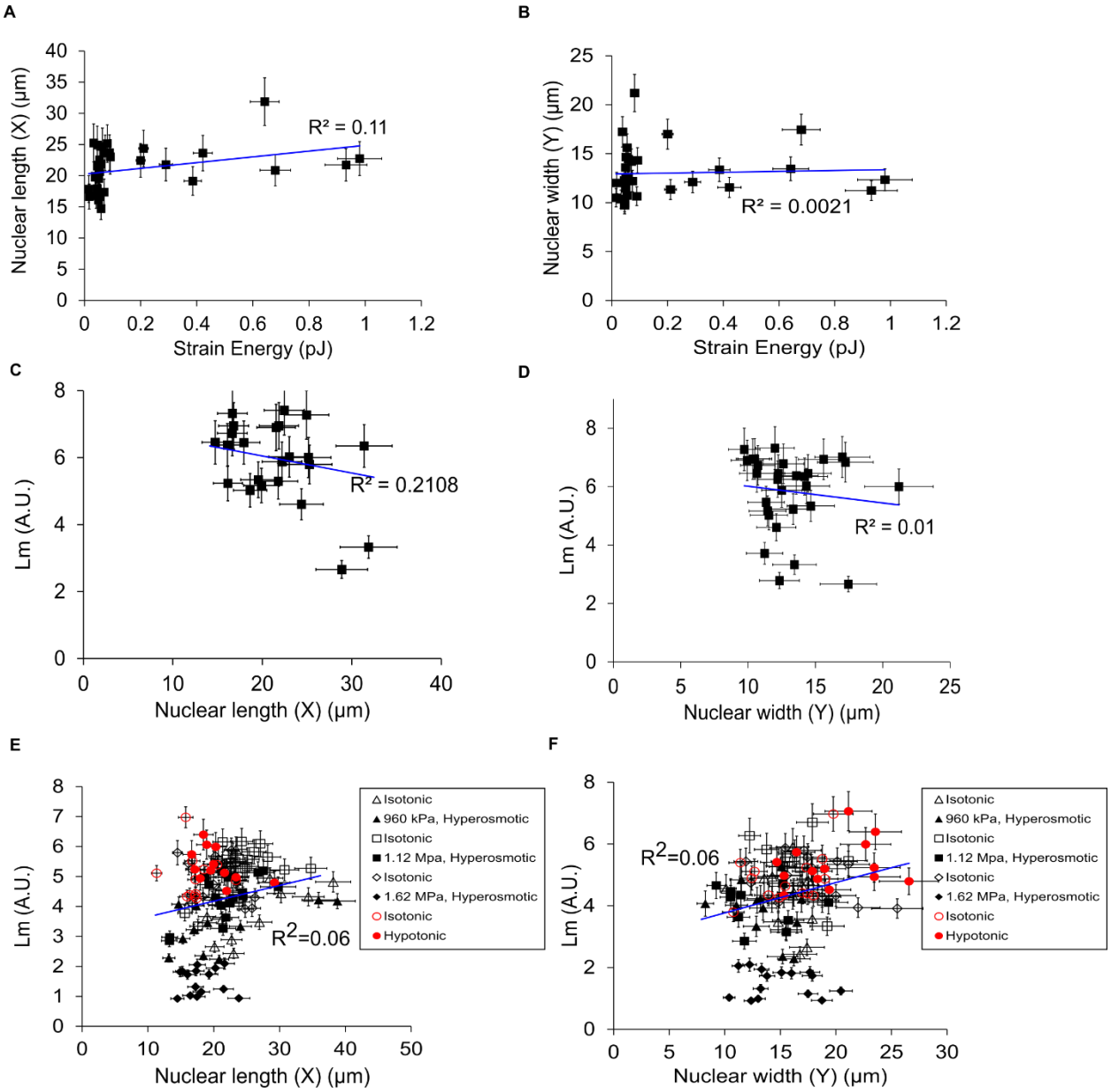

Figure S2. Lamin A/C redistribution is highly sensitive to the nuclear vertical compression. A) Quantification of nuclear length (X) as a function of strain energy for the cells cultured on PDMS substrate with Young' s modulus of 18 kPa (n>10 cell), B)

Quantification of nuclear width (Y) as a function of strain energy for the same cells as in (A), C) Quantification of Lm as a function of nuclear length for the same cells as in (A), D) Lm as a function of the nuclear width (Y) for the same cells as in (A), E) Analysis of Lm distribution as a function of nuclear length (X) before (open markers), after (solid markers) applying hyper-osmotic and hypo-osmotic shocks (n>10 cells per each condition), F) Lm as a function of the nuclear width (Y) for the same cells as in (E). Error bars indicate standard deviation (SD).

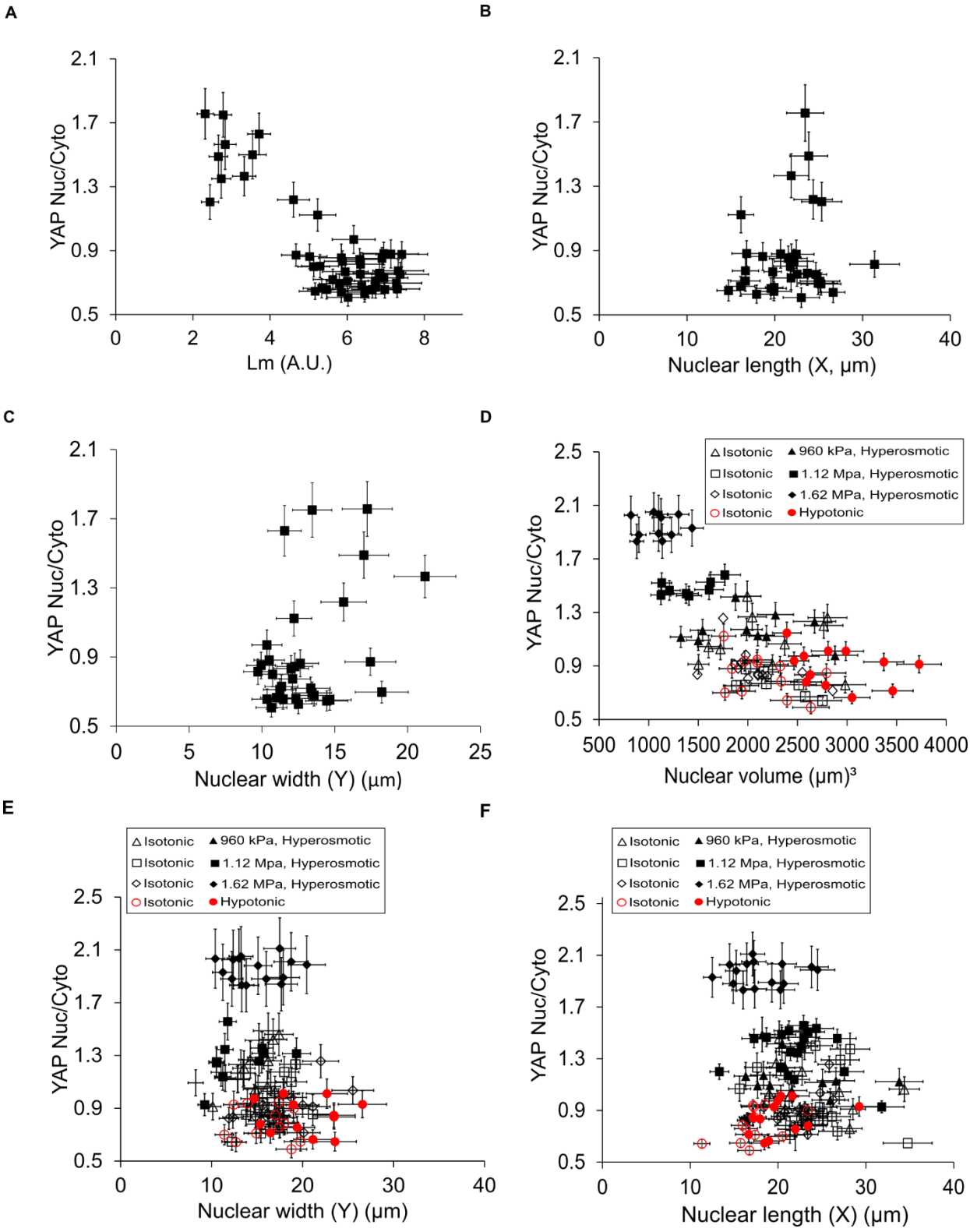

Figure S3. Lamin A/C redistribution through nuclear vertical compression effectively impacts YAP localization. A) YAP ratio as a function of Lm for the cells cultured on PDMS substrate with Young's modulus of 18 kPa (n>10 cells), B) YAP ratio as a function of nuclear

length for the same cells as in (A), C) YAP ratio as a function of nuclear width for the same cells, D) YAP ratio as a function of nuclear volume before (open markers) and after (solid markers) applying different hyper-osmotic and hypo-osmotic shocks (n>10 cell per each condition), E) YAP ratio as a function of nuclear width for the same cells as in (D), F) Quantification of YAP ratio as a function of nuclear length for the same cells. Error bars indicate standard deviation (SD).

3.8 Materials and methods

3.8.1 PDMS substrates fabrication

To measure the effects of cell contractile work on lamin A redistribution, we fabricated PDMS substrates as described previously [120,121]. In brief, we mixed 1:1 weight ratio of component A and B of commercial PDMS (NuSil® 8100, NuSil Silicone Technologies, Carpinteria, CA), and then added 0.45 weight percent of Sylgard 184 PDMS as a crosslinker to make PDMS substrates with a Young's modulus of 18 kPa [120,121]. 170 µl of PDMS mixtures then were placed on clean 24*24 mm cover glasses, and then baked at 100 °C for two hours. To measure cell contractility, cured PDMS substrates were coated with a ~1µm thick layer of PDMS with embedded custom-synthetized fiduciary particles and cured at 100 °C for an hour. Cell-induced bead displacements were measured using confocal microscopy (Leica TCS SP8) and used to quantify cell traction stress and strain energy.

3.8.2 Surface modification

In order to culture cells on PDMS substrates, we applied Sulfo-SANPAH (ThermoFisher Scientific) solution diluted in 100 mM HEPES on top of the substrates and placed them under UV for 2 minutes. To make PDMS bioactive, we then removed dark orange Sulfo-SANPAH solutions and replaced them with 5 µg/ml fibronectin, followed by incubating the substrates 9-12 hours at room temperature. Fibronectin solutions were then removed and bioactive PDMS substrates were rinsed 2-3 times with 1% PBS. After UV sterilization, cells were cultured on top of the substrates and incubated 12 hours at 37 °C to adhere on top of the PDMS substrates.

3.8.3 Traction Force Microscopy

To measure cell contractility and contractile work, we applied traction force microscopy (TFM) as previously described [120,121]. In brief, we cultured transfected cells on fluorescent bead coated PDMS substrates and both cells and fluorescent beads were imaged using confocal microscope with low magnification (x10/NA 0.4 air objective). Cell-induced deformations of the underlying

elastic substrate were quantified from bead displacement. To measure bead displacement, cells were detached at the end of imaging to acquire a reference image of and compare bead positions with cell adhered image. Traction stress and strain energy were calculated from bead displacement and the substrate's Young's modulus [123].

3.8.4 Cell culture

All measurements were performed on NIH 3T3 *Mus musculus*, mouse cell line which was obtained from ATCC. The cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Wisent) supplemented with 10% fetal bovine serum (FBS) (Wisent) and 1% Penicillin-Streptomycin antibiotic (P/S) (Thermo Fisher). The cells were incubated at 37 °C in 5% CO2 environment and allowed to adhere on fibronectin-coated PDMS substrates 12-18 hours before imaging.

3.8.5 Transfection and quantification of lamin A and YAP distribution

To examine the distribution of lamin A between the inner nuclear membrane and the nucleoplasm, we transfected NIH 3T3 cells with GFP tagged lamin A chromobody (Chromtek), using GenJet transfection reagent (Signagen) as per manufacturer recommendations. Previously, it has been shown that GFP lamin A chromobody accurately labels total lamin A in the nucleus without interfering with its function or localization between the inner nuclear membrane and the nucleoplasm [125]. 18 hours after transfection, cells were cultured on UV-sterilized fibronectin-coated PDMS substrates, and they were allowed to attach to the surfaces over 12 hours incubation time. To maintain a controlled culture environment during confocal imaging, substrates with adhered cells were transferred to a custom-made heated stage adjusted at 37 °C and perfused with 5% CO2, and mounted on the confocal microscope.

To measure the lamin A distribution, images of GFP-lamin A in the nucleus were acquired with confocal microscopy (Leica SP8, x63/1.4NA oil immersion objective). We then quantified normalized lamin A localization in the nuclear membrane by performing image segmentation with MATLAB code which created nuclear membrane and nuclear interior masks, and quantified the ratio of lamin A fluorescence in the nuclear membrane to lamin A fluorescence in the nuclear interior and referred it to as Lm.

To examine the role of lamin A redistribution between the nuclear membrane and the nucleoplasm in YAP localization between the cytoplasm and the nucleus, NIH 3T3 cells were cotransfected with GFP-lamin A chromobody, iRFP-YAP (a gift from Xavier Trepat, Institute for bioengineering of Catalonia (IBEC), Barcelona)), and EBFP2-Nucleus-7 (nuclear localization signal, Addgene, plasmid #55249) to visualize the nucleus, and they were imaged at the same time. To specify YAP localization, we used the principle metric of nuclear to cytosolic YAP ratio similar to previous studies. We quantified the ratio of iRFP-YAP fluorescence in the nucleus to iRFP-YAP fluorescence in the cytoplasm using MATLAB code, and we refer it to as YAP Ratio (YR).

3.8.6 Hyper-osmotic and hypo-osmotic shocks

To examine which modes of nuclear deformation effectively determine lamin A localization and YAP distribution, we applied positive hyper-osmotic pressure adding different concentrations of PEG400 (Table 1) and negative hypo-osmotic shock by adding 66% water (-0.56 MPa osmotic pressure). To analyze how nuclear compression and swelling mediated by applied pressures impact lamin A distribution and YAP localization, we quantified nuclear volumetric deformation ($-\Delta V/V_0$), change in Lm ($-\Delta Lm$) and YR ($\Delta YR/YR_0$) by comparing values in isotonic condition and after applying different pressures.

PEG concentration wt%	Osmotic pressure (MPa)
2.5	0.96
5	1.12
10	1.62

Table 1. Quantified osmotic pressures yielded from applying different concentrations of PEG400.

3.8.7 Nuclear area and Nuclear volume measurements

To measure how contractile work impacts cells' nuclear area, we acquired XY images of EBFP-Nucleus of the cells cultured on the fibronectin-coated PDMS traction substrates (coated with monolayer of fluorescent beads) using confocal microscopy under high magnification (x63/1.4NA oil immersion objective) at the same time we performed TFM. We then analyzed nuclear area of each cell with specified contractile work using FIJI software. We performed thresholding of each nucleus and used "analyzed particles" in FIJI to quantify nuclear area.

To measure nuclear volume, we acquired XYZ stacks of EBFP tagged nuclei with a z-step size of 0.5 μm using confocal microscopy with a x63/1.4NA oil immersion objective. We quantified 3D nuclear volume via thresholding of the acquired stacks based on top and bottom of each nucleus using FIJI software. The number of voxels of the threshold area was quantified and multiplied by the size of each voxel using FIJI software. To examine nuclear volume mediated by cell contractile work or external pressure, we measured nuclear volume at the same time we performed TFM or applied different pressures.

3.8.8 Nuclear vertical and lateral axis measurements

To measure the nuclear lateral (major) and vertical (z) axis, we acquired XYZ stacks for different EBFP-tagged nuclei. We then performed "z-project" which summed all the stacks in Z direction using FIJI software. Z-projections then were fitted by an ellipse, and the lateral and vertical axes of each nucleus were calculated using "analyze particle" in FIJI. To specify the effects of contractile work or extracellular forces on the nuclear vertical and lateral axes, we acquired XYZ stacks while we measured TFM or applied different pressures. To measure nuclear lateral and vertical deformation, we calculated ($-\Delta X$) and ($-\Delta Z$), respectively, by comparing nuclear vertical and lateral axes before and after applying different positive and negative pressures. To determine how nuclear vertical and lateral deformation impact YAP distribution and lamin A localization we performed these measurements for GFP tagged lamin A(source) and iRFP-YAP(source) transfected cells and examine how nuclear vertical and lateral change mediated by either intracellular contractile work or extracellular positive (compression) and negative (swelling) forces affect YAP localization and lamin A distribution.

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Chapter 4: General Discussion & Conclusion

The detailed mechanobiology of how cells biochemically transduce mechanical signals is reshaping our basic understanding of life and pathology. In particular, YAP has emerged as a key mechanosensitive transcriptional factor in diverse organisms, and in broad human development and pathology, impacting virtually every process from cancer metastasis to organ development and stem cell differentiation. Numerous prior works have related YAP to substrate stiffness, applied stress, nuclear membrane stretching, and the cytoskeletal tension. In this work, I showed that all of these parameters are indirect or inaccurate, and I discovered an entirely new mechanosensitive mechanism which directly regulates YAP nucleocytoplasmic translocation.

In this thesis, I revealed a previously unknown relationship between cell contractility, nuclear deformation, Lamin A localization, and YAP nuclear translocation. For the first time, to my knowledge, I showed that YAP translocation between the cytoplasm and the nucleus is dynamic over time, and that substrate stiffness and cell area do not play a crucial role in YAP localization in NIH 3T3 fibroblast cells. This work demonstrated for the first time that cell contractile work (not stress) varies during cell movement on a specific substrate and promotes YAP nuclear localization independent of substrate stiffness. By modulating nuclear stiffness, I also showed that nuclear deformation rather than applied stress determines YAP localization in a nuclear stiffness-dependent way. Previous study has introduced the nuclear membrane tension and the force-induced nuclear pore opening as the principle mechanism regulating YAP nuclear translocation[90]. In this thesis, however, I depicted that nuclear swelling which increases the nuclear surface area and increases the nuclear tension does not trigger YAP nuclear entry which overturns the dogma of the field. I discovered that not only lamin A expression level but lamin A distribution across the nucleus is sensitive to the nuclear flattening. I also found that force-induced delocalization of lamin A from the nuclear membrane uniquely describes YAP localization independent of nuclear mechanics and across all investigated experimental conditions. This thesis, for the first time demonstrates that lamin A redistribution, not nuclear membrane tension, is the unique effector of YAP localization. Understanding the mechanical stimuli that regulates YAP is of profound widespread interest in cell biology, as it impacts diverse aspects of eukaryotic life. This thesis provides a comprehensive examination of the main mechanical regulatory mechanism of YAP localization, which can pave the way for better understanding of cellular mechanosensation. This

work offers a paradigm shift improving mechanobiology as a tool; regulating YAP activity in physiology and pathology should not be pursued through indirect means such as substrate stiffness or applied stresses, but nuclear deformation and lamin A distribution. I believe these discoveries are truly monumental, impacting virtually every mammalian eukaryotic process from stem cell differentiation to cancer progression, making it of critical biological importance for all cell biology researchers. This thesis offers the potential to reshape how we understand basic cell biophysics in medicine. Furthermore, a clear understanding of the link between nuclear stiffness, nuclear deformation, peripheral and interior lamin A/C, and YAP localization would potentially resolve complex challenges in therapeutic strategies targeting different diseases associated with impaired nuclear mechanics and cellular dysfunction, including cancer progression [132,168,172,173], aging disorders [59,131], Emery-Dreifuss muscular dystrophy[57], and Hutchinson-Gilford progeria syndrome[60]; As a single example, if nuclear deformation and/or lamin A loss lead to oncogenic YAP activity[82,132,168,174], my findings offer a novel clear path of stiffening nuclei and/or increasing lamin A organization to retard metastatic progression. Finally, my findings also provide a better understanding of the mechanoregulatory mechanism directing stem cell engineering and homeostasis. In this study, the interplay between YAP, nuclear volume, and nuclear deformation may lead the notion of cell compression-mediated osteogenic differentiation as a result of YAP activation [81,103,133]. In addition, cell deformation and shape changes involved in developmental processes, including gastrulation[175] or in the inner cell mass[176] likely induce nuclear deformations which may regulate YAP translocation, as a crucial driver in hemostasis. More generally, I believe that this work, potentially, can control nucleocytoplasmic translocation of other mechanoresponsive transcription factors, including MRTF-A[177] or b-catenin[178] targeting different genes in response to force transmission to the nucleus.

A further challenge for the future will be represented by the need to investigate YAP activity via examining target gene expressions and YAP phosphorylation under proposed mechanistic activators in this thesis, including hyperosmotic shocks, lamin A reorganization, or nuclear deformability which have been shown to potentially increase YAP nuclear entry. Moreover, the molecular mechanism disassociating lamin A/C from the nuclear membrane under physical forces still remains unknown. Finally, to build more reliable models for pathological situations and to identify problems more effectively in vivo condition, future work needs to scale up mechanobiology investigations into a 3D setting.

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