New perspectives on acto-myosin networks in mammalian cells

ANOOP VADAKAN CHERIAN

Dissertation zur Erlangung des Doktorgrades an der

Fakultät für Chemie und Pharmazie

der Ludwig-Maximilians-Universität München

Anoop Vadakan Cherian
Manarcadu, Indiaen. December 2012

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2012

Erklärung

Diese Dissertation wurde im Sinne von § 7 der Promotionsordnung vom 28. November 2011 von Herrn Prof. Dr. Don C. Lamb betreut.

EidesstattlicheVersicherung

Diese Dissertation wurde eigenständig und ohne unerlaubte Hilfe erarbeitet.

München, am 12. 11. 2012

Anoop Vadakan Cherian

Dissertation eingereicht am: 12. 11. 2012

1. Gutachter: Prof. Dr. Don C. Lamb

2. Gutachter: Prof. Dr. Michael Schleicher

Mündliche Prüfung am: 19. 12. 2012

Acknowledgement

First and foremost owe my deepest gratitude to Dr Roland Wedlich-söldner, who provides me generously support from the beginning to the end. His Guidance and support help me to finish my thesis. It has been an honor to work with him. I appreciate his contribution and sincerely thank for providing such a great working atmosphere in the lab.

I am grateful to Prof. Don C. Lamp, who agrees to become my Doctoral thesis supervisor. Heartfelt thanks to Dr. Alvaro Crevenna who help me by discussing the results and data analysis. Also I would like to thank to Ms. Gisela Beck for her great technical support. I am grateful to all the present and previous members of the laboratory of cellular dynamics and cell patterning, Max-Planck institute of Biochemistry for providing great support and vibrant lab atmosphere.

I am grateful for time spent with my friends in Munich especially Ranga, JuhiSardana, Siju K. Purayil, Nagarjuna, Kirti, Rochelle, Krishna and Jyoti and rest of the Indian group.

Especially I would like to thank to my wife Sruthy Maria Augustine for her support in the good and the bad time. Last but not the least; I would like to thank my family for all their love and encouragement. I pay my gratitude for my loving, supportive, encouraging parents who have raised me and supported me in all my endeavors

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Summary

This thesis is split into three parts that describe work on various aspects of actin and acto-myosin networks in mammalian cells. The three sections are summarized and discussed each in turn.

A) The cytoskeleton is a central player in the detection, transmission, and generation of mechanical forces that determine individual cell shape as well as collective cellular behaviors underlying tissue organization. Numerous morphogenetic processes are actively driven by actin dynamics, including cell polarization, migration, division, and differentiation. Mammalian cells exhibit actin-based protrusions of varying density and shape on their non-adherent surfaces. However, not much is known about their distribution, dynamics, and biological roles. Here we demonstrate that apical surfaces of non-confluent epithelial cells are shaped by characteristic elongated actin structures, which are called dynamic microvilli to reflect their undulating shape and motions. Dynamic microvilli exhibit complex rearrangements that largely depend on the activity of type II myosin. We found that myosin II is organized into a network of filaments covering the apical cell surface. Dynamic microvilli and myosin filaments undergo coordinated pulsating rearrangements that drive rapid changes in cell shape.

B) Studies showed that cytosolic actin assemblies play an important role in mechanical signal transduction from the cell periphery to the nucleus and also keep the nuclear shape. Gene regulation and chromatin organization also depends on the nuclear shape. However, no such structures showed before, which physical link between plasma membrane and nucleus. Using different image techniques like live cell fluorescence imaging and transmission electron microscopy, we showed the presence of cytoplasmic acto-myosin network in mammalian epithelial cells. Cytoplasmic acto-myosin network is a bridge between both the nucleus and the plasma membrane. Nucleus start shrinking after de-polymerization of actin or inhibition of myosin II, suggest that the cytoplasmic acto-myosin structures exert force on the nuclear envelope. Moreover, we observed an imbalance in nuclear positioning and strong deformations of the nucleus upon coincident disruption of the actomyosin and microtubule network, suggesting the opposing roles of these two networks. Taken together, these results provide evidence for the structural basis of cellular mechanotransduction.

C) Myosin II and actin play a prominent role in cell division. Moreover, myosin II plays a role in spindle assembly and centrosome dynamics during mitosis, and cortical actin provides attachment sites for astral microtubules during cell division. Previous studies showed that in mammalian cells, actin and myosin II- based cortical flow regulates centrosome separation. Here we show that, in addition to its cortical function, actin and myosin II network also form a dense three-dimensional network surrounding the chromosomes and spindle during mitosis. We also demonstrate that this cytoplasmic acto-myosin network plays an important role in chromosome positioning during early mitosis (prophase and pro-metaphase). We propose that cytoplasmic acto-myosin networks play direct and essential role during spindle assembly and chromosome segregation.

1. Introduction

1.1. Actin

The mechanical interaction with the environment is very important for cell function. Cells have to change their shape, move from one place to another, divide, and rearrange their internal components in response to external stimuli and changes. In order to carry out these remarkable functions, cells rely on a system of filaments, called the cytoskeleton. The cytoskeleton is composed of three different polymers - actin, microtubules, and intermediate filaments. Although these polymers have distinct mechanical properties, dynamics, and biological roles, they work together in order to control cell shape, cell movement, and cell strength.

Actin is a 42-kDa protein and one of the most abundant proteins found in eukaryotic cells. Actin is involved in many cellular processes such as cell division, cell migration, intracellular transport, and cell morphogenesis. Through polymerization and association with myosin motor molecules, actin is also one of the major force generators of cells. In vertebrates different actin isoforms have been identified. Nonmuscle cells of humans express β and γ isoforms of actin and four different α and β isoforms of actin. The average concentration of actin inside the cell is around 100 μM [1]. The physiologically relevant forms of actin are filaments. Actin filaments are also known as microfilaments. Single actin filaments consist of two proto-filaments arranged in a right-handed double helix. Filamentous form of the actin is called as F-actin, whereas the monomeric form of the actin is called G-actin (from globular). Actin is an ATPase, therefore it can be nucleotide-free, or with ATP,

ADP-Pi or ADP bound to its nucleotide state. Actin kinetics and thermodynamics depend critically on its nucleotide state.

Actin gene is present in all eukaryotic families and it has almost similar physiological function. Actin genes are extremely well conserved throughout evolution. Comparison between the nucleotide sequences of the actin gene between *Homo sapiens* and *Saccharomyces cerevisiae* shows 80.2% sequence similarity with a 95% sequence identity at the protein level. Recent studies show that actin homologues exist in the prokaryotic systems [2, 3]. Sequence similarity of these proteins to actin is relatively low although show very similar fold. Some of these proteins are also able to polymerize into filamentous structures and essential for maintaining cell morphology [4].

Actin filaments are essential components of the cytoskeleton. Actin filaments underlying the plasma membrane provide mechanical stability. Some actin filaments project from the cell membrane and some of these projections are dynamic in nature, such as lamellipodia and filopodia. Cortical actin plays an important role during cell division, actin assembles on the contractile ring and help for the cell division [5]. In addition, actin filaments can also form bundles, which can be for example found in microvilli on the surface of the intestinal epithelium. Microvilli help to increase the surface area of the apical cell and also enhance nutrient absorption. The fundamental and highly conserved property of actin filaments is to provide mechanical stability to the cells. In mammalian cells, actin plays a major role in cell migration. Actin filaments, together with myosin II, generate force in order to push the cell membrane forward [6, 7]. F-actin polymerization has been studied extensively with *in vitro* experiments using purified actin [8, 9]. Actin polymerization is a two phase process, with the first phase known as the nucleation phase, and the

second phase known as elongation phase. During actin nucleation process, actin monomer forms dimers and trimers. These dimer and trimer are thermodynamically unstable. This process making nucleation the rate-limiting step of actin polymerization [9]. Addition of a subunit to the trimer is more favorable than subunit dissociation, making this the elongation phase [9]. Rate of elongation is proportional to the concentration of the G- actin in the solution. During elongation G-actin are attached to the ends of an actin filament in a particular orientation. Due to this particular arrangement and the asymmetry of the actin monomer in the actin filament helps to attain a polarity for the actin filament. Growing end of the actin filament is termed as barbed end (+) and the other is pointed end (-) (Fig. 1-1) [10]. Finally the system reaches a steady state. In steady state, the rate of addition of the monomer is equal to the rate of dissociation of the monomer. Concentration of the free subunit in the steady state is called the critical concentration Cc= k_{off}/k_{on} (this is for each end) and the particular Cc will depend on the nucleotide.

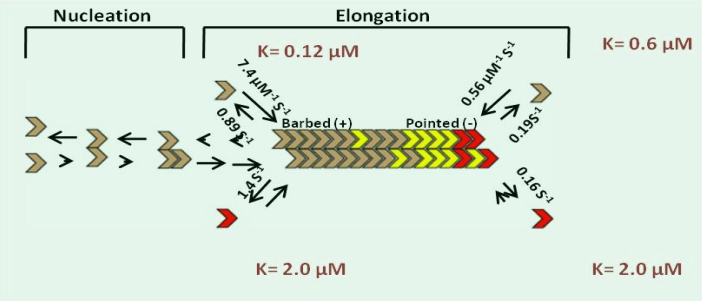


Figure 1-1. Polymerization cycle of F-actin. Schematic diagram showing two stages of F-actin polymerization are. Yellow L-shapes indicate ATP-bound actin subunits. Red L-shapes indicate ADP actin, while yellow indicate ADP-Pi states. Association and dissociation rates are shown. K is critical concentration (Kd) of the actin monomers for each end and nucleotide condition. Image adapted with modification from Nicholson-Dykstra *et al.*, 2005.

1.2. Actin binding proteins

Polymerization and depolymerization of the actin filament in the live cells are controlled by more than 60 families of actin binding protein. These proteins are divided into different families that bind to the monomer, sever filament, cap filament end, nucleate filament, and crosslink filament. Actin binding protein will not function alone; two or more proteins interact to control the actin dynamics.

1.2.1. Actin monomer binding proteins

The main role of these proteins is to maintain the amount of un-polymerized actin in the cytoplasm by binding to the actin monomer and cooperate with the capping proteins. These proteins also help to regulate the binding of actin to the nucleotide [7].

1.2.1.1. Profilin

Profilin is an actin binding protein and play an important role in the dynamic turnover and restructuring of the actin cytoskeleton and It is a small globular protein of 125-139 residues [11]. Binding affinity of the profilin to cytoplasmic ATP- actin monomer is higher than the cytoplasmic ADP-actin monomer and muscle actin [12]. Profilin also bind to the polyproline sequence on the formins and to help the processive elongation of the actin filament. Profilin can block the elongation and nucleation of the actin filament by binding on the barbed end of the actin monomer. Binding affinity of profilin to the actin filament is low, because binding site on the barbed end of the actin is hidden

in the filament. After profilin actin complex bind to the barbed end, profilin suddenly dissociates from the profilin actin complex [7].

1.2.1.2. Cofilin

Binding affinity of the cofilin is high to the ADP-actin compared to the ATP-actin and cofilin also help to inhibit nucleotide exchange. The main role of cofilin is to attach to the ADP- actin filament and destabilizes the filament [13, 14].

1.2.2. Actin filament nucleation factor

Nucleating factors like Arp2/3 complex, formin, and spire helps to generate different actin structure in the mammalian cells (Fig. 1-2, Fig. 1-3). The Arp 2/3 complex first attaches to the side of a pre-existing actin filament and nucleates a new filament 'branch' at an angle of ~70^{o} [15]. This process generates branched network of actin filaments [7]. Repeated branching of actin filament leads to the formation of dendritic network of actin filament.

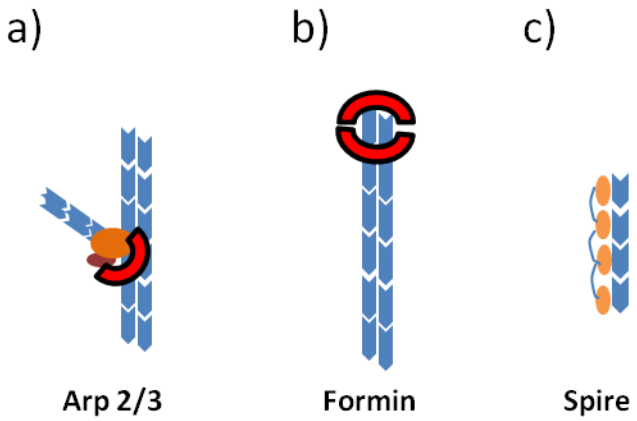


Figure 1-2. Actin nucleators. a) Arp2/3 complex (color). b) Formin (red circle). c) Spire. Image adapted with modification from, Chhabra *et al.,* 2007.

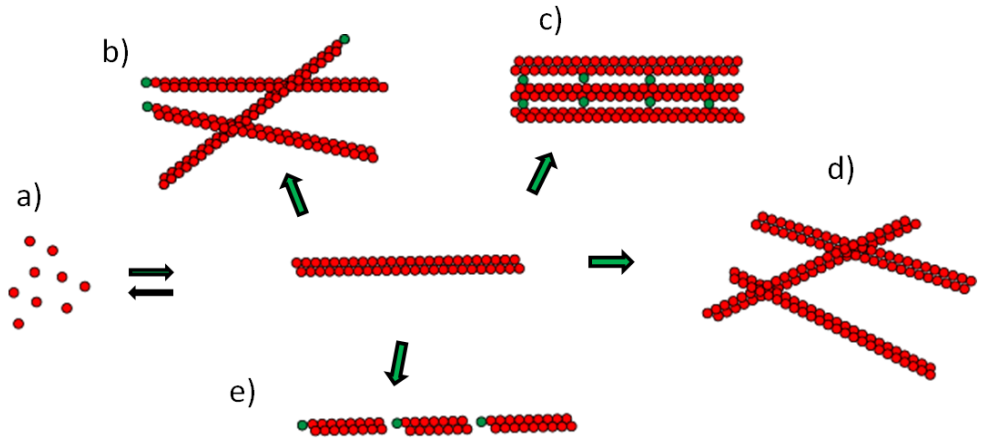


Figure 1-3. Organization of F-actin via actin binding proteins. a) monomer sequestration, b) capping of filaments, c) parallel or anti-parallel bundling of filaments, d) filament cross linking, e) filament severing. Image adapted with modification from book chapter Alberts, 2008.

Formin is a single polypeptide and it has multiple domain of proteins [16]. All formin are dimeric and FH2 domain in the formin is responsible for the nucleation of the actin. After the nucleation, FH2 domain remains in the barbed end of the actin filament and processivly move together with the barbed end. Actin filament formed with the help of formin- nucleated is not branched [16]. Spire is the other class of actin nucleators that were initially found in Drosophila [17]. Spire protein is made of single polypeptides, this polypeptides contain four Wasp homology (WH2) motifs and this motifs help to nucleate actin [18]. Similar like formin spire-nucleated actin filaments are also formed straight bundle. Once the actin filament is formed, various types of regulation factors, which are controlled by various signals, help to form various actin structures described previously.

1.2.3. Capping proteins

Capping protein usually can bind to barbed or pointed end of the actin filament. Addition of the capping protein on the barbed or pointed end will block the addition and dissociation of the actin subunit. Recent studies showed that capping protein also can stimulate the formation of new actin filament [14].

1.2.3.1. Gelsolin

Gelsolin bind to the side and barbed end of the actin filament and block the association and dissociation of the actin subunit. Gelsolins can also able to bind the actin dimer [14].

1.2.3.2. Tropomodulin

Tropomodulin protein is attached to the pointed end of the stable actin filament in different cell type such as red blood cells, muscle, and cells of other higher organism. Tropomodulin bind to the pointed end with the help of tropomyosin [14, 19].

1.2.4. Actin filament severing protein

Gelsolin, fragmin/severin and ADF/cofilin are the Main classes of protein helps to sever the actin filament. In the presence of micro molar concentration of Ca^{2+}, domain 2 of the gelsolin attached to the side of actin filament and domains 1 attached between the subunit of actin filament disrupt the filament.

Fragmin and severin also sever the actin filament in the presence of Ca^{2+}. ADP/cofilin severs and depolymerize the actin filament independent of Ca^{2+} [14, 20].

1.2.5. Actin filament cross linking proteins

Cross linking proteins help to attach two actin filaments and help to stabilize the higher order assembly of the actin filament [6, 21]. Depending on the protein and length of the actin filament, they can arrange into random network or regular bundle of actin filaments. Alpha- actin is one of the actin cross linking proteins in the cell [22]. It shows different function in different cell. Alpha-actin is an important cross linking protein found in the cortical actin network, it localizes with the stress fibers and also localized to the Z-disc of striated muscles. Fimbrin and villin, the actin cross linking proteins are usually observed in the microvilli [23]. Actin bundles in the microvilli are stabilized by these proteins. Cross linking proteins like spectrin helps to attach the actin filament to the membrane proteins [24, 25].

1. 3. Actin binding proteins (ABPs) and their involvement in disease

Actin filament plays a significant role in maintaining the shape and mechanically stability of the cells. It also has an important role in the cell division, cell migration, and organelle transportation. Actin filaments can assemble into higher order structure such as actin bundles or actin fibers. Assembly and dynamics of the actin bundle or fibers are controlled by different actin binding proteins (ABP) such as Arp2/3, cofilin and profilins. Impaired function can lead to problems in different cellular function such as,

1) polymerization and assembly dynamics; 2) formation of filament assembly and network; 3) interaction with membrane and ECM; and 4) actomyosin based contractility. Disruption of any of these cellular functions is associated with a variety of human disease such as Wiskott- Aldrich syndrome (WAS), cancer, deafness**,** neurological defects, and muscular dystrophy.

1.3.1. Pathogenic Bacteria

Many of the bacteria enter the cells hijack cellular actin binding protein and use this protein to propel through the cytoplasm. *Listeria, Shigella, Rickettsia, Burkholderia* and *Mycobacterium* are some of the bacteria that use the cytoplasmic actin to propel through the cytoplasm. Usually bacteria will not destroy the ABP function, rather use the ABP for the propulsion through the cytoplasmic and make a protrusion on the cell membrane and enter into the adjacent cell [26]. Not all bacteria enter into the host cells, some of them like Enteropathogenic *E. coli* and enterohemorrhagic *E. coli* do not enter the host cell. These bacteria make a large membrane protrusion filled with actin filaments, called “pedestals” [26-28]. Surface proteins on the *Listeria* and *Rickettsia* can recruit Arp 2/3 complex and promote nucleation of actin [26].

1.3.2. Cell motility

Studies showed that a mutation in the gene encoding WASp is the major reason for the Wiskott- Aldrich syndrome (WAS). Patients with WAS suffer from immunodeficiency and reduced platelet number and size. Another characteristics of WAS are defect in actin polymerization and cell adhesion and cell motility in cells like macrophages, dendritic cells, and lymphocytes

[29]. Cancer cells are more migratory than the normal cells. Studies showed that certain ABPs such as cofilin and its upstream regulators (LIM kinase and PAK), capping proteins, Arp2/3 subunits and N-WASp are up regulated in the cancer cells [30]. Studies also show that actin-severing proteins like gelsolin has displays decreased expression in cancer cells [31, 32].

1.3.3. Deafness

Detection of sound and maintain the balance of the human body mainly depends on the stereocilia in the inner ear. Stereocilia is specialized microvilli like projection and made up of long parallel actin filament. Ion channel, cell adhesion receptor, and signaling complexes are connected to stereocilia through the actin fibers. Actin bundling proteins radixin or espin play an important role in hearing. Mice lacking radixin and espin shows degeneration of a stereocilia and it will lead to hearing loss [33, 34]. Studies showed that one of the reason for the inherited deafness in human is, mutations in the actin binding protein espin.

1.4. Different faces of actin

1.4.1. Lamellipodia

Lamellipodia are surface attached sheet-like membrane protrusion formed when the cells are starting to migrate. The electron microscope images showed the thickness of the lamellipodia ~ 100-300 nm and made up of network of actin filament [35-37] . These structures are less adherent, highly dynamic, and devoid of organelles [38]. Activation of the nucleation and elongation factors on the lamellipodium tips helps to polymerize the actin fibers towards the plus end and it will exert a pushing force on the cell membrane [39-41]. Polymerization of actin filament at the leading edge and

turnover at the back of the lamellipodium gives rise to the retrograde flow of the actin network in the lamellipodia [42-44]. A study showed that, lamellipodia undergoes pauses and retraction [35], in addition to retrograde flow, related with reorientation of the actin filament [45]. Evidences showed that filopodia also can generate from lamellipodia actin filaments [46]. Electron microscope picture reveal that lamellipodia are made up of branched actin networks [15]. *In vitro* experiments have demonstrated that branching of the actin filament is catalyzed by the activated Arp2/3 complex. Changing the activity of the Arp2/3 complex using either an over expression of central acidic (CA) or WH2 central acidic (WCA) constructs block the lamellipodia assembly [47-49]. Inhibition of Arp2/3 by using microinjected antibody , on the dendritic branching of actin, also showed the inhibition of lamellipodia protrusion [50]. Significant loss of lamellipodia in mouse cells was observed after inhibition of the subunit of Arp2/3 complex [51]. Arp2/3 complex is the dominating nucleator in the lamellipodia region and the Arp2/3 complex is used as a marker for lamellipodia [52, 53].

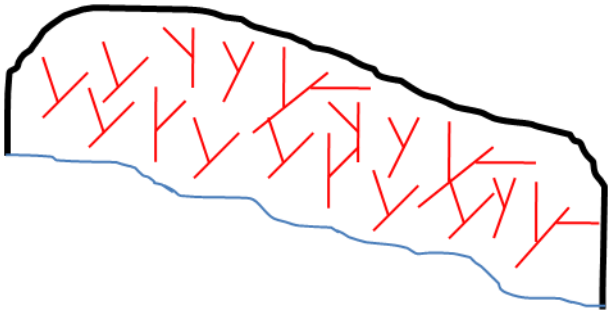


Figure 1-4. Lamellipodium. a) Red color showing the branching of actin. Image adapted with modification from Chhabra *et al.,* 2007

1.4.2. Filopodia

Filopodia are cytoplasmic protrusion from the leading edge of many migrating cells such as fibroblast, and neuronal growth cone. The electron microscopic pictures showed that filopodia are made up of long actin filament [54-56]. Usually filopodia are present in the leading edge of migratory cells. With the help of cryo-electron microscopy studies in the Dictyostelium showed that short filaments are also present in the filopodia and the tip of the filopodia filaments arranged in a meshwork pattern [57]. Depending on the cell types, after nucleation, the filopodia start to elongate with an elongation rate of 1 to 5 µm/min [58]. The elongation rate and the direction of elongation of filopodia can be altered during the time of filopodia elongation. The elongation rate of filopodia depends on the number of the actin monomers adding to the tip of the filopodia and the retrograde flow is related to the de-polymerization of actin at the base [59]. Different types of molecules are involved in the bundling of the actin fiber inside the filopodia, fascin being one of the main molecules that bundle actin filaments and also Eva/Vasp proteins are co-localized within filopodia. Interestingly, recent studies show that filopodia can even sense the chemical gradients [60].

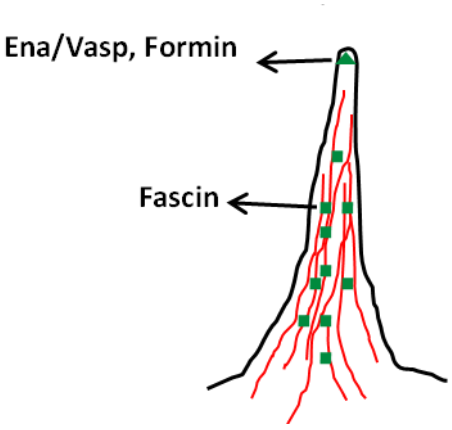


Figure 1-5. Filopodia. Cartoon of a filopodia. Image adapted with modification from Chhabra *et al.,* 2007.

1.4.3. Microvilli

Microvilli are microscopic protrusions on the plasma membrane. Microvilli are covered with cell membrane and it contains the cytoplasm and actin filaments, and cellular organelles are usually not see in the microvilli. Microvilli are predominantly present on the surface of intestine and kidney epithelium [61, 62]. However, it is also observed on the cells in sensory organs such as inner ear, taste buds, olfactory receptor and other epithelial cells. Recent studies show that microvilli also help to segregate plasma membrane proteins [63, 64]. Each microvillus contains long parallel actin filaments arranged in a bundle. Around 20-30 actin filament are cross linked by the actin binding proteins fimbrin and villin [61]. In addition, myosin 1a connects actin filaments with the surrounding cell membrane. The barbed ends of the actin filaments face the tip of microvilli and are capped.

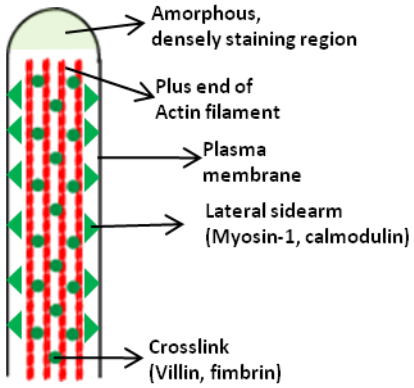


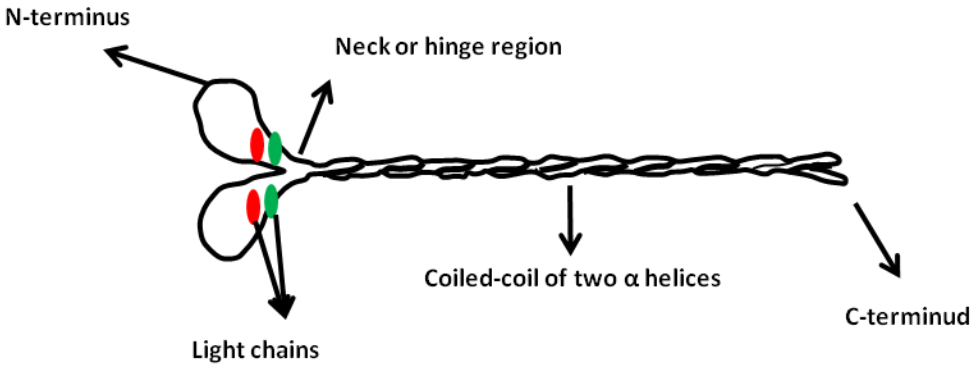
Figure 1-6. Microvilli. a) Cartoon of a microvilli. Image adapted with modification from book chapter Alberts, 2008.

1.5. Non-muscle myosin II

Myosin is actin-based motor proteins which play an important role in generates force in eukaryotes. Previous studies have revealed that there are at least 25 different classes of myosins which play important roles in various cellular processes [65, 66], such as organelle transport, cytokinesis, cell polarization, intracellular transport, and signal transduction [67]. Myosin II is one of the main classes of the myosin family and its main function is to generate force inside the cell together with the actin. Myosin II, which is present in all eukaryotic non muscular cell, are called non-muscle myosin and myosin II, which is present in muscle cells, are called muscular myosin. Muscular myosin-II has specific functions like skeletal muscle development and differentiation. Also myosin II has a role in maintaining the tension inside the smooth muscles [68-70]. Non-muscle myosin II is made up of three pair of peptides, a pair of heavy chains of 230 kDa, a pair of 17 kDa essential light chains (ELC), and a pair of 20 kDa regulatory light chains (RLC) (Fig. 1-8). Globular 'head' domain of the myosin-II is followed by a neck region which acts as a lever arm for producing motion. Myosin-II exerts tension on actin filament by coupling a conformational change with ATP consumption. In this way, chemical energy is transformed into mechanical work. Two essential light chains (ELC) and two regulatory light chains (RLCs) are also attached to the neck domain of the myosin-II (Fig. 1.7). Neck domain of the myosin II is followed by the long α-helical coiled coil domain. This coiled coil domain helps

to the dimerization between the heavy chains. The end of the coiled coil domain terminates short non-helical tail. Previous studies have identified three isoforms of non-muscle myosin-II:NMIIA, NMIIB, and NMIIC. ATP hydrolysis ratio of NMII A is higher than the other three isoforms of myosin II [71]. Recent studies showed that NMIIB play an important role in the smooth muscle contraction [69]. N-terminus of the myosin II is involved in the enzymatic and motor activity. C-terminal domain of the myosin II includes the coiled coil and non-helical part of the myosin II heavy chain that help to form myosin filaments and its intracellular localization [67].

Myosin II filament can produce tension on the actin filament by walk on the actin filament. Acto-myosin contraction is a cyclic process. At the starting of the cyclic process, myosin II is attached to the actin filament, this state will exist only for a short time. Myosin will detach from the actin as soon as ATP is bound to the myosin II head. After ATP binding on the myosin II leads to the detachment of myosin II from actin filament. This will reduce the affinity of myosin II head from actin and myosin head can now move freely along with the filament. ATP attached to myosin II head undergoes hydrolysis and forms ADP and inorganic phosphate (p_i), ADP and (p_i) that are tightly attached to the myosin head domain. Hydrolysis of ATP to ADP creates large conformational changes of myosin II and the head moves to a distance of 5 nm. Myosin II will attach to the new site on the actin filament and the attachment of myosin II to the actin filament cause release of Pi. This process will trigger the power stroke and myosin II head will regain the original conformation (Fig. 1-8).



1- 7. Structure of non-muscular myosin II. Image adapted with modification from book chapter Alberts, 2008.

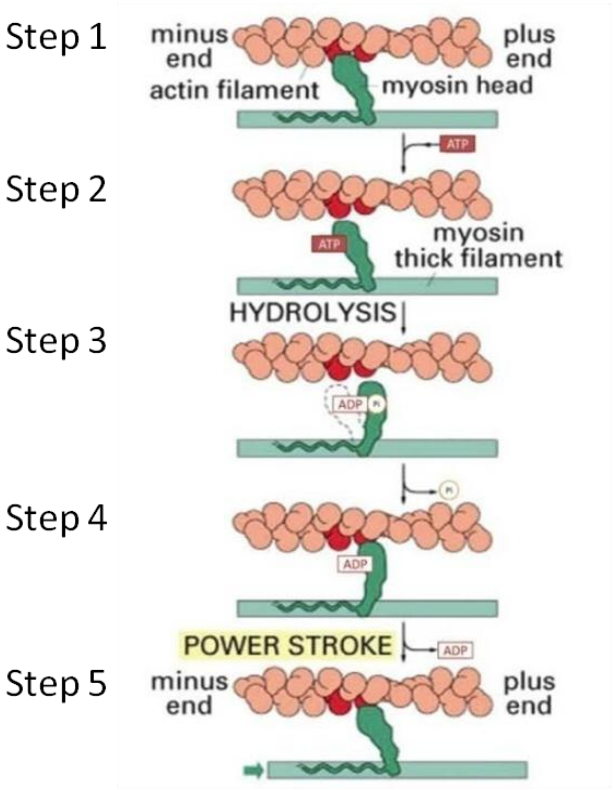


Figure 1-8. Force generating mechanism of myosin II.Step1. Beginning of the cycle myosin II attached to the actin. **Step2**: Binding of the ATP change the conformation of the myosin II and myosin II detached from the actin filament. **Step3**: ATP hydrolysis into ADP and inorganic phosphate (p_i) Hydrolysis of ATP also create a large conformation change in the myosin II head and displace along the actin filament about 5nm. **Step4:** Myosin II bind to the new site of the actin filament will cause of release of p_i, this will generate the power stroke. **Step5**: Myosin II comes to the original position. Image adapted with modification from book chapter Alberts, 2008.

2. Dynamic microvilli and myosin II filaments organize the apical cell cortex in non-confluent epithelial cells

2. 1 Introduction

The cytoskeleton is a central player in the detection, transmission, and generation of mechanical forces that determine individual cell shape as well as collective cellular behaviors underlying tissue organization. Whereas microtubules and intermediate filaments pervade the cytosol, actin filaments are largely restricted to the cell periphery or cell cortex [72], where they facilitate mechanical stabilization and remodeling of cell shape. Numerous morphogenetic processes are actively driven by actin dynamics, including cell polarization, migration, division and differentiation [73, 74]. To perform its diverse functions, actin is assembled into bundles and networks of filaments that are in turn connected to the extracellular matrix or neighboring cells via adhesion receptor complexes [75-77]. Mechanical forces are generated by actin polymerization and depolymerization or contraction mediated by myosin motors [78].

In contrast to most other cell types, epithelial cells (ECs) organize into adherent groups that form boundary layers for tissue compartments in multi cellular organisms. Because of their exposed location, ECs need to generate as well as withstand significant mechanical stress during tissue remodeling in development, growth and wound-healing response. It is therefore of interest to understand specific structural and dynamical aspects of cortical

organization and remodeling that enable cells to generate and sustain such forces.

Individual cells within closed epithelial layers are polarized along an apical-basal axis. This is reflected in the organization of cytoskeletal elements, membrane trafficking and ion transport [79], and it also includes cortical actin organization. At the basal surface of single-layer epithelia, actin forms focal adhesions and stress fibers. These acto-myosin bundles, consisting of anti-parallel actin filaments cross linked by myosin II, connect adhesion sites and exert pulling forces on the underlying basement membrane [80]. The most prominent actin structure in many polarized ECs is a lateral circumferential ring of actin filaments, which are connected to cadherin-based adherent junctions [81]. This junctional actin is thought to stabilize cell-cell contacts and to be the principal structural source of force transmission within the epithelia [82]. Finally, the apical surface of epithelia is decorated by numerous microvilli. These membrane protrusions consist of bundles of parallel actin filaments and extend up to several microns from the cell surface. In the brush border of epithelia in the small intestine and kidney, up to 15,000 short (0.2-2 µm) microvilli per cell [83] are thought to increase cellular surface area for nutrient uptake and ion exchange. These microvilli are interconnected at their base by a dense meshwork of actin, spectrin and myosin filaments called the terminal web [84, 85]. Outside of highly specialized tissues, ECs often form actin-containing apical protrusions at considerably lower density and with diverse morphologies, including tube-like protrusions [86, 87], ridges [88] and ruffles [86, 89]. Organization, molecular composition and biological functions of these protrusions are largely unknown [90].

During developmental processes such as gastrulation [91] as well as during wound-healing, ECs undergo drastic shape changes to facilitate large-scale tissue morphogenesis. In many of these processes, ECs undergo epithelial-mesenchymal transition (EMT), where they acquire morphological features that are reminiscent of mesenchymal cells or mesenchymal stem cells [92-94]. Specifically, cells down-regulate cell-cell and cell-matrix contacts [95, 96] and change their typical epithelial apical-basal polarity to a front-rear polarity that is characteristic of migratory cells [79]. Notably, actin is organized in lamellipodia and filopodia at the leading edge of such cells [97]. EMT can occur during embryonic development such as in neural crest cell delamination and migration [94] and has also been proposed to underlie the metastatic potential of epithelial tumors [98].

While there has been significant progress in understanding the molecular basis for the regulation of EMT [99], our understanding of the changes in the apical cortex organization in this process remains very limited [100, 101]. In particular, we lack information on the dynamics, and biological role of apical actin-based protrusions during epithelial reorganizations.

2.2. Results

2.2.1. Cortical actin organization in ECs

So far, people studied the organization and dynamics of actin on the basal region of the cells. Here, we characterize and compare the structural organization and dynamics of actin at the cortex of confluent/polarized vs. non-confluent/migratory ECs. We show that the apical surface of confluent

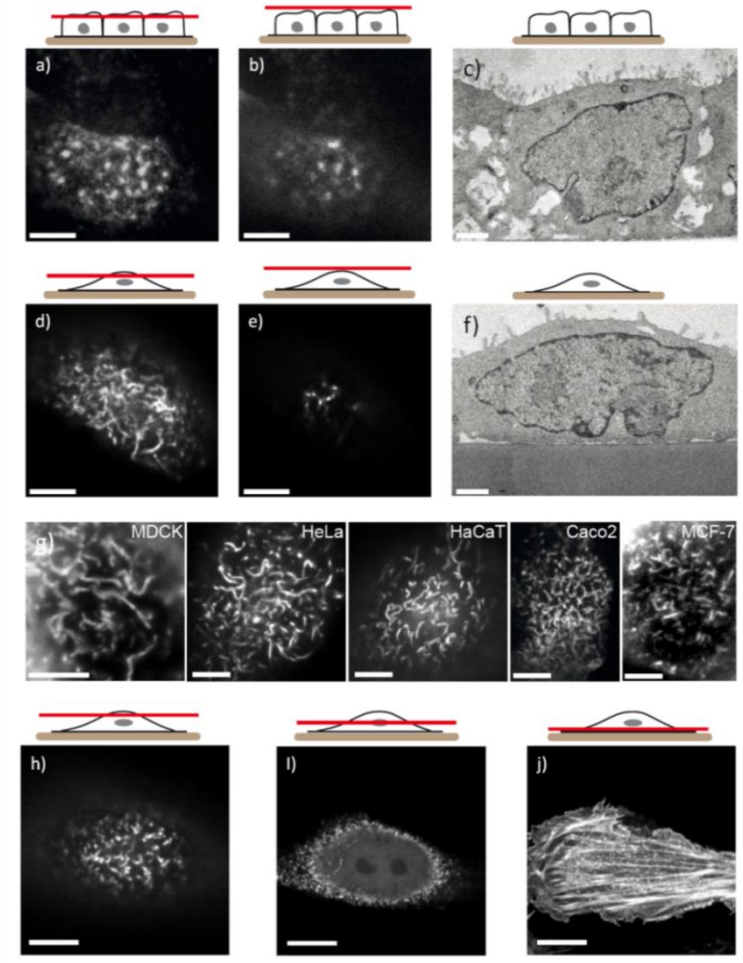
ECs is decorated by dense arrays of static microvilli, whereas non-confluent ECs form a smaller number of highly dynamic microvilli-like actin protrusions. These structures exhibit complex reorganization through bending, fission and fusion events. Individual dynamic microvilli are mechanically coupled in an isotropic network, and the dynamics and inter-connectivity of dynamic microvilli depend on the activity of type II myosin. Interestingly, myosin II itself is also assembled into a pre-stressed network of filaments that is integrated with the network of dynamic microvilli. We demonstrate that formation of dynamic microvilli and apical myosin II networks is induced during cells undergoing EMT. We propose that the identified apical acto-myosin network provides a readily available reservoir for active actin microfilaments to support rapid changes in cell shape and promote membrane relocalization during cell migration. Dynamic microvilli could be useful in identifying early stages of wound healing and EMT during cancer invasion and metastasis or developmental processes.

To investigate cortical actin organization in ECs, we stably transfected Madine Darby Canine Kidney (MDCK) cells with the F-actin marker Lifeact-GFP [102]. This revealed the apical surface of confluent cells to be covered with dense arrays of microvilli (Fig. 2-1a, c) that extended 1–2 µm above the plasma membrane (Fig. 2-1b, c). The presence of microvilli in these cells was confirmed by electron microscopy (EM), which allowed their unambiguous identification despite small size and high density (Fig. 2-1c). In contrast, when we observed non-confluent cells, we found a large number of elongated actin structures at the apical surface (Fig. 2-1d). Note that we use the term *apical* to describe the cell surface distant from the glass substrate, irrespective of the polarization state of cells. Formation of elongated actin structures is not

attributable to Lifeact-GFP expression because similar structures were observed using actin-mRFP in live cells (Fig. 2-2a) or Rhodamine-phalloidin in fixed cells (Fig. 2-2b). In contrast to the relatively straight and extended nature of filopodia, classical microvilli, and stress fibers, the observed apical actin structures were typically curved or wavy (Fig. 2-1d) and are reminiscent of microvilli-like protrusions and ridges described previously [87, 88, 103]. Dynamic microvilli extending parallel to the apical surface, non-confluent ECs also contained a low number of microvilli-like protrusions perpendicular to the surface that could clearly be distinguished by spinning disk (Fig. 2-1e) and electron microscopy (Fig. 2-1f).

Next we tested whether dynamic microvilli were cell-line specific by imaging a variety of cell lines transiently transfected with Lifeact-GFP. Interestingly, all non-confluent cells of epithelial origin examined, including MDCK, Hela, HaCaT, CaCo2, and MCF7 cells (Fig. 2-1g), exhibited dynamic microvilli on their apical surfaces. Optical sectioning by confocal spinning disk microscopy revealed that dynamic microvilli were restricted to the cell cortex (Fig. 2-1h-j) and absent from the basal cell surface, which instead contained stress fibers, lamellipodia and focal adhesions (Fig. 2-1j). Because MDCK cells serve as a model system for the study of EC polarization [79], we chose to focus on this cell line for further characterization of the structure, dynamics, and origin of the observed dynamic microvilli. First, in order to investigate the relation between dynamic microvilli and elongated apical cell protrusions described previously [86, 88], we sought to determine the extent to which apical actin structures extended away from the cell surface. Optical sectioning and 3D rendering revealed that the apical cortex of MDCK cells contained dynamic microvilli that extended parallel to the surface (Fig. 2-3a, Dynamic microvilli)

as well as microvilli-like protrusions that were oriented perpendicular to the membrane (Fig. 2-3a, microvilli). Re-constructions indicated that dynamic microvilli formed structures with considerable depth up to 1 µm. This became also apparent from overlays of several planes taken at 500 nm apart (Fig. 2-3b, red shows most basal plane, followed by green and blue). In the apical areas shown, it was evident that some dynamic microvilli were restricted to the lowest plane (Fig. 2-3b, arrows). While others extended to more apical planes either diagonally (Fig. 2-3b, arrow) or perpendicular to the surface



F**igure 2-1.Actin organization at the apical surface of ECs**. a-f) Apical actin organization and surface topology of confluent (a-c) and non-confluent (d-f) MDCK cells. Lifeact-GFP labels dense clusters of microvilli in confluent cells (a, b), whereas curved and elongated dynamic microvilli are typically observed in non-confluent cells (d). Electron micrographs show the reduced number of surface protrusions in non-confluent cells (c, f). g) Lifeact-GFP labels dynamic microvilli on the apical surface of different non-confluent EC cell lines (MDCK, HeLa, HaCat, Caco2, MCF-7)0. (h-j) Lifeact-GFP labels dynamic microvilli at the apical (h) and lateral (i) surfaces of dome-shaped non-confluent MDCK cells. Basal surfaces instead exhibit stress fibers and lamellipodia (j). Position of image plane indicated in schematic representations as red line. Scale bars: 5 µm, c, f: 2 µm.

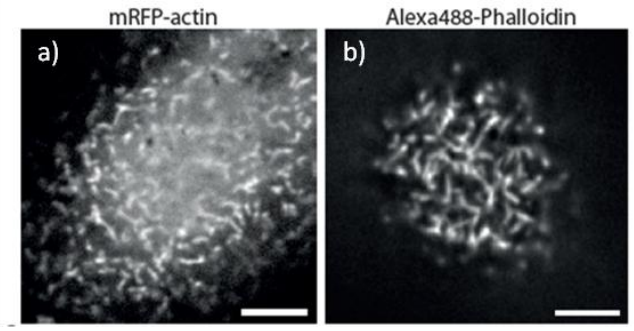


Figure 2-2.microvilli in MDCK cells. a) Localization of mRFP-actin in a live cell. b) Alexa488-phallodin labeled actin structures in a formaldehyde fixed cell. Scale bars: 5 µm.

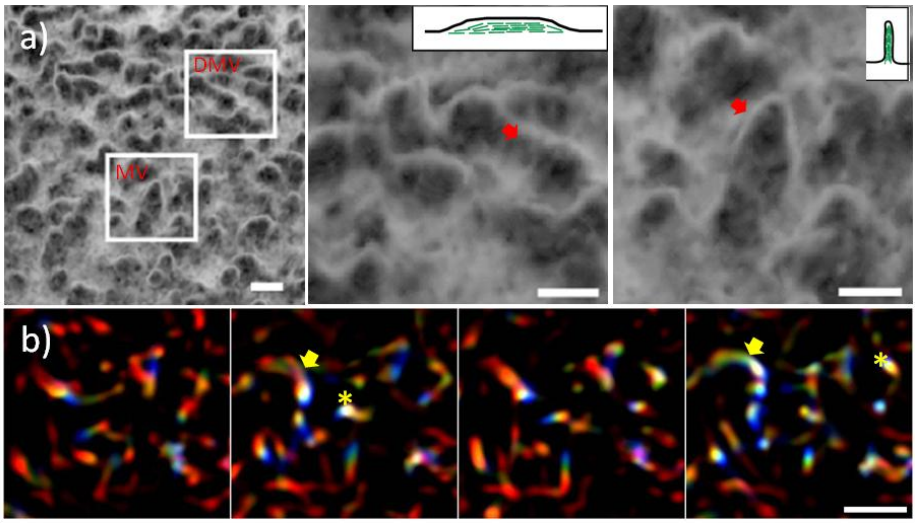


Figure 2-3. Topology of dynamic microvilli. a) Rendered representation of an image stack covering the apical surface of non-confluent MDCK cell. Examples of an dynamic microvilli (DMW) and microvilli like protrusion are shown with red arrows in zoomed images. Proposed orientation of actin filaments is shown in the schematic. b) Color coded projection of three focal planes taken 500 nm apart. Dynamic microvilli restricted to the lowest plane are indicated with arrows and microvilli-like structures extending perpendicularly from the surface are indicated with asterisks. Blue: top plane, green: middle plane, red: bottom plane. Scale bars: 2 µm

(Fig. 2-3b, asterisks). The latter structures often spanned 3 planes, corresponding to a depth of 1.5 µm. The elongated protrusions observed in several epithelial cell types by scanning EM [86, 104] suggest that dynamic microvilli might help to shape the plasma membrane. Indeed, we found that fluorescent collagen-coated beads that were bound to the apical surface of MDCK cells moved in concert with underlying dynamic microvilli, suggesting integration or association of dynamic microvilli with the overlaying plasma membrane(Fig. 2-4a,b).

In summary, we identified dynamic microvilli as a characteristic actin structure on the apical cortex of non-confluent ECs. When cells become confluent theses dynamic microvilli are turned into dense arrays of microvilli that protrude from the cell surface.

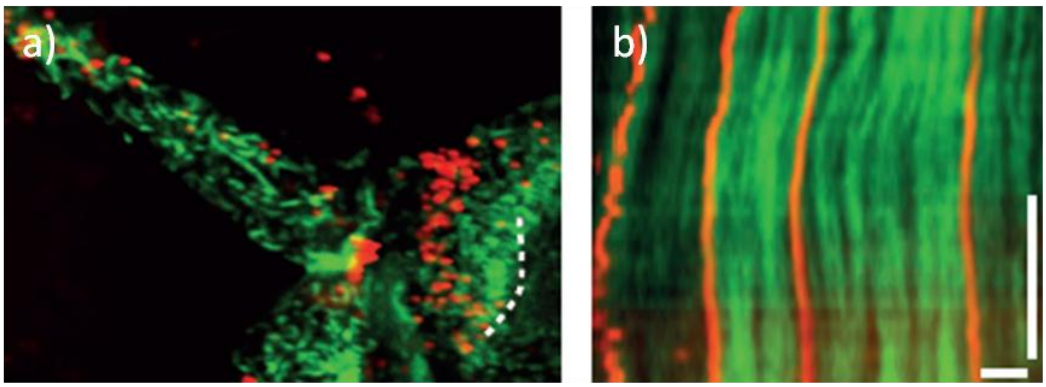


Figure 2-4. Joint movement of Dynamic microvilli and collagen coated beads on the cell membrane. **a)** Colocalization of red fluorescent collagen coated beads with Dynamic microvilli labeled with Lifeact-GFP. **b)** Joint movement is indicated by the kymograph taken along the dotted line. Scale bars: 200 µm. Time bar: 5 s.

2.2.2. Dynamics of microvilli

To further characterize dynamic microvilli, we investigated their dynamics using live-cell imaging and quantitative image analysis. Time-lapse movies, kymographs, and image correlation analysis of microvilli in confluent MDCK cells showed limited lateral motion over time-scales of 100 sec (Fig. 2-5a, c, d, decay rate: $0.2 \pm 0.1 \times 10^{-2} s^{-1}$, average ± s.e.m., n=22). This is in contrast to dynamic microvilli, which were morphologically dynamic and exhibited lateral mobility over large distances within the same time-scale (Fig. 2-5b). Dynamic microvilli typically reorganized within minutes with a decay rate of $2.4 \pm 0.7 \times 10^{-2} s^{-1}$ (average ± s.e.m., n=8, Fig. 2-5c, d). Lateral dynamic microvilli motions and reorganization involved changes in overall shape and connectivity to neighboring dynamic microvilli. Dynamic microvilli continuously changed in both their length and spatial position. We observed growth (Fig. 2-5e) and shrinkage (Fig. 2-5f) of individual dynamic microvilli. In addition, dynamic microvilli exhibited several types of motion that are not observed for other actin structures, such as fusion (Fig. 2-5g), and splitting (Fig. 2-5h). These structural rearrangements were responsible for the reorganization of dynamic microvilli observed on the time scale of one to several minutes (Fig. 2-6i).

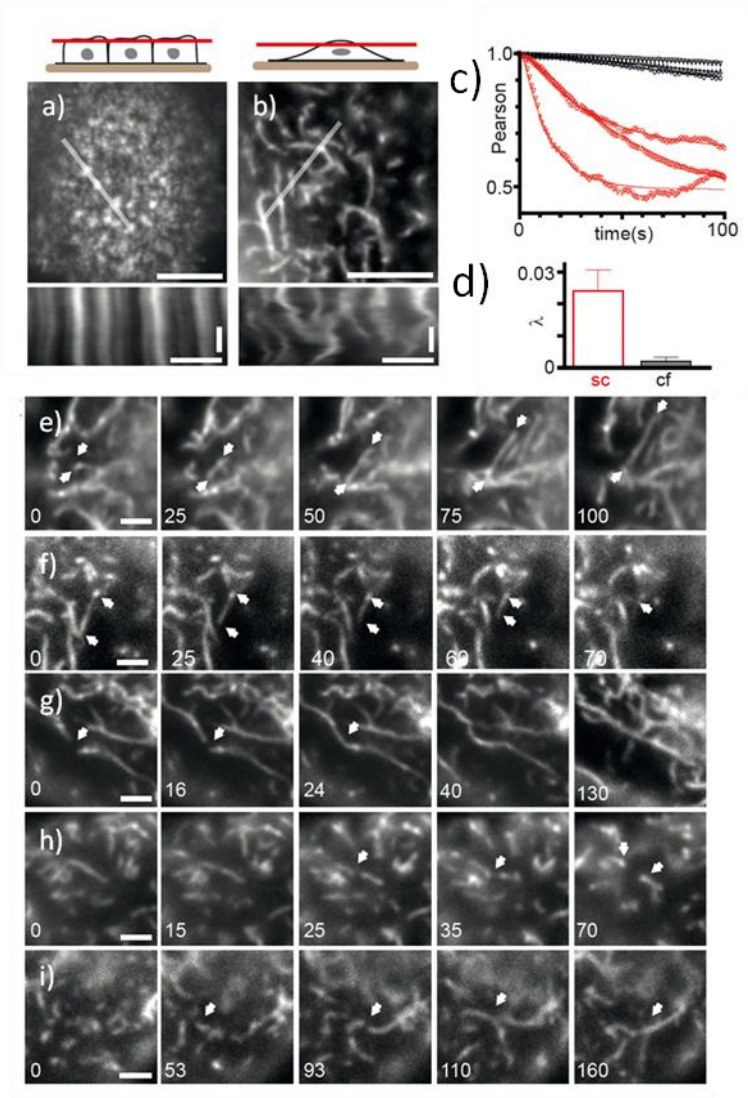


Figure 2-5. Dynamic microvilli. a-d). Dynamics of dynamic microvilli on the apical surface of MDCK cells expressing Lifeact-GFP (left panel). Kymographs indicate that microvilli clusters do not exhibit noticeable displacement within the observation time (Right panel) (a), whereas dynamic microvilli exhibit extensive lateral motion (b). The time-scale of dynamic microvilli rearrangements is quantified by the autocorrelation of the Pearson correlation coefficient between successive images (c, d). Actin signal in non-confluent cells (sc) de-correlates rapidly (red data points and curve fits), whereas the signal in confluent cells (cf) is largely constant (black data points and curve fits). e-i) Examples of dynamic microvilli in MDCK cells exhibiting growth (e), shrinkage (f), fusion (g), splitting (h) or reorganization (i). Arrows indicate dynamic microvilli of interest. Time in s. Scale bars: 2 µm, Time bars: 100 s.

While dynamic microvilli dynamics were in part observable in single imaging planes, the full extent of their structural dynamics and reorganization could only be captured using 4D imaging (Fig. 2-6a, b). However, 3D reconstructions revealed protrusion and collapse of microvilli like structures as well as considerable rearrangement of dynamic microvilli across multiple planes.

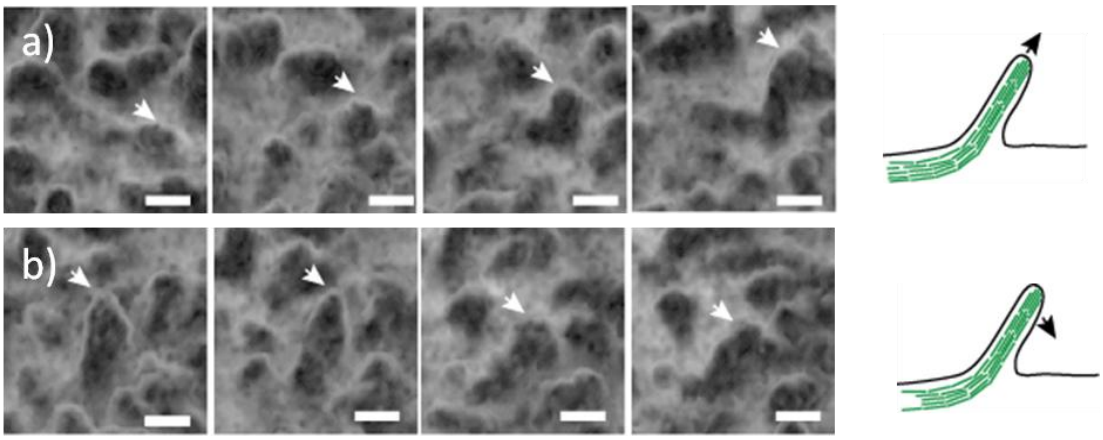


Figure 2-6. Dynamic microvilli. Time series showing extension (a) and collapse of individual dynamic microvilli (b). White arrows indicate structures of interest. Images are rendered representations of 3D stacks (planes with 300 nm distance). Schematics show assumed orientation of actin filaments. Time in seconds. Scale bars: 2 µm

Majority of dynamic micovilli movements that we observed did not occur along the dynamic microvilli long-axis but rather as lateral mobility of entire dynamic microvilli. Indeed, kymographs of longer time-courses revealed that dynamic microvilli exhibited slow lateral oscillations (Fig. 2-7a). It was also apparent that neighboring dynamic microvilli moved in a coordinated manner (Fig. 2-7a, kymograph 1), whereas distant dynamic microvilli were not

correlated in their motion (Fig. 2-7a, kymograph 2). Using spatio-temporal image correlation spectroscopy we analyzed how the degree of correlation between dynamic microvilli movements decayed with increasing separation between dynamic microvilli (Fig. 2-7b). The characteristic de-correlation length-scale R_0 of dynamic microvilli in non-confluent MDCK cells was 3.1 ± 0.8 µm (average ± s.e.m., n = 16, Fig. 2-7c) with some dynamic microvilli exhibiting correlated motions despite separations of up to 10 µm (Fig. 2-7a, kymograph 1).

The correlated motion of neighboring dynamic microvilli suggested a physical linkage into a network that spans the apical cell surface. In addition, the observed oscillatory motions indicated the presence of active processes driving dynamic microvilli motility (Fig. 2-7a). To gain further insight into the nature of force generation driving dynamic microvilli motility, we treated cells with low doses of the G-actin sequestering drug Latrunculin A (Lat A). Addition of 2 µM Lat A induced disruption of basal stress fibers within less than 15 min. Dynamic microvilli, however, were only affected after 15–30 minutes of incubation with Lat A. This argued against a major contribution of actin polymerization to the observed actin dynamic microvilli dynamics, despite the occasional dynamic microvilli elongation via polymerization noted above. After prolonged treatment (>30 min) with Lat A, dynamic microvilli became concentrated into a ring around the apical cell periphery, which subsequently began to narrow and eventually rupture at one or more locations (Fig. 2-7d). Importantly, cell integrity was not affected by rupture of the dynamic microvilli network (Fig. 2-7d, cytosolic background). The joint motion after rupture indicated that dynamic microvilli were physically coupled and subject to mechanical stress. In summary, we found that individual

dynamic microvilli exhibit complex rearrangements and are physically coupled with each other.

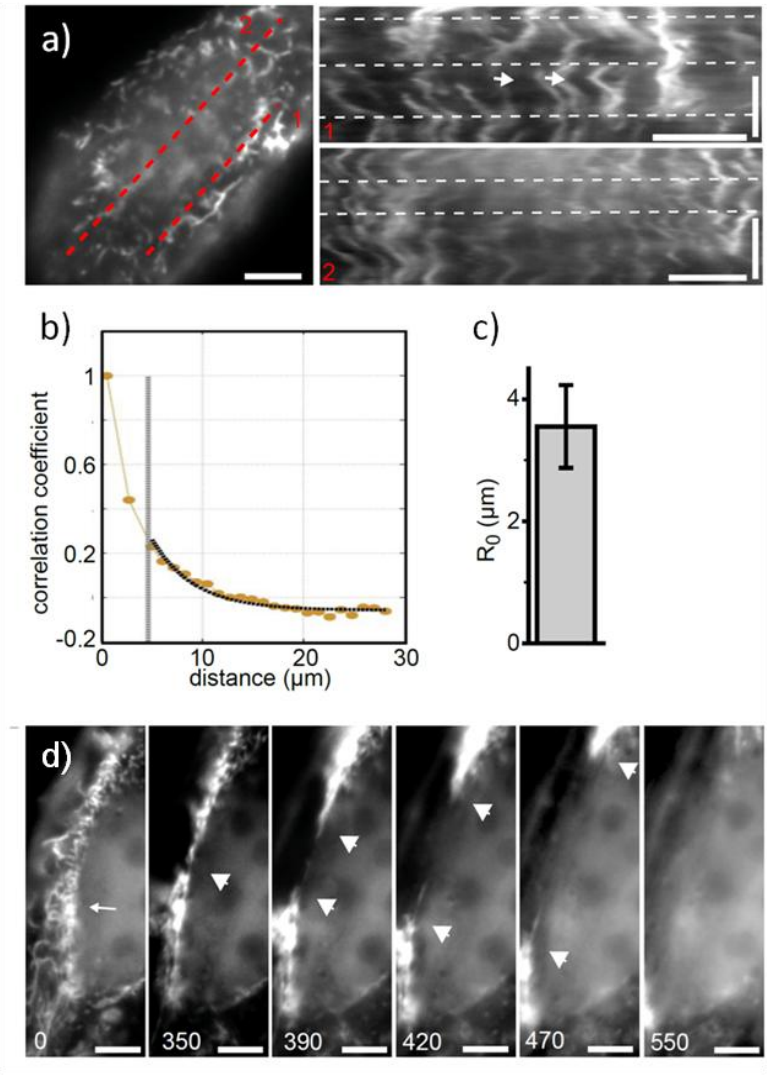


Figure 2-7. Lateral coordination of dynamic microvilli. a) Coordinated movement of neighboring dynamic microvilli indicated in a kymograph along line 1 and non-correlated movement of distant actin structures along line 2. Arrows indicate coincident change in direction of motion. b, c) STICS analysis of dynamic microvilli with a typical curve showing the spatial correlation of flow fluctuations (b) and characteristic correlation length-scale R_0 (c). The dotted grey line in B represents the

size of the STICS window and was used as cutoff for fitting. d) Sequence showing rupture of dynamic microvilli bundles after treatment with 2 µM Lat A. Note that the drug was added 30 min before start of the shown sequence. Time in s. Scale bars: 3 µm. Time bars: 500 s.

2.2.3. A cortical myosin II network drives dynamic microvilli organization and dynamics

With some exceptions [105, 106], generation of tension and contractile forces in actin networks is driven by molecular motors of the myosin family [78]. The most likely source of contractile forces in the observed actin dynamic microvilli network is therefore non-muscle myosin II. To directly test the role of this molecular motor on dynamic microvilli structural dynamics, we treated non-confluent MDCK cells with 50 µM of the specific myosin II inhibitor blebbistatin [107]. Remarkably, this led to a rapid reduction of dynamic microvilli dynamics (Fig. 2-8a). Dynamic microvilli reorganization in blebbistatin-treated cells was slowed down to $(1.42 \pm 0.15) \times 10^{-2}$ s^{-1} (average ± s.e.m., n=14, Fig. 2-8c, d) with some structures becoming completely immobilized (Fig. 2-8b). The fact that inhibition of F-actin polymerization by Lat A treatment did not immediately perturb dynamics but myosin II inhibition by blebbistatin did, suggest that myosin II activity and not F-actin polymerization/ depolymerization dynamics is essential to drive oscillation and reorganization of the dynamic microvilli network. To reveal the structural basis for myosin II mediated reorganization of dynamic microvilli, we generated MDCK cells stably expressing GFP fusions of myosin light chain (MLC) or myosin heavy chain A (MHCA). In confluent cells, MLC localized to patches (Fig. 2-8e), consistent with the previously reported localization of myosin II at the base of microvilli [108]. In contrast, we found that MHCA localized to filamentous structures at the apical surface of non-confluent

MDCK cells (Fig. 2-8g, h). These filaments were reminiscent of myosin filaments or mini filaments that have been described previously [109, 110]. However, unlike isolated filaments, cortical myosin II in MDCK cells formed a continuous branched network of filaments (Fig. 2-8f, g) (insets in Fig. 2-8f, g). Projections of 3D stacks revealed that myosin II networks spanned the apical surface of dome-shaped non-confluent cells (Fig. 2-8h). However, in contrast to dynamic microvilli, myosin networks were largely flat and therefore visible in a single optical plane. While individual myosin II filaments did not exhibit the characteristic bending or extension-shrinking behavior of dynamic microvilli, the entire network moved in an oscillatory pattern that was qualitatively similar to the slow dynamic microvilli oscillations described above (Fig. 2-8i). Myosin filaments remained characteristically straight instead of exhibiting bending motions like dynamic microvilli further suggested that the myosin filaments may be under tension. Importantly, spatially distant regions of the myosin II network appeared to move independently of one another suggesting that forces generated by myosin-driven contractions dissipated within the network.

To investigate the turnover of myosin subunits within filaments we employed fluorescence recovery after photobleaching (FRAP). For both MHCA and MLC we saw continuous and rapid fluorescence recovery of apical myosin filaments (Fig. 2-9a, b). The half-time of fluorescence recovery (MHCA: 1.6 ± 0.3 s, n = 19; MLC: 1.9 ± 0.7 s, n = 11; Fig. 2-9e) was somewhat slower than for cytosolic myosin (0.6 ± 0.1 s, n = 11; Fig. 2-9c, e) but significantly faster than for basal stress fibers (5.8 ± 2.2 s, n = 14; Fig. 2-9d, e).The observed oscillatory movements of the dynamic microvilli and myosin II networks with comparable spatial correlations in the networks suggested a mechanical coupling between myosin II filaments and dynamic microvilli. This hypothesis

is also consistent with the observed loss of dynamic microvilli organization and dynamics upon blebbistatin treatment, and conversely suggests that the myosin network could also depend on the presence of actin filaments to transmit and sustain forces generated by tension in the network. Indeed, treatment of MLC-GFP expressing MDCK cells with Lat A rapidly induced disruption of myosin II filaments into small patches that were evenly distributed along the cortex (Fig. 2-9f). During myosin II disassembly under Lat A treatment we observed that some filaments recoiled from an initial breakage point, suggesting that the network may be under mechanical tension (Fig. 2-9f, g). To further examine this recoil behavior we used laser ablation to cut individual myosin filaments at the apical cell surface. Ablation using low laser intensity was equivalent to FRAP, with the MLC-GFP signal recovering rapidly (Fig. 2-9h, j). In contrast, at higher laser intensity, that resulted in several myosin II filaments being severed along a line. we observed a rapid expansion of the lesion within seconds (Fig. 2-9i, j). The lesion mainly expanded perpendicular to the cut direction, consistent with a preferential loss of connections transverse to the cut and internal tension in the network (Fig. 2-9j). Interestingly, we observed slow and uniform recovery of myosin II structures within 1 min following ablation, suggesting that myosin II was capable of efficiently assembling into new filaments and to seal the laser-induced cortical lesion (Fig. 2-9i, j).

To examine how dynamic microvilli and myosin II filaments integrated structurally, we observed the two structures simultaneously in live cells. We found that dynamic microvilli overlapped with myosin networks but that individual filaments were not aligned with one another (Fig. 2-10a). However, numerous connections between myosin II filaments and dynamic microvilli were found to consist of alternating stretches of myosin II and actin (Fig. 2-

10b). These joint or hybrid filaments moved either as a single, continuous structure (Fig. 2-10c, asterisk), or as distinct, separate fragments (Fig. 2-10c, arrow). We also observed how bending of dynamic microvilli occurred when connected myosin filaments selectively pulled on one end of the dynamic microvilli (Fig. 2-10d, asterisk). In some instances the end of a myosin filament was seen to move along dynamic microvilli (Fig. 2-10e, asterisk). Taken together, these qualitative observations illustrate interactions that may give rise to the observed acto-myosin network dynamics.

In summary, we identified an isotropic network of actin and myosin bundles at the apical cortex of non-confluent EC. Dynamic microvilli and myosin filaments are interconnected and interdependent in their organization. The combined network is under tension, exhibits myosin-driven oscillatory contractions and is able to rapidly self-assemble after laser-ablation.

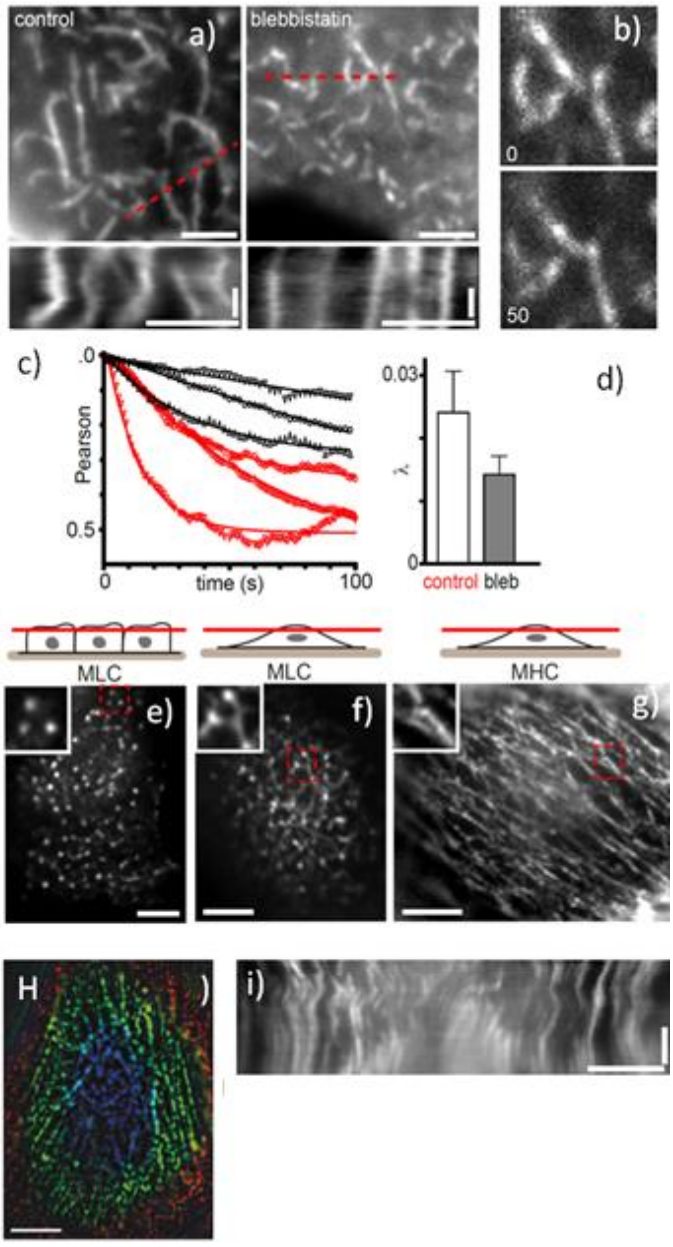


Figure 2-8. Role of myosin II in actin organization. a) Kymographs showing inhibition of dynamic microvilli movement after treatment with 50 µM blebbistatin for 30 min. b) Example of dynamic microvilli becoming completely immobile after blebbistatin treatment. c, d) Reduced de-correlation of actin signals after treatment with blebbistatin (black curves). Red control curves and constants identical to Fig. 3c, d and shown for comparison.e-h) Labeling of myosin II at the apical surface of

stably transfected MDCK cells. A GFP fusion to myosin light chain (MLC) localizes to patches in confluent cells (e) and networks in non-confluent cells (f). Networks are also seen with a GFP fusion to non muscle myosin II heavy chain A (MHCA). h) Color overlay of three focal planes separated by 1 µm showing a MHCA-GFP network covering the apical and lateral cell surface. Blue: top plane, green: middle plane, red: bottom plane. i) Kymograph showing coordinated movement of MHCA-GFP. Scale bars: 3 µm. Time bars: 50 s.

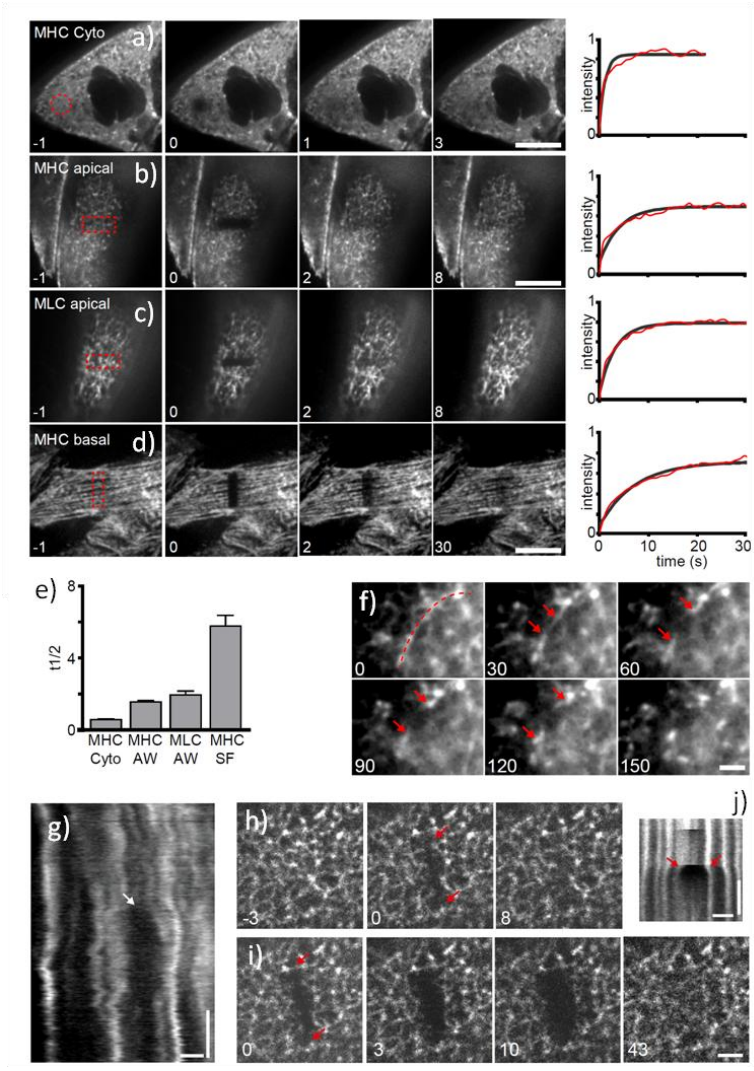


Figure 2-9. Myosin dynamics. a-d) FRAP of myosin II heavy chain (MHCA) in the apical network (b, c), in the cytosol (a) and in basal stress fibers (d). Shown are images before bleaching (-1 s) and at indicated times after bleaching. Bleached regions are indicated as red dotted boxes. Exemplary recovery curves are shown next to the images (data in red, curve fits in black). e) Recovery half-times for conditions in (a-d), shown as mean ± SD. f, g) rupture of a myosin II filament after treatment with 2 µM Lat A. Arrows indicate site of rupture. Kymograph (g) taken along dotted red line. h-j) FRAP (h) and ablation (i) of filaments within the apical myosin II network. Arrows indicate position of laser. i) Note the expansion of the lesion and distortion of neighboring filaments after ablation (i, j). All times in seconds. Scale bars: 3 µm. Time bars: 25 s.

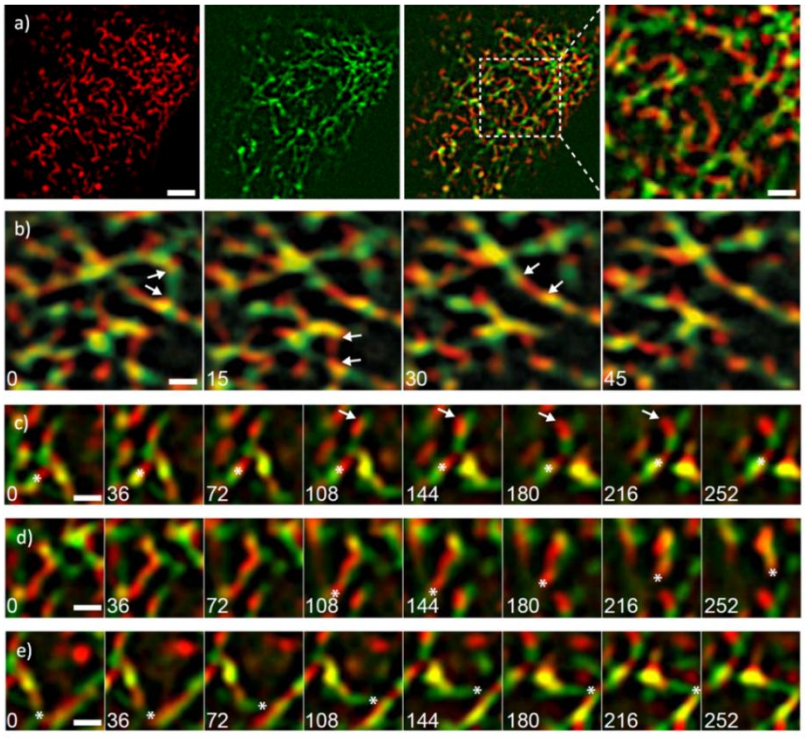


Figure 2-10. Coordination of dynamic microvilli and myosin II networks. a-e) Colocalization of transiently transfected actin-mRFP and MLC-GFP in non-confluent MDCK cells. a) Overview of overlapping actin and myosin structures with zoomed area. b) Alternating actin and myosin stretches (arrows) within a joint network. c-e) Coordinated motions of dynamic microvilli and myosin filaments. Structures referred to in the text indicated as arrows and asterisks. Scale bars: 3 µmin (a), 1 µm in (b-e).

2.2.4. Dynamic microvilli during wound-healing and EMT

To obtain more information about the biological role of dynamic microvilli, we analyzed the physiological conditions under which dynamic microvilli are formed. Usually, we detected prominent dynamic microvilli dynamic microvilli in non-confluent epithelial cells. These cells were dome-shaped, weakly connected to neighboring cells, and are able to migrate either as individual cells or small clusters. In contrast, confluent cells are tightly connected to their neighbors and only exhibit very slow collective movement. We therefore speculated that dynamic microvilli may support migratory behavior of ECs. We tested this hypothesis using scratch assays, an established *in vitro* model for wound healing [111]. We found that 24 h after wound induction cells moving into the scratch contained apical dynamic microvilli (Fig. 2-11a), whereas cells further away from the wound were covered with microvilli (Fig. 2-11b).

We next tested the effect of hepatocyte growth factor (HGF) on dynamic microvilli. HGF induces scattering of MDCK cells, characterized by dissociation of cell-cell junctions and induction of cell migration. This treatment is also used to mimic EMT in ECs [112]. As in the scratch assay, cells treated with 100 ng HGF formed prominent dynamic microvilli. After 12 h of incubation, abundant and elongated dynamic microvilli were found in all cells (Fig. 2-11c). At the same time the typical apical myosin II networks were formed (MLC-GFP, Fig. 2-11d).

To determine how quickly after HGF addition dynamic microvilli formed, we observed cells after shorter incubation times. Prior to HGF addition, highly

confluent cells contained dense arrays of microvilli on their apical surface (Fig. 2-11e, 0 min) and focused patches of junctional actin at lateral cell-cell contact sites (Fig. 2-11f, 0 min). After only 90 min incubation with HGF, a large number of dynamic microvilli had formed on the apical surface, while short actin bundles protruded from cell junctions (Fig. 2-11e, f, 90 min). This phenotype became even more pronounced after prolonged incubation, when cells were apically covered with dynamic microvilli, while the smooth actin staining at cell-cell junctions had turned into a meshwork of loosely connected bundles (Fig. 1-11e, f, 270 min). These junctional actin bundles resembled dynamic microvilli in shape and also shared their characteristic dynamical behavior including correlations in movements of neighboring bundles and oscillatory motions (Fig. 2-11g). To further address a possible connection

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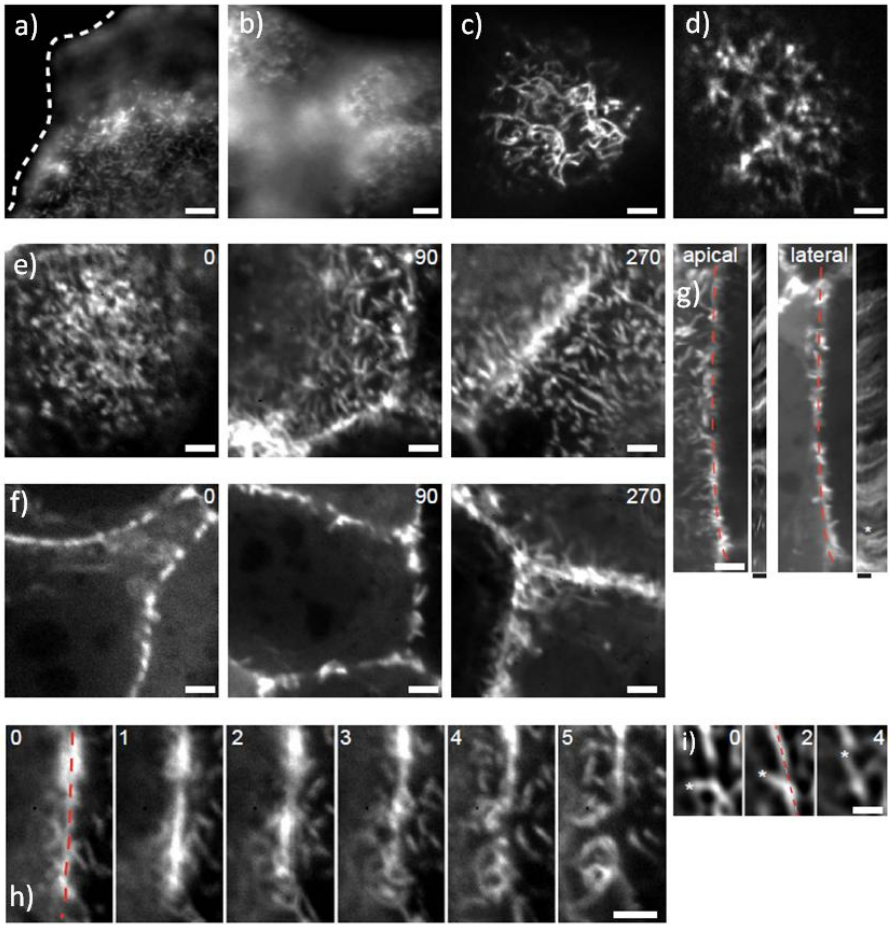

Figure 2-11. Dynamic microvilli are typical for migratory MDCK cells. a, b) Dynamic microvilli appear in leader cells migrating into a wound (a) but not in cells distant from the wound. Wounds were generated by mechanical scratching of confluent MDCK layers. Cells are shown 12 h after wound induction. c-g) Apical and junctional actin structures in MDCK cells after induction of EMT with 100 ng HGF. Apical dynamic microvilli are induced within 90 min after addition of HGF to confluent cells (c) and resemble actin structures emerging from lateral cell-cell junctions. Coordinated movement of actin structures can be observed on the apical surface and junctional regions (e). Dynamic microvilli and junctional actin structures appear continuous and rapidly reorganize (f) with individual dynamic microvilli emerging from junctions (f) or becoming aligned along the junctional surface (g, dotted line indicates orientation of cell contact). Time: min in c, d; s in f, g. Scale bars: 3 µm.

between HGF-induced actin bundles at junctions and dynamic microvilli we directly observed actin reorganization at the interface between cell-cell junctions and the apical surface. We found that dynamic microvilli constantly moved in and out of the junction area. On the one hand, arrays of dense junctional actin frequently disassembled into individual dynamic microvilli (Fig. 2-11h). At the same time dynamic microvilli became oriented along and incorporated into cell-cell junctions (Fig. 2-11i).

In summary we found dynamic microvilli to be a characteristic feature in all stages of MDCK physiology with migratory character. In addition, dynamic microvilli are in continuous exchange with actin at junctions during HGF induced morphological transition

2.2.5. Dynamic microvilli and myosin II network sensitive to mechanical stress

The cytoskeleton has an important role in the transduction of mechanical signals into biochemical signals. Several studies have investigated the

remodeling of stress fibers on the basal region of the cell [113]. However, little is known about the remodeling of actin on the apical region of the mammalian cells. Usually dynamic microvilli were seen on the apical region of the MDCK cells. To date remodeling of myosin II on apical surface of the cell after mechanical stress has not been studied. Previous studies have shown that myosin II activity depends on mechanical activity in the cell. To test whether the dorsal acto-myosin network was sensitive to mechanical stress, we gently placed a small iron weight coated with plastic (3 mN / cm^2) on top of the cells, which could then easily be removed with the help of a magnet. We found that within 20-30 min dynamic microvilli and MLC GFP became fragmented into small pieces. Upon removal of the weight the myosin II network rapidly reappeared, while actin gradually reassembled into longer dynamic microvilli (Fig. 2-12a, b). Interestingly stress fibers were not affected by the mechanical stimulus. This shows that the cortical network of alternating dynamic microvilli and myosin II filaments are sensitive to mechanical strain and at the same time capable of rapidly assembling into an isotropic and flexible network.

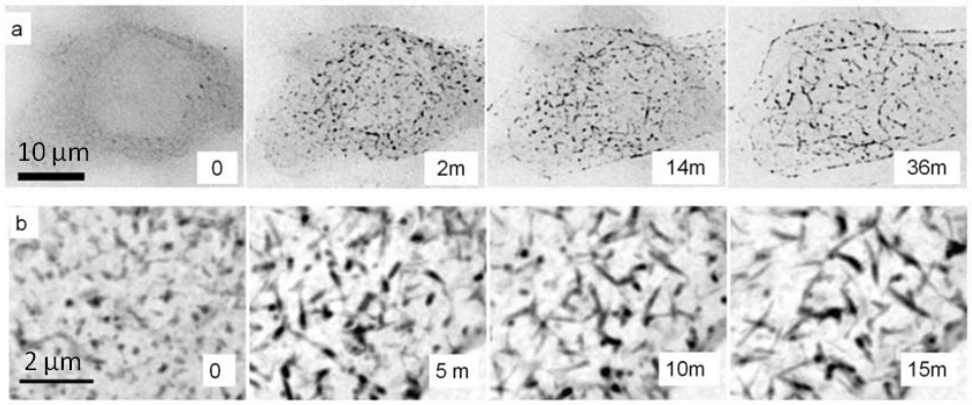

Figure 2-12. DM and myosin II network on the apical region of the MDCK cells sensitive to mechanical stress: a,b) Reformation of myosin II network(a, myosin II

labelled with GFP) and actin (b, actin labelled with Lifeact GFP) networks after weight-removal. Scale bars: 10 µm (a), 2 µm (b). Time in min.

2.3. Discussion

2.3.1. Dynamic microvilli: a new cortical structure characteristic for non-confluent ECs

Dynamic microvilli are relatively large cortical structures found on the apical surface of all epithelial cell lines that we tested and they are easily visible with commonly used actin probes. Why then have they not been featured more prominently in the literature before? One reason is certainly that many studies focused on polarized ECs in confluent sheets, where dynamic microvilli are absent. In addition, actin organization has traditionally more often been studied at the basal surface of cells, where the molecular pathways controlling formation of lamellipodia, filopodia and stress fibers have been elucidated in great detail [114-117]. In recent years an increasing number of publications have also addressed the organization and role of actin during cell-cell junction formation - again mostly focusing on confluent EC monolayer. Finally, the early characterizations of apical actin structures were largely restricted to the highly specialized but biochemically accessible brush border epithelium with its prototypical organization of microvilli and terminal web [84, 85, 118]. There are a number of reports and images where dynamic microvilli or related structures can be clearly distinguished. Scanning electron micrographs from the 1970s clearly show elongated and bent apical protrusion in certain epithelial cell lines or under specific culture conditions [84, 86, 89]. More recently, AFM has been used to reveal dynamic microvilli or ridge-like topological structures on the apical surface of MDCK and melanoma cells [88, 119]. In addition, Ezrin or its adapter EBP50 localize to

plasma membrane protrusions that strongly resemble short dynamic microvilli [87, 103]. Many other individual images of apical cell surfaces include structures that probably correspond to dynamic microvilli. Perhaps most importantly, protrusions with characteristics of dynamic microvilli have been also described for apical surfaces of epithelia in developing embryos [104].

Various names have been used to describe elongated apical actin structures or surface protrusion, including microvilli, microvilli-like, surface folds, plicae and ridges. However, in none of the previous studies was dynamics of apical actin and its lateral connectivity examined in detail. Therefore, the rapid lateral actin reorganization and interconnection with myosin filaments shown here, was so far not appreciated. Dynamics of the microvilli which is primarily driven by an underlying myosin network and it will leads to the typical undulating motion of individual dynamic microvilli. As indicated by their 3D organization, dynamic microvilli frequently protrude from the apical surface but also extend parallel to the plasma membrane. In contrast to the dense arrays of microvilli seen in confluent epithelial layers , individual dynamic microvilli can be clearly distinguished and exhibit more rapid dynamic rearrangement .

2.3.2. Lateral dynamics and connectivity

Organization and dynamics of dynamic microvilli combine several characteristics that are unique among known actin structures. The undulating shape of individual dynamic microvilli stands in contrast to the straight organization of actin bundles in microvilli, filopodia, and stress fibers [23] and also markedly differs from the dendritic organization in lamellipodia [7]. This curved shape is very likely the result of pulling forces exerted by myosin II . In

addition, dynamic microvilli are randomly oriented and have an isotropic distribution. Maintenance of such a random distribution in cells often requires constant energy input [120], which in the case of dynamic microvilli is again probably provided by myosin-mediated pulling forces. Finally, although individual dynamic microvilli exhibit dynamics through growth and shrinkage , they are mechanically connected and undergo coordinated lateral motions with a characteristic length-scale. These coordinated dynamics are typical for networks of flexible polymers. Therefore, dynamic microvilli present an easily accessible cellular system to address fundamental questions regarding actin-myosin interaction or properties of bio-polymer networks that could until now only be studied *in vitro* [121].

2.3.3. A cortical myosin II network

One major finding of our study was the identification of an extensive network of myosin filaments covering the whole apical surface of ECs. As this myosin network is structurally interlinked with dynamic microvilli it constitutes a particularly interesting case of an active bio-polymer network where molecular motors utilize chemical energy to change topology of a semi-flexible polymer network [122, 123]. This rapidly changing topology is directly reflected in the modular rearrangements of dynamic microvilli, where short actin bundles constantly change orientation, split or fuse with each other. Such dynamics on the level of whole actin bundles could provide a very efficient mechanism for large scale cortex reorganization without the need for stable reference points such as provided by focal adhesions on the basal surface or adherents junctions at cell-cell contacts. Despite its apparent flexibility in organization, the joint acto-myosin network appears to be under considerable tension as seen after Lat A treatment or laser ablation experiments. It will be interesting

to determine, whether the organization of the apical acto-myosin network is linked to cytoskeleton organization and adhesion structures at the surfaces interacting with substrate and neighboring cells. Our results clearly indicate that the cell has to integrate forces over its whole surface to allow accurate responses to environmental cues.

2.3.4. Function and outlook

We found a strong correlation between formation of dynamic microvilli and apical myosin II networks on the one hand and motile and “mesenchymal-like” phenotype of ECs on the other hand. This might simply be a result of changes in actin organization upon reduced cell-cell adhesion or altered substrate adhesion. However, the identified network could also actively support morphological changes typical for the observed situations. One interpretation of dynamic microvilli in low confluence cultures and in HGF-treated cells is that epithelial cell plasticity requires a reservoir of active actin microfilaments to perform rapid changes in cell shape and promote membrane re-localization in migration. This would be consistent with a previously proposed role for membrane invaginations and ruffles in increasing endocytic uptake through macro pinocytosis [89, 124]. In addition, an increase in actin dynamics could also help to release surface tension in domains close to cell-cell contact. The close association of dynamic microvilli with the apical plasma membrane also suggests a direct influence of the apical acto-myosin network on lateral segregation and mobility of membrane proteins – either through direct attachment and transport [125] or as diffusion barrier and fence [126].

3. Cytoplasmic acto-myosin assemblies determine nuclear shape

3.1. Introduction

Shape and size of the cell nucleus are crucial in regulating genome function [127-129]. Recent studies have shown that cytoskeleton has a role in maintaining the nuclear shape and size, little known about the molecules that are involved in this process [130-134]. SUN (Unc-84, Sad 1p) and KASH (ANC-1, Klarsicht, Syne Homology) domain of the proteins can interact with cytoskeleton filament and establish a physical connection between the nucleus and cytoskeleton. SUN domain-containing proteins are present on the inner nuclear membrane and the KASH domain-containing proteins are connected on the outer side of nuclear membrane. These proteins help to make a connection between the cytoskeleton outside the nucleus and the components inside the nucleus. Recent studies showed that the complex called LINC (Linker of nucleo- skeleton and cytoskeleton) directly anchors the cytoskeleton to nuclear membrane. The LINC complex is composed of three different proteins: nesprins, sun, and lamin [130, 132, 133] (Fig.3- 1). Laser ablation of heterochromatin induced drastic reduction in nuclear volume without affecting the cell shape. Inhibition of cytoskeleton using different drugs also showed the same result. After inhibition of actin (cytochalasin D) and myosin II (blebbistatin), nuclear volume reduced drastically. However in the case of microtubule inhibition, volume of the nucleus increased significantly. These experiments show that cytoskeletal filaments play a significant role in maintaining the nuclear shape [135]. There are different types of signaling cascades involved in the focal adhesion like protein kinase

C, tyrosine phosphorylation, and phosphorylation of the focal adhesion kinase [136-138]. Paxillin is one of the predominant protein in the focal adhesion and it is a marker for the focal adhesion kinase in the Src kinase pathways [139]. Recent studies showed that endothelial glycocalyx has no effect on the focal adhesion protein paxillin [140, 141]. These studies propose that stress fibers in basal region are weakly attached between peripheral actin band and actin cortical web through weak actin fibers. These weak actin fibers help to transmit fluid shear force from the apical region of the cell to the basal region. These studies suggest the presence of actin fibers inside the cytoplasm. The growing evidence suggest that, there is a direct mechano-chemical connection between the cell surface and the nuclear membrane [130, 132, 133]. Different molecular mechanisms play an important role in transmitting the force from the cell membrane to the nuclear membrane. Studies show that when the integrins were pulled with ligand coated micropipette or magnetic particles, there was a drastic displacement in the internal structures like the mitochondria and the nucleus. Studies showed that, pulling on the integrins will cause realignment of the molecules on the stress fibers attached to the nuclear membrane. Force applying on the integrins was also able to displace the mitochondria and the nucleus up to 20 μm [142, 143]. These results suggest the presence of actin network inside the cytoplasm.

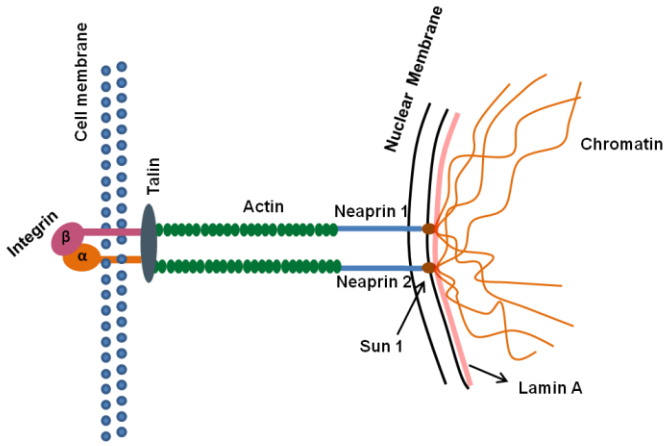

Figure 3-1. Cytoplasmic actin network. Adapted with modification from Vicente-Manzanares *et al*, 2009.

So far, an active physical link between the nucleus and cell membrane is still not clear. In this study, we showed for the first time, the presence of a cytoplasmic actin network with the help of TEM imaging. Furthermore, we also show the physical link of the cytoplasmic actin bundle with the cell membrane as well as nucleus. With these experiments together, show that active physical link between the nucleus and cell membrane has an important role in maintaining the nuclear shape.

3.2. Results

3.2.1. Cytoplasmic acto-myosin network

Maintaining the nuclear shape is very important for genome regulation and function [127, 128]. So far, how living cells maintain nuclear shape is still poorly understood. Recent studies proposed the existence of a cytoplasmic network of actin and myosin II inside the cytoplasm of the mammalian cells which can hold the nucleus in a pre-stressed state [135, 144]. Evidence showed that cytoplasmic actin network could physically connect on the cell, membrane as well as the nucleus. Inhibition of actin using Lat A and inhibition myosin II using blebbistatin reduces the area of the nucleus [135]. To study the contraction of the nucleus after the inhibition of the actin and myosin II, we made a stable MDCK cell line of the Lamin-A mCherry. We made time laps movies of the lamin A mcherry labeled nucleus after adding Lat A or blebbistatin. The time laps movies showed contraction of the nucleus after the

inhibition of actin or myosin II (Fig. 3-2a, b, c and d). These experiments illustrate that actin and myosin II plays a major role in keeping the nucleus shape. Further, we checked the presence of actin in the cytoplasm of MDCK cells using Phalloidin. Interestingly, we observed a dense network of actin inside the cytoplasm of the MDCK cells (Fig. 3-3a). One of the prominent proteins function together with actin is myosin II. We next checked whether the myosin II network is present in the cytoplasm. To visualize myosin II, we made a stable cell line of the myosin II heavy chain (MHC) GFP. Interestingly, we observed the presence of myosin II, network inside the cytoplasm (Fig. 3-3b). To study the dynamics of the myosin II, we made time laps movies and these movies showed that myosin II, inside the cytoplasm is always rearranging. To study the distribution of the actin and myosin II in the cytoplasm, we labeled the myosin II heavy chain with GFP and stained the actin with phalloidin. Co-localized images showed that, myosin II network inside the cytoplasm co-localized with actin (Fig. 3-3c). Our experiments suggest that cytoplasmic actin and the myosin II together generate force, and these forces can lead to the deformation of the nucleus shape.

Resolution of the fluorescent images is not enough to study the distribution of the cytoplasmic actin network of the cells. Therefore we performed TEM to study the cytoplasmic actin network in detail. We observed both straight and bend actin bundles as well as scattered single actin fibers in the cytoplasm (Fig. 3-3d, e). In the figure (Fig. 3-3f), different colors represent different curvature of the actin bundle inside the cytoplasm; green straight, brown=4.72 μm^{-1}, red =1.34 $\mu m^{-1}$and black represented the scattered single actin fibers inside the cytoplasm. Actin, intermediate filament and microtubules are the three polymers present in the cytoplasm of the mammalian cells.

Distinguishing the actin and intermediate filament with EM is very difficult because the size of these filaments is similar. However, we can easily distinguish the actin and microtubules because the size of the microtubule is bigger than actin. To confirm the presence of the cytoplasmic actin filament, we treated MDCK cell with lat B. After treatment with lat B, our data showed that length of and density of the actin bundles were drastically reduced (Fig. 3-4a, b).

Previous studies have reported the physical link between the cytoplasmic actin and the mitochondria. However, there was no study that showed the physical link between the cytoplasmic actin and the mitochondria. We stained the mitochondria with mito- tracker red and actin labeled with lifeact GFP. Live-cell imaging showed that the mitochondria were co-localizing with cytoplasmic actin (Fig. 3-5e) and we also confirmed the physical link between the mitochondria and cytoplasmic actin using TEM analysis (Fig. 3-5a, b).

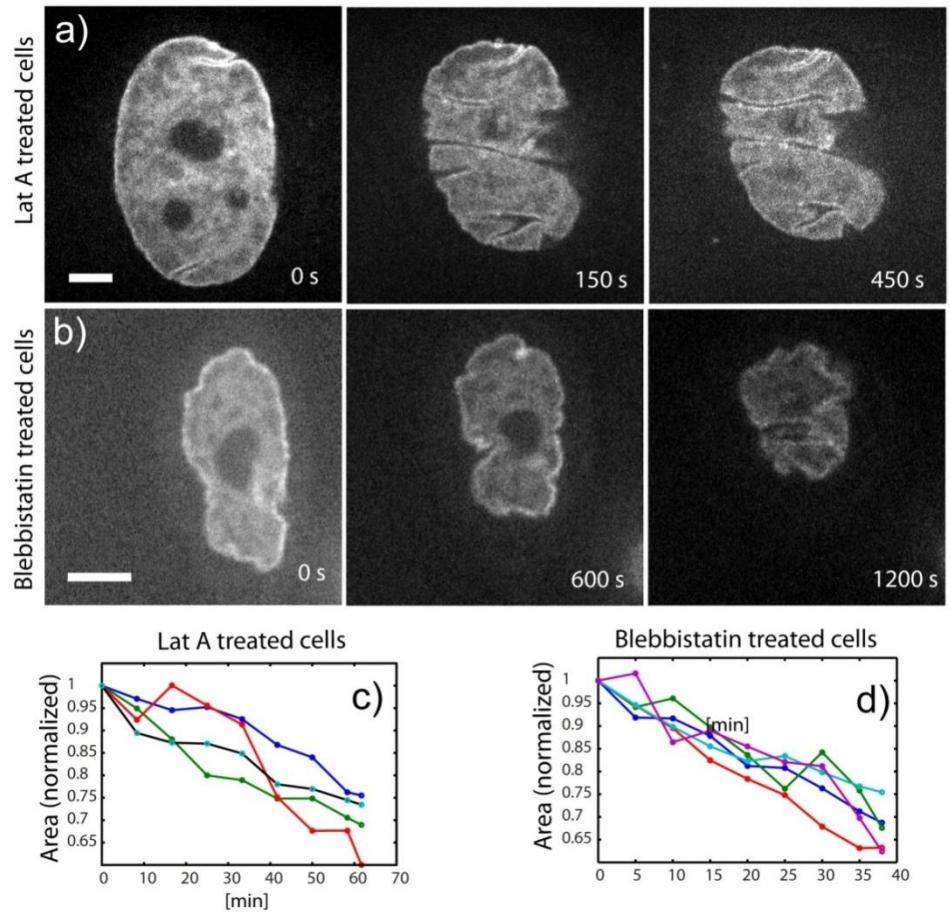

Figure 3-2. Cytoplasmic Acto-myosin network maintain the nuclear shape. a), c) Nuclear deformation after treatment with Lat A (Actin de-polymerizing drug). b), d) Nuclear deformation after treatment with Blebbistatin (Which reduce the activity of myosin II). Scale bar: 5µm.

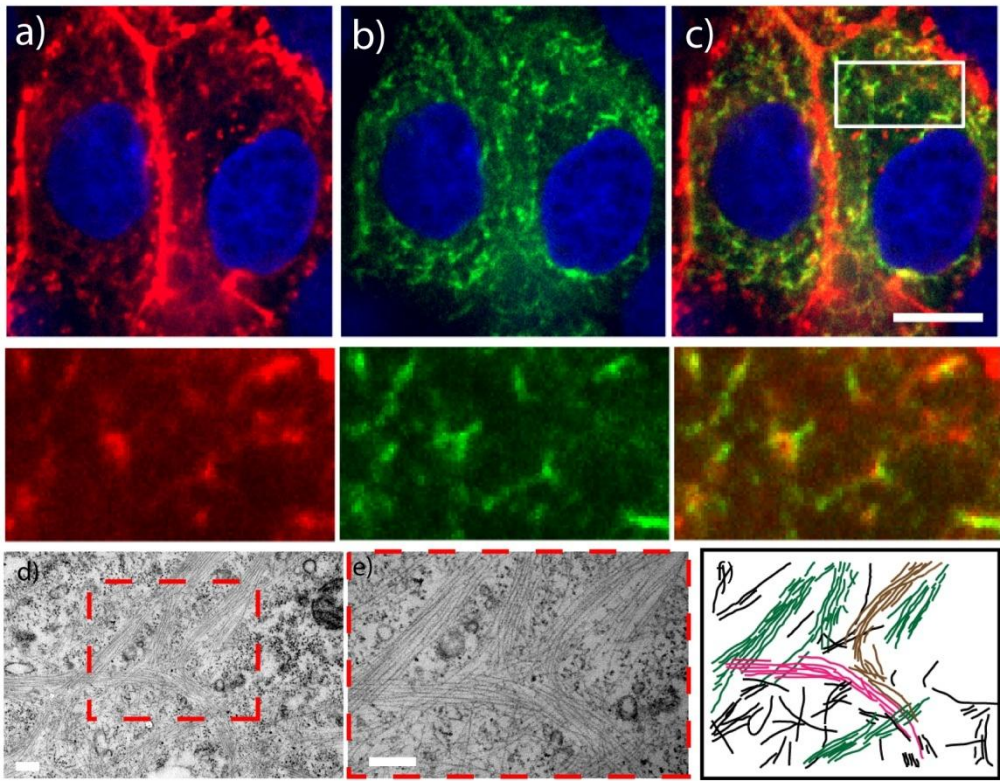

Figure 3-3. Network of the actin and myosin II in the cytoplasm. a) Actin stained with Rhodamin- phalloidin. b) Myosin II labeled with GFP-MHCA (myosin II heavy chain) and nuclei stained with hoechst, in blue. Bottom: Magnified area from (c) illustrating co-localization of the cytoplasmic actin and GFP-MHCA. d) TEM micrograph of the cytoplasmic actin network. e) Magnified area from (d). f) Drawing of identified actin filaments. Red: curvature 1.34 μm^{-1}, brown: curvature 4.72 µm-1, green: straight bundles, black: single actin filaments. Scale bar: c)10 µm, d),e) 200 nm.

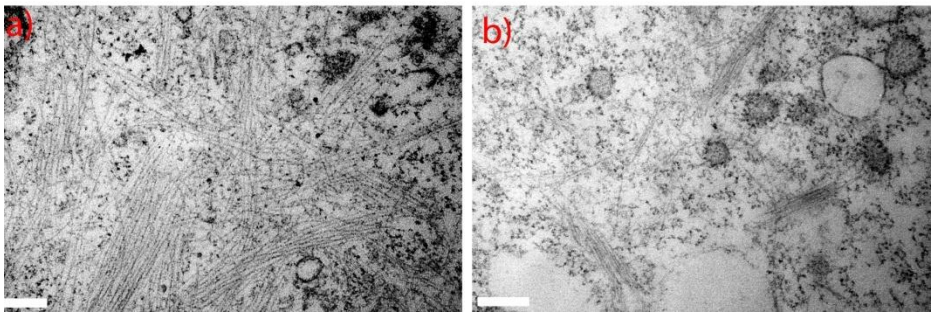

Figure 3-4.Cytoplasmic actin network. a) Non treated cells, b) Length and density of cytoplasmic actin network reduced after lat B treatment. Scale bars: 200 nm.

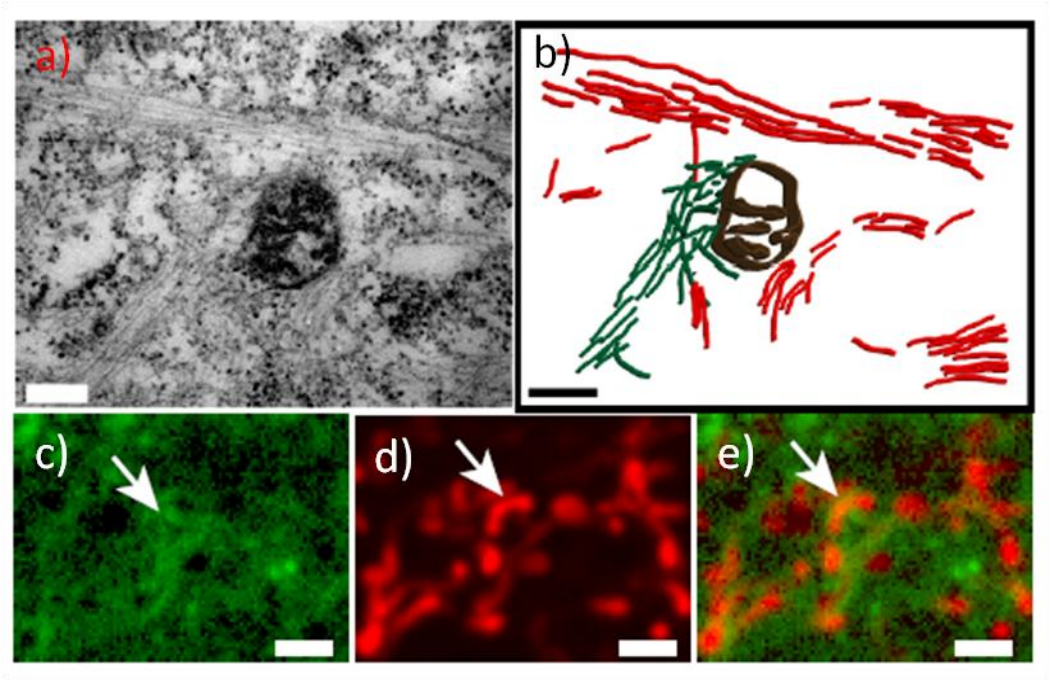

Figure 3-5. Actin bundle physically attached to the mitocondria. a) TEM picture and (b) drawing of actin bundle attached to mitochondria. c) Cytosolic actin labeled with lifeact GFP, arrow pointing the cytosolic actin bundle. d) Mitochondria stained with mitotraker red, arrow pointing the mitochondria. e) Color combined images of (c) and (d), arrow pointing the co-localization cytosolic actin and mitochondria. Scale bar: a) 200 nm, c)-d) 2 µm.

3.2.2. Cytoplasmic actin network attached to the cell membrane through actin bundle

In our study, we showed a cytoplasmic actin network inside the cytoplasm of the MDCK cells. We observed that cytoplasmic network of the actin in both fixed and live cell imaging. Then we checked whether cytoplasmic actin network is attached to the cell membrane. For demonstrating the link between the cytoplasmic actin and cell membrane, we performed three different experiments 1) live cell microscopy, the actin were labeled with lifeact GFP, 2) Fixed cell microscopy, actin was showed with phalloidin, 3) TEM imaging. In all three methods, we showed cytoplasmic actin network attached to the cell membrane through actin bundles (Fig. 3-6a, b, c, blue arrow pointing the actin bundle). It appears that the actin bundle attached to the cell membrane through the adherens junction. TEM picture clearly demonstrate that, actin bundle from the membrane merge to the cytoplasmic actin network (Fig. 3-6d, e).

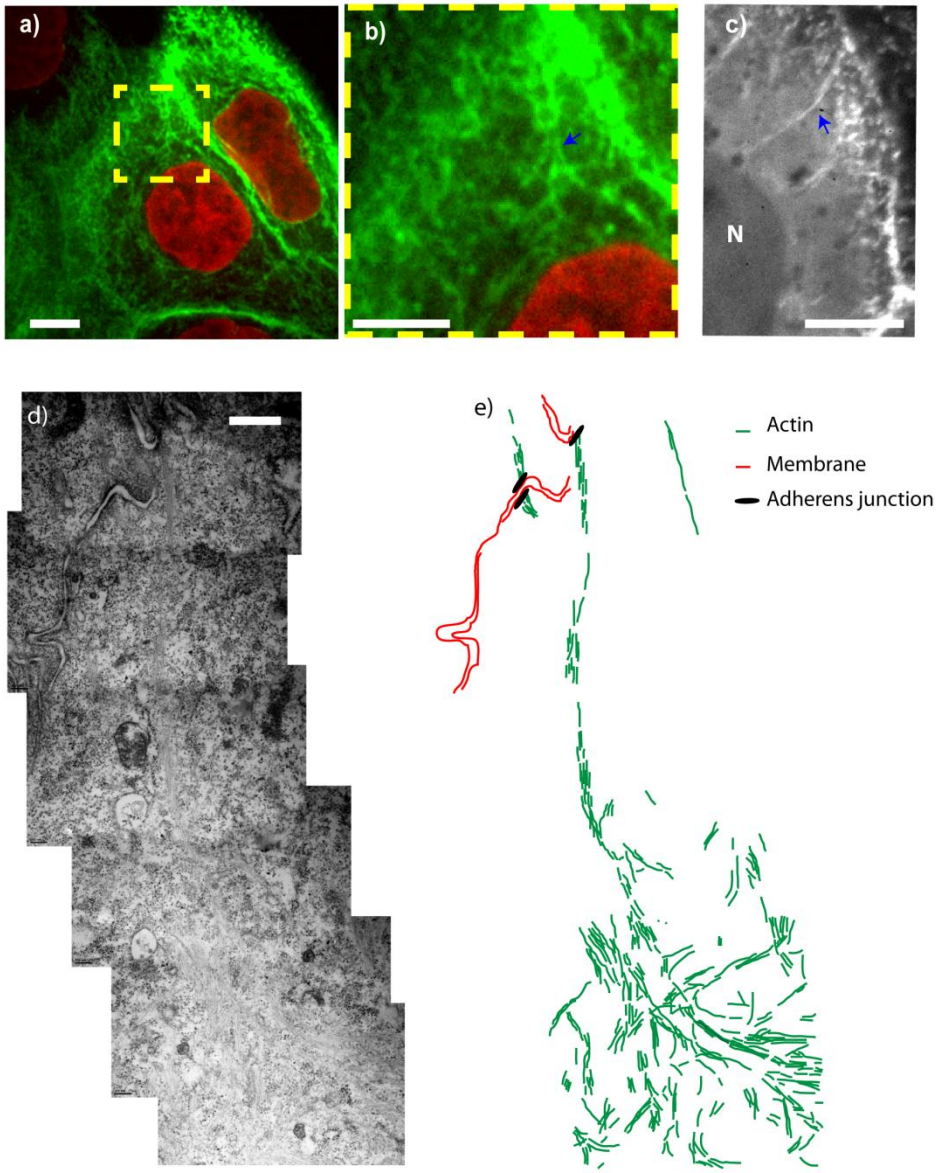

Figure 3-6. Cytoplasmic actin network connected to the cell membrane. a) Actin stained with Alexa 488-phalloidin. b) Magnified area from (a) showing cytoplasm actin network connecting between the nucleus and cell membrane. c) MDCK cell label with Lifeact-GFP showing actin bundles connected between the nucleus and cell membrane. d) TEM micrograph showing cytoplasmic actin bundle connected to the cell membrane, e) Drawing of identified actin filaments (Green). Scale bar: a), c) 10 µm. d) 0.5 µm.

3.2.3. Cytoplasmic actin network attached to nucleus

Our data demonstrate the cytoplasmic actin network attached to cell membrane through actin bundles. As a new step we checked, whether cytoplasmic actin network is attached to the nucleus as well. To show the connection between the cytoplasmic actin network and nucleus, we added 2 µM of Lat A into cells that were labeled with lifeact GFP. We observed the tethering movement of the cell cortex, and some of the cytoplasmic actin stayed together with the nucleus (Fig. 3-7a, b, c, d). These results are in agreement with the previous hypothesis that the cytoplasmic actin may make physicaly link the nuclear membrane and the cell membrane. Fluorescent images show that cytoplasmic actin was placed close to nucleus. Using fluorescent images, it was difficult to demonstrate the physical link between the cytoplasmic actin and the nucleus because of the low resolution of the fluorescent images. Then we performed TEM images to demonstrate the physical connection of the cytoplasmic actin on the nucleus. Interestingly, we observed that the actin bundle was physically connected to the nucleus. These actin bundles are also connected to the cytoplasmic actin (Fig. 3-8a, b (red arrow pointing actin bundle, nucleus attachment), c, d,). We suggest that the actin bundle needs different proteins to attach the nuclear membrane. In other words these proteins can form ‘Nuclear focal adhesion (NFA)’ together with the actin bundle. The TEM picture showed that the thickness of the actin bundle is ~ 1 -2 µm. It appears that actin fibers inside the actin bundle that are attached to nucleus are closely packed compared to the actin bundle in the cytoplasm. The distance between the actin fiber in the actin bundle attached to the nucleus is less (~10nm) compared to the distance between the actin fibers in the cytoplasmic actin bundle (~15nm) (Fig. 3-8e, f). Further

analysis of the TEM image showed that some of the actin bundle is placed very close to Nuclear Pore Complex (NPC) (Fig. 3-8g), May be a cytoplasmic actin bundle physically connected to the NPC. Taken together, our data showed that cytoplasmic actin network physically connect to the nuclear membrane.

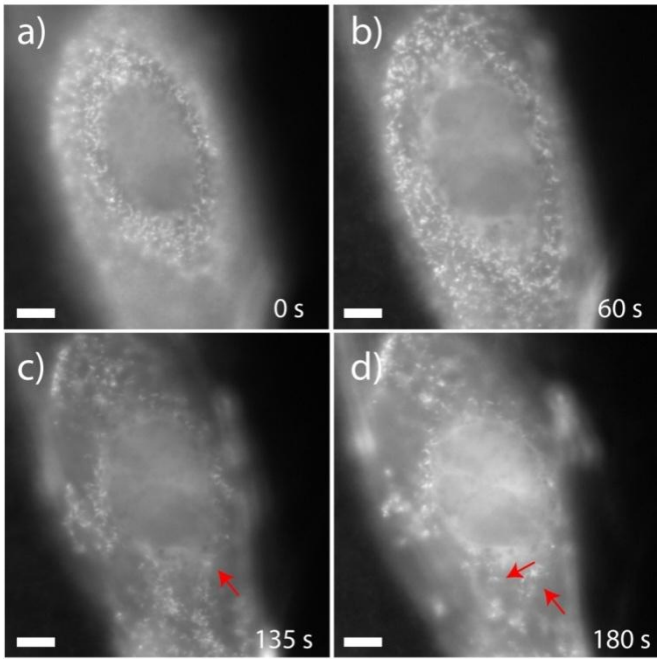

Figure 3-7. Cytoplasmic actin attached to the nucleus. Cells are labeled with lifeact GFP. Sequence showing rupture of dynamic microvilli bundles after treatment with 2 μM Lat A. Note that the drug was added 3 min before start of the shown sequence. Red arrows point to the actin bundle attached to the nucleus(c, d). Scale bar: 5 μm.

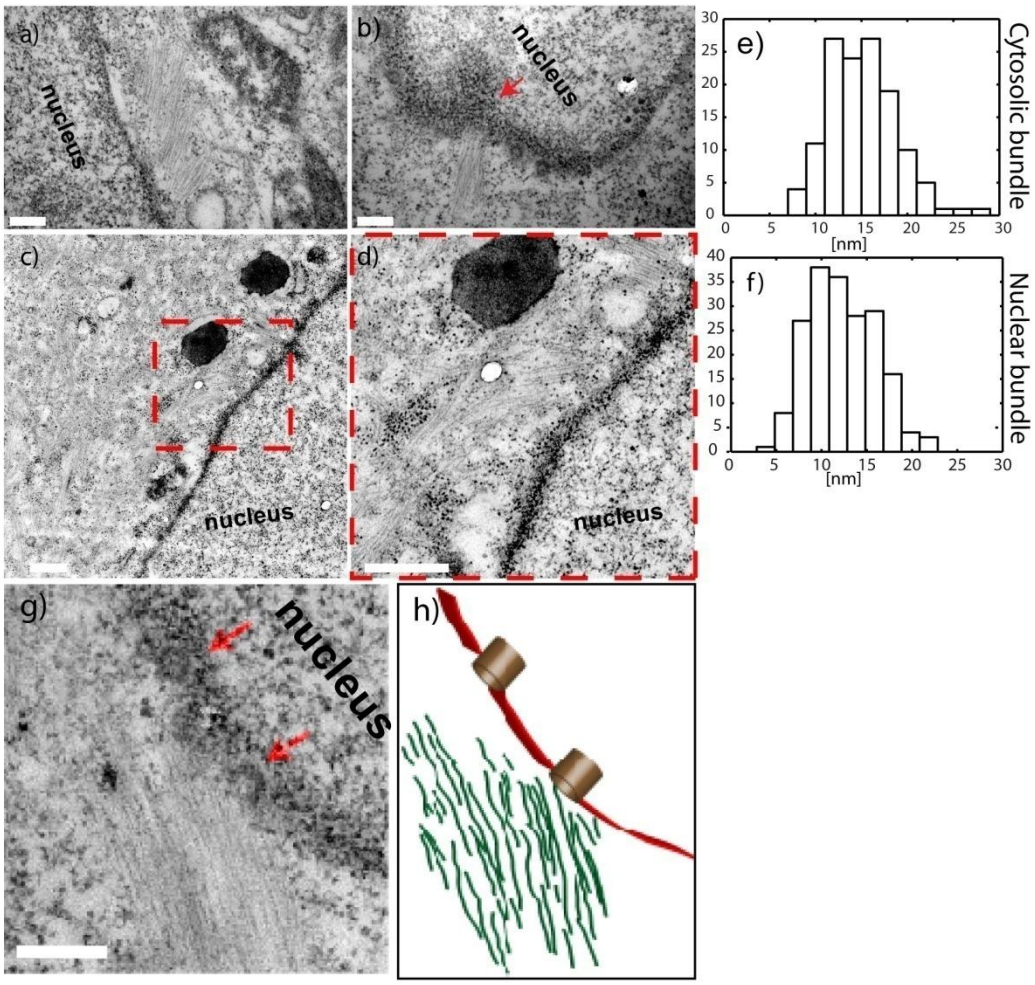

Figure 3-8. Cytoplasmic actin network connected to the nucleus. a, b) TEM image showing the actin bundle attached to the nucleus, the red arrow points to the actin bundle attached to the nucleus. c, d) Actin bundles from the nucleus attached to the cytoplasmic actin network. e-f) Quantifications of the average inter filament distance of the cytoplasmic actin bundle and actin bundle attached to nucleus. g) Actin bundle in-proximity to the NPC. h) Drawing of the identified actin filaments. Scale bar: a), b), g) 200 nm. c), d) 500 nm.

3.2.4. Cytoplasmic actin and myosin II maintain the nuclear shape

Our study revealed the presence of a 3D network of actin and myosin II in interphase cells. In order to determine the function of this cytoplasmic actin and myosin II network, we de-polymerized actin using Lat B. We hypothesized that the cytoplasmic actin network would partially de-polymerize in a concentration of Lat B used. The partial de-polymerization of the cytoplasmic actin network in interphase would create an imbalance in the nucleus. Based on our hypothesis, we used low concentrations of Lat B (0.5 µM). However, the Lat B treated cells did not show the predicted imbalance suggesting that the balance of the cytoplasmic actin network might be sustained by microtubules. Hence, we simultaneously de-polymerized actin and microtubules using 0.5 µM Lat B and 2 µM nocodazole and interestingly observed an imbalance of the nucleus and deformation of the nucleus shape (Fig. 3-9a, d, e). Then we checked the distribution of cytoplasmic actin and myosin II network after treating the cell with 0. 5 µM Lat B, both cytoplasmic actin and myosin II form patches inside the cytoplasm and these patches co-localize (Fig. 3-9f, g, h). Formation of these patches might have been the reason for the imbalance of the nucleus and its shape deformation. After we simultaneously de-polymerized actin and microtubules using a high concentration of Lat B (2 µM) and 2 µM nocodazole, the observed oscillation was completely abolished and nucleus shape deformation resembled non treated cells (Fig. 3-9a-c,d). This experiment demonstrates that the cytoplasmic network of actin and myosin II can exert force on the interphase nucleus in cells. Low Lat B concentration partially destroys the cytoplasmic actin network in interphase, leading to a force imbalance within the network. Uneven force distribution inside the network will push and pull the nucleus in

interphase and lead to nucleus oscillation and nucleus shape deformation. In high Lat B concentrations the cytoplasmic actin network is completely destroyed, hence, no uneven force acts on the actin network resulting in the absence of nucleus oscillation and nucleus shape deformation. These experiments suggested that the cytoplasmic actin mesh work has a role in nuclear positioning and maintaining the nuclear shape during the interphase.

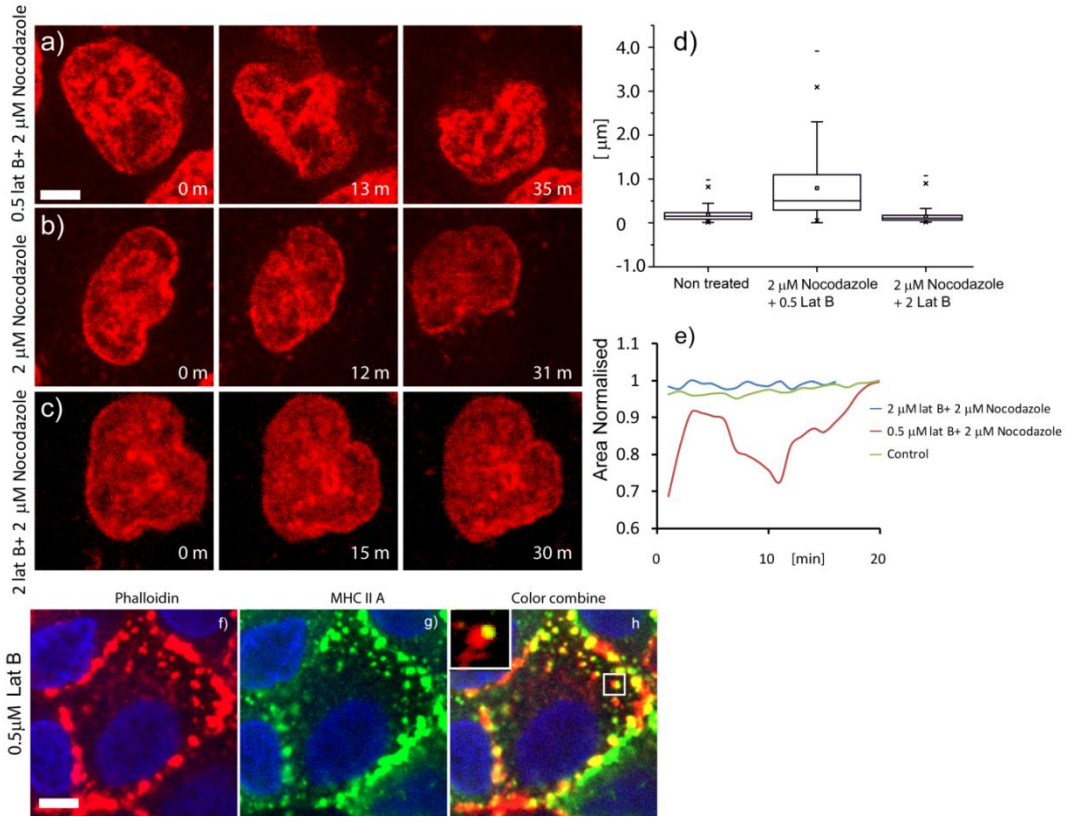

Figure 3-9. Cytoplasmic actin and myosin II maintain the nuclear shape. a) - h) Nucleus are labeled with hoechst. a) Deformation of nucleus shape. b- c) Nuclear shape unchanged. d) Nucleus displacement within 30 s (n= 10 cells). e) Change of cross section area. f- g) Formation of patches of actin (f) and myosin II (g) after treatment of 0.5 µM Lat B, cells are stained with phalloidin (f, red), cells are labeled with Myosin heavy chain A (g, green). h) Color combine images of (f) and (g). Scale bar: 5 µm

3.3. Discussion

Mechanical force plays an important role in shaping the tissue and organs in our body [142] . In a single cell, mechanical perturbation on the membrane takes only milliseconds to reach the nucleolus. Only stress wave can propagate in this fast manner. Three different types of cytoskeletal polymers, actin, intermediate filaments, and microtubules are present in a vertebrate/mammalian cell. Although the presence of actin in the cytoplasm is controversial, it has been suggested that actin bundles in cytoplasm may help to connect the membrane to the nucleus. Studies also showed that different nuclear membrane proteins like Nesprin 1 and Nesprin 2 are able to attach the actin fibers to the nucleus [130, 132, 133]. Yet, there are no previous studies visualizing actin fibers in the cytoplasm. In our studies we showed that there is an extensive network of cytoplasmic actin and myosin II in the cytoplasm of the mammalian cell. And these two networks co-localize and can exert force on the nucleus or other organelles inside the cytoplasm. We were able to resolve the cytoplasmic actin network through EM analysis and we showed that these structures are attached to the nucleus. We observed actin bundles that were either connected to adherens junction in the cell membrane or connected with the nucleus on one end and extended into the cytoplasm with the other end. Some of the actin bundles connected with the cell membrane also interact with actin bundles attached to the nucleus, thereby indirectly connecting the nucleus with the cell membrane.

This is in line with previous studies, where it showed that adherens junction help to connect two cells and actin bundles are attached to adherens junction. Our study suggests those adherens junctions are physically connected with the help of actin bundles. Figure (3-10) depicts that actin bundles from the

neighboring cellsare attached to the same adherens junction and the same actin bundle is physically connected to the nucleus of the respective cell (Fig.3-8d). Our data suggests that actin bundles in the cells help to connect the nucleus of one cell to the nuclei of the adjacent. These connections may not only help to transfer mechanical stimulus from the cell membrane to the nucleus within one cell but also to nuclei of other cells within the epithelial sheet. In conclusion, the nucleus within the epithelial sheet is physically connected through cytoplasmic actin.

We hypothesize that cytoplasmic actin plays an important role in the epithelial sheet migration. We also suggest that cytoplasmic actin could play a significant role in EMT. During EMT most of epithelial junctions are destroyed leading to disruption of the physical link of actin between the nucleus and the cell membrane. This might change the mechanical properties of the nucleus.

Our studies demonstrate the existence of a cytoplasmic actin network. While previous studies showed that Nesprin 1/2 on the outer nuclear membrane (ONM) can bind to cytoplasmic actin, no studies showed the physical link between cytoplasmic actin and the nucleus. Nesprin is a rod like protein which protrudes 150 nm away from ONM with actin attached to its amino terminus [145]. In our experiments, we observed actin bundles directly attached to the nuclear membrane. Antibody staining shows that Nesprin distributes equally in the cell membrane. If Nesprin is involved in actin bundle attachment on the nuclear cell membrane, we should see Nesprin localize into distinct patches. We hypothesize additional proteins other than Nesprin involved in the attachment of the actin bundle to the nucleus. These proteins

on the nucleus could form a complex together with actin bundles and it could be named as 'Nuclear Focal Adhesion (NFA)'.

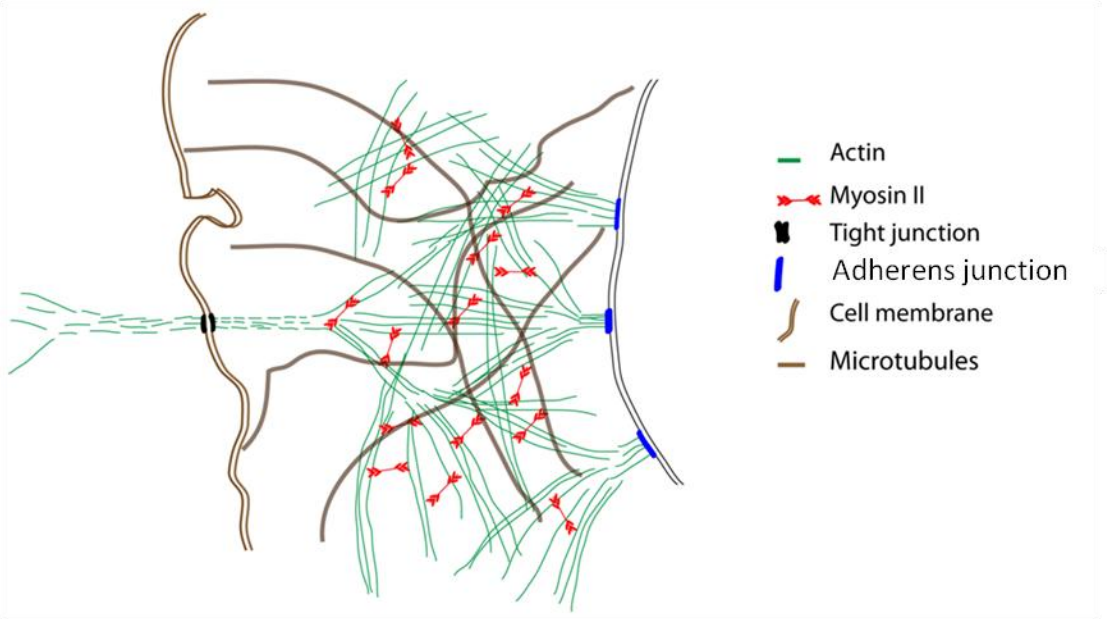

Figure 3-10. Cytoplasmic acto-myosin network. Arrangement of actin network in an epithelial cell, actin bundle from the cell membrane and actin bundle from the nucleus attached to the cytoplasmic actin network. Actin bundle from the nucleus extend to the cytoplasmic actin network, similarly actin bundle from the membrane merge to cytoplasmic actin network. Cytoplasmic actin makes indirect connection between the nucleus and cell membrane. Actin bundle from the nearby cell attached on the same adherens junction, hence actin bundle from the two cells physical connected and this actin bundle indirectly attached to the nucleus of the respective cells.

4. Cytoplasmic acto-myosin network maintaining chromosome positioning during mitosis

4.1. Introduction

Microtubules and acto-myosin assemblies are involved in different aspects of cell motility and cell division [146]. Microtubules and microtubule motors drive chromosomes segregation and spindle formation [147]. In addition, cortical actin influences spindle positioning and orientation as well as cytokinesis [5, 148, 149]. Myosin II and actin localize in a contracting cortical ring at the division site. Mutation of myosin II affects cytokinesis in a wide range of cells from amphibian eggs, slime mold and, fission yeast [150, 151]. Inhibition of myosin II after metaphase will prevent cells from progressing into anaphase and cytokinesis in yeast and *Dictyostelium*, In contrast, myosin II appears not to be required for spindle assembly. Myosin II deletion using RNA-i in cultured drosophila cells showed that myosin II is important for mitosis [152, 153]. Studies performed in the mammalian cell culture showed that actin and myosin II based cortical flow regulate the centrosome separation and positioning [5]. Orientation of the spindle network also depends on the cortical acto-myosin contraction [5]. During the centrosome separation, actin acts as an attachment site for the astral microtubules [154-156]. In Drosophila embryos, dynamics of actin polymerization help the centrosome separation and it helps to position the centrosome in cell migration, cell polarity maintenance, and asymmetric cell division [157-159].

Previous studies showed that dynamics and positioning of the chromosomes depends on the cortical actin and myosin. Here we show that, in addition to its cortical function, cytoplasmic actin and myosin II network also form a dense three-dimensional network surrounding the chromosomes and spindle during mitosis. We also demonstrate that this cytoplasmic acto-myosin network plays an important role in chromosome positioning during mitosis.

4.2. Results

4.2.1. Cytoplasmic actin network mitotic cells

Previous studies have shown that cortical myosin II and actin have an important role in the mitotic spindle assembly in cells [147]. Disruption of actin and myosin II using different drugs or RNAi revealed problems in metaphase spindle assembly [152, 153]. Recent studies have shown the presence of sub- cortical structures of actin in mitotic cells [160]. With the help of phalloidin we study the distribution of actin in mitotic phase (M-phase) of Hela and MDCK cells. Interestingly, we observed a dense flexible actin network around the chromosomes during the M-phase. These flexible actin fibers were connected and formed a 3D actin network around the spindle in M-Phase (Fig. 4-1a-f). The observed actin network was also attached to the cortex of cells (Fig. 4-1b, yellow arrow). In prophase dense 3D actin network was distributed around the chromosomes and some of the actin fibers expanded into the chromosomes (Fig. 4-1a, yellow arrow). The actin fibers remained distributed around the spindle in both metaphase and anaphase, while in anaphase some of the actin fibers were also observed between chromosomes. In Our fluorescent images, we were not able to see the distribution of actin fibers in detail due to the lack of optical resolution. Since

we were not able to resolve details of actin fiber distribution in fluorescent images, we decided to perform EM. We used MDCK cells because they displayed a higher density of the cytoplasmic actin 3D network compared to Hela cells. EM pictures revealed the presence of actin fibers in close proximity to the chromosomes, forming a network around each chromosome in prophase (Fig. 4-1g-k).

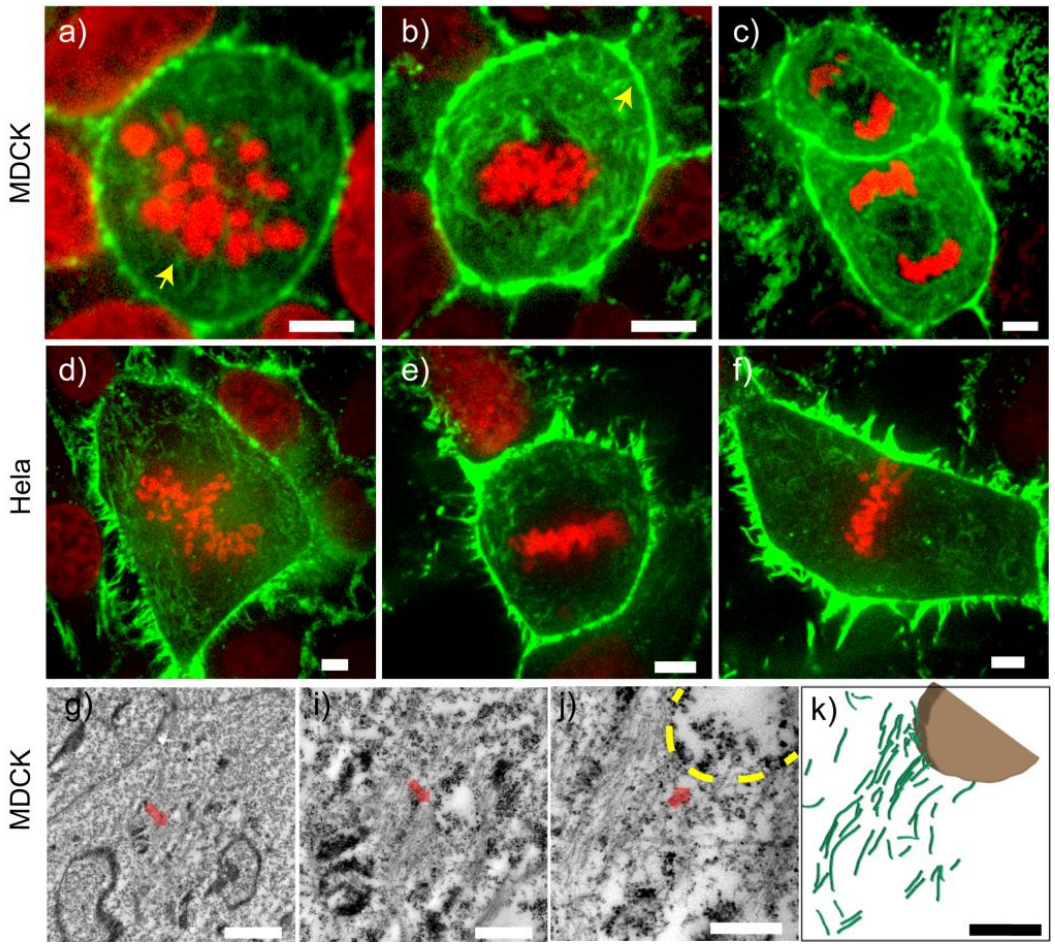

Figure 4-1. Cytoplasmic actin network surrounding chromosomes during mitosis. Actin stained with Alexa488-phalloidin (Green), Chromosomes stained with Hoechst (red). a-c) Actin network around chromosomes in MDCK cells.d-e) Actin network around chromosomes in Hela cells. g-j) EM picture showing actin filaments around chromosomes, red arrows point to chromosomes, yellow dotted line outlining chromosomes. Scale bar: a)-f) 5 µm, g) 2 µm, i) 1 µm, j) 200 nm.

4.2.2. The Cytoplasmic actin network maintains position of the chromosomes in M- phase

Our study revealed the presence of a 3D network of actin in M-phase cells. In order to determine the function of this cytoplasmic actin network, we de-polymerized actin using Lat B. We hypothesized that the cytoplasmic actin network would partially de-polymerize in a concentration of Lat B. The partial de-polymerization of the cytoplasmic actin network in M-Phase would create an imbalance in chromosomes dynamics. Based on our hypothesis, we used different concentrations of Lat B, ranging from 0.1 µM to 4µM. We observed oscillating M-phase chromosomes at low concentrations of Lat B and found increased dynamics with higher concentrations of Lat B. Interestingly, the highest dynamics was observed using 0.3 µM Lat B even higher concentrations, dynamics of the chromosome was completely abolished and dynamics of the chromosomes similar to the dynamics of chromosomes of the non treated cells (Fig. 4-2a-c).This experiment demonstrates that the isotropic meshwork of actin can exert force on M-phase chromosomes. Low Lat B concentration partially destroys the actin network in the M-phase, leading to a force imbalance within the network. Uneven force distribution inside the network will push and pull the chromosomes in the M- Phase and it will lead to the dynamics of the chromosome (Fig. 4-2d). High Lat B concentrations the cytoplasmic actin network is completely destroyed, hence, no uneven force acts on the network resulting in the absence of chromosome dynamics. These experiments, suggested that the cytoplasmic actin mesh work has a role in chromosome positioning during the M-phase.

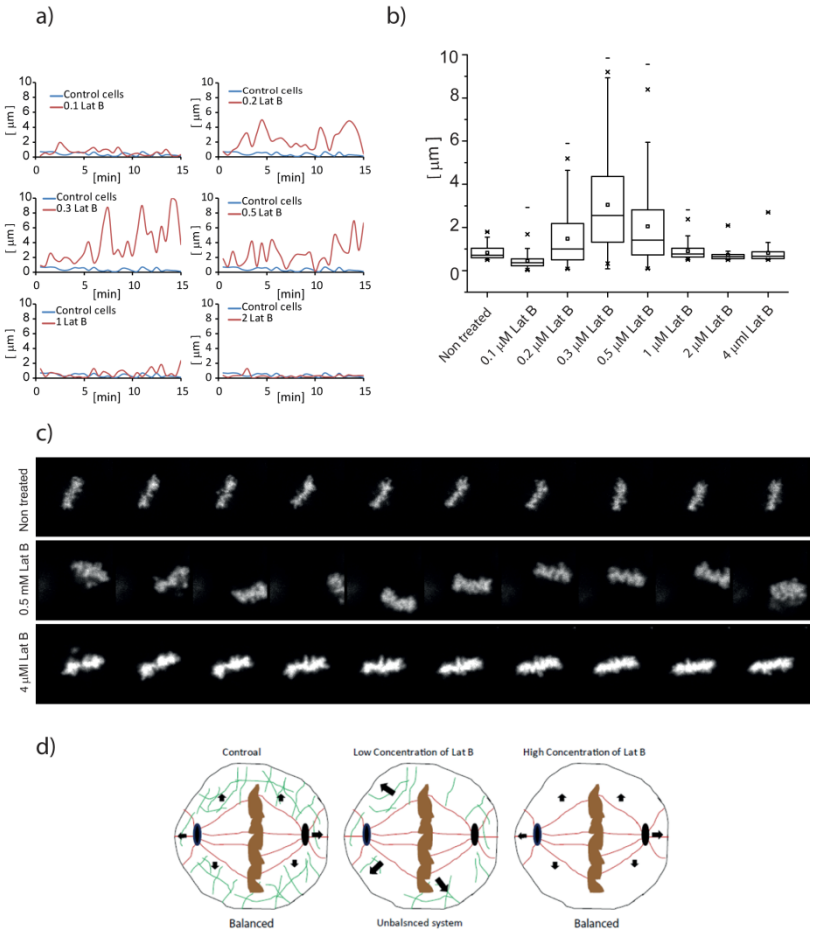

Figure 4-2. Cytoplasmic actin meshwork positions chromosomes in M- phase. a) Chromosome dynamics in Lat B. b) Chromosome displacement within 30 s (n=15 cells). c) Dynamic of chromosomes in different concentration of Lat B. d) Cartoon illustrating the distribution of cytoplasmic actin network in M-phase in different Lat B concentrations.

4.2.3. Cytoplasmic myosin II network in M-phase

Section (3.2), we characterized that cytoplasmic actin network in the mammalian cells. In addition to actin, myosin II is one of the predominant cytoskeletal components. It has been shown that Myosin II plays an important role in cell division. Hence, we decided to investigate the myosin II distribution in the cytoplasm of M-phase cells. We used a stable cell line of myosin II heavy chain GFP (MHC II GFP) to visualize the myosin II distribution in the M-phase of MDCK cells. Interestingly we found that, similarly to actin (section, 3.2), myosin II network around the chromosomes and that this network is also attached to the cell membrane (Fig.3-3a-c). Next, we checked whether actin co-localizes with the myosin II network. In order to visualize the cytoplasmic actin and myosin II network simultaneously, we stained actin using rhodamine phalloidin in cells expressing MHC II GFP. We found, that cytoplasmic actin and myosin II completely co-localize (Fig.3-3a-c). In addition, we performed time lapse microscopy of MHC II GFP expressing cells and found, that myosin II forms rods (approx. 2 µm in length) when the cell reaches M-phase (Fig. 3-3d, e), these myosin II rods assemble during prophase and then form a network around the chromosomes (Fig. 3-3d, e). In addition, we observed that some of the myosin II rods are also present inside the chromosomes during the prophase (Fig. 3-3f). In metaphase, the myosin II network formed around the spindle together with actin. In anaphase, myosin II and actin redistributed around the metaphase spindle and some of the myosin II rods were also observed between the chromosomes together with actin. Next, we investigated the role of the cytoplasmic myosin II network in M-phase. Therefore, we inhibited myosin II with blebbistatin. Our experiments showed that partial inhibition of the cytoplasmic actin network creates a force

imbalance inside this network, leading to the dynamics of chromosomes. Then we treated cells with a low concentration of Lat B, in addition to blebbistatin, and interestingly found that the dynamics of chromosomes was completely abolished (Fig. 3-3g). This experiment suggested that the myosin II network together with actin plays an important role in the positioning of M-phase chromosomes.

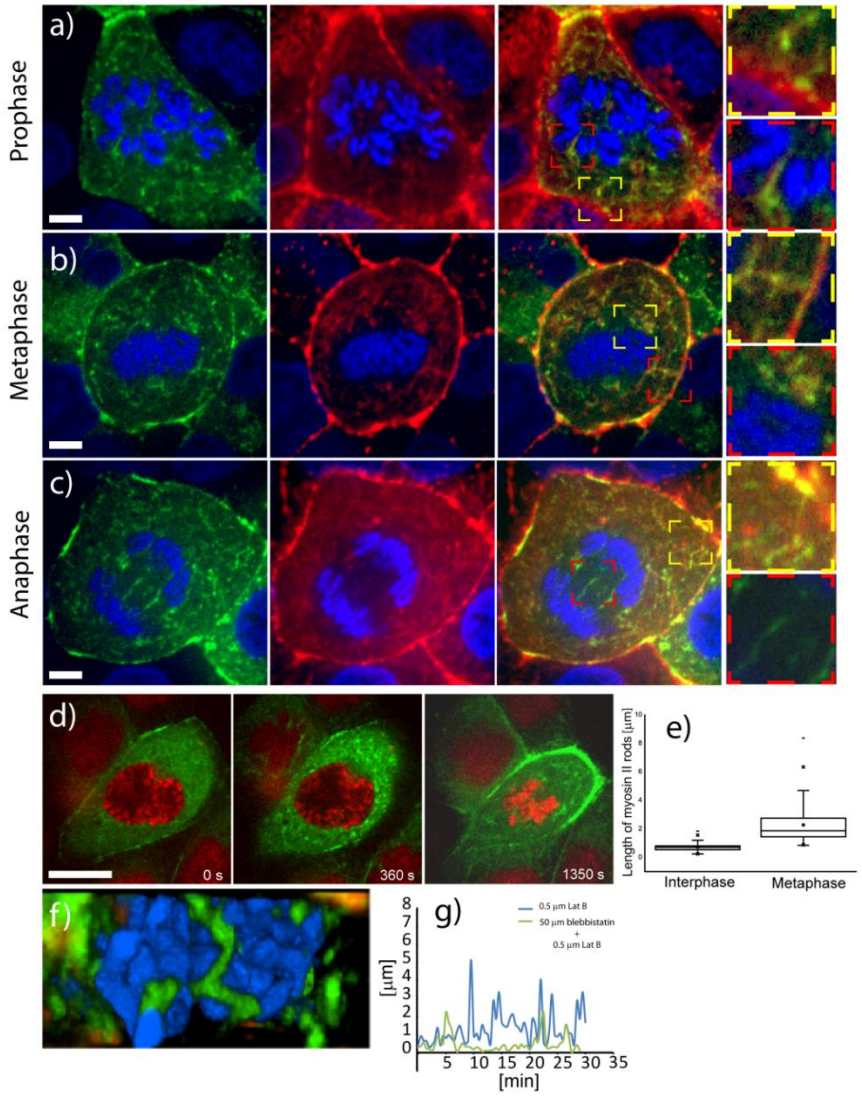

Figure 4-3.Cytoplasmic myosin II networks. a)-c) Cells are labeled with MHCII GFP (Green), actin stained with rhodamine phalloidine (Red), chromosomes stained with Hoechst. Yellow box showing cytoplasmic actin and myosin II attached to the cell membrane. Red box represents cytoplasmic actin and myosin II network. d) Images from a time-lapse movie showing the formation of myosin II rods in M-phase. e) Length of myosin II rods in interphase and M-phase. f) Myosin rods inside the chromosome networks. Arrow points to myosin II network. g) Dynamics of chromosomes after actin and myosin II inhibition. Scale bar: a)-c) 5 µm, d) 10 µm.

4.2.4. Cytoplasmic actin network position chromosomes in anaphase

Microtubules play an important role in the chromosome separation and once chromosomes have been transported to the poles, the metaphase spindle disappears in late anaphase. Role of microtubules during M-phase is well characterized. Our studies revealed that the cytoplasmic actin network plays an important role in chromosome positioning and dynamics in early M-phase. Next, we investigated the role of the cytoplasmic actin network during the anaphase. To study the role of the cytosolic actin network in chromosome dynamics in anaphase, we treated cells with different concentrations of Lat B. Low Lat B concentrations (0.1 µM) generated a force imbalance on chromosomes and hence to the dynamics of the chromosomes in the late anaphase. Increasing concentrations of Lat B (section, 3.2**)** resulted in increased chromosome dynamics in late anaphase. We observed that chromosomes displayed maximum dynamics in 0.3 µM Lat B, where as increasing the concentration of Lat B further reduced the dynamics of chromosomes in the late anaphase. In 2 µM Lat B, dynamics of chromosomes in the late anaphase similar to the non-treated cells (Fig. 4-4a-f). Our observation suggests that low concentrations of Lat B partially de-polymerize the cytosolic actin network, resulting in imbalanced chromosome positioning in late anaphase. High Lat B concentrations de-polymerized the

cytoplasmic actin network completely, resulting in the absence of imbalanced chromosome positioning. During separation, chromosomes are balanced in both low and high concentrations of Lat B, suggesting that the cytosolic actin network plays no role in chromosome separation in the anaphase. The chromosome imbalance observed in low concentrations of Lat B indicates the presence of cytoplasmic actin network in anaphase.

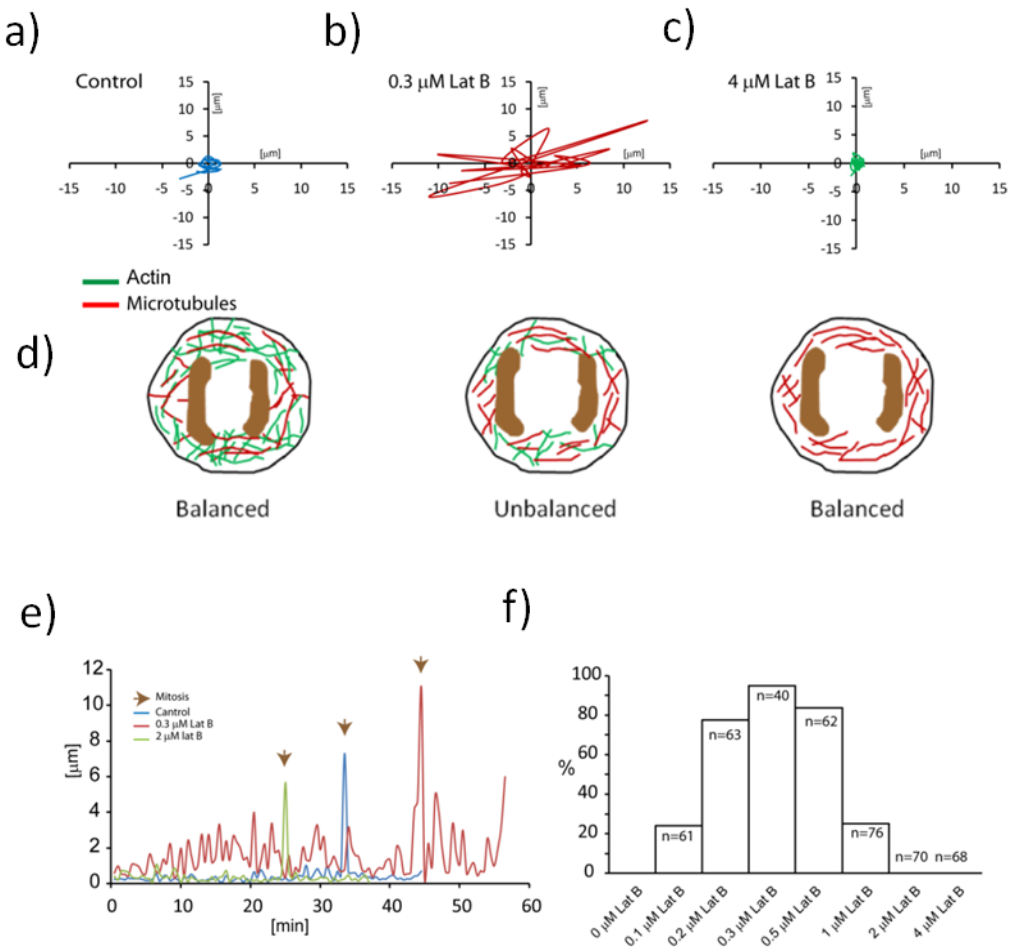

Figure 4-4. Cytoplasmic actin mesh work holding the chromosome in anaphase. a-c) Chromosome Dynamics in different concentration of Lat B in late

anaphase. d) Cartoon illustrating the distribution of cytoplasmic actin mesh work in M-Phase, in different concentrations of Lat B. e) Displacement of chromosome in different concentration of Lat B. f) Chart illustrating the percentage of chromosome imbalance in different Lat B concentrations.

4.2.5. Cytoplasmic actin and microtubules hold chromosomes together in prophase and pro-metaphase

In section (3.4), we showed that actin and myosin II form a network around the metaphase spindle and that the actin network is also present within the chromosome network in prophase. We hypothesize that the cytoplasmic actin network forms a scaffold-like structure in order to hold the chromosomes together in prophase. To test this hypothesis, we partially de-polymerized the cytoplasmic actin network by treating the cells with low concentration of Lat B. We observed oscillating chromosomes inside the cytoplasm possible due to force acting on the chromosomes. We did not see any disintegration of chromosomes in 0.5 µM Lat B (Fig. 4-5a). Depolymerization of microtubules using 2 µM nocodazole also did not lead to disintegration of chromosomes (Fig.4-5b). Next, we simultaneously de-polymerized actin and microtubules using 0.5 µM Lat B and 2 µM nocodazole and interestingly observed disintegration of chromosomes (Fig. 4-5c, d). Within 10 min, chromosomes started to disintegrate and most of the chromosomes were disintegrated from the chromosome network within 20 min. These results suggest that cytoplasmic actin and microtubules play a significant role in holding the chromosomes together in prophase.

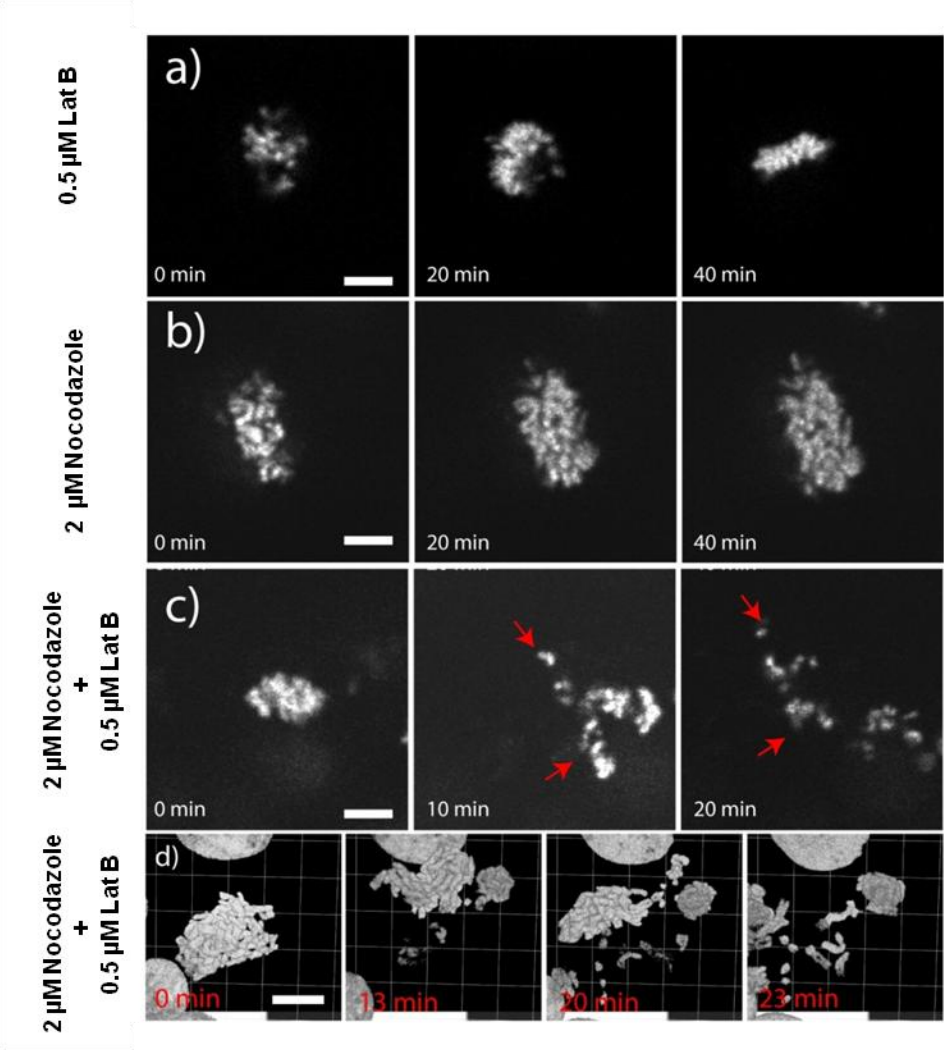

Figure 4-5. Cytoplasmic actin holding the chromosomes in prophase together with microtubules. a-d) Histone B labeled with RFP. a) Hela cells treated with 0.5 µm Lat B. b) Hela cell treated with 2 µm nocodazole. c) Hela cells treated with 2 µm nocodazole and 0.5 µm Lat B, the red arrow pointing points to disintegrated chromosomes. d) Image from 4D movie showing the disintegration of chromosomes after 2 µm nocodazole and 0.5 µm Lat B treatment. Scale bar: 5 µm. Time in minutes.

4.3. Discussion

The morphology of the cell changes drastically during the mitotic phase (M-phase). Actin filaments and myosin II which are present in the cortex play an important role in the drastic morphology change of the cells. Studies showed that cortical flow generated by the actin and myosin II interaction is required for centrosome separation and positioning [5, 161]. Moreover, proper orientation of the mitotic spindle needs cortical actin [162, 163]. When the cell reaches the mitotic phase, drastic rearrangement of cortical actin and myosin II networks take place. Our experiments show that actin and myosin II networks are present not only in the cortex but also in the cytoplasm and these networks play a role in maintaining the chromosome position in M-Phase. When the cell reaches prophase, actin and myosin II fibers form a network around the chromosomes, these acto-myosin fibers and myosin rods are also observed inside the chromosome~~s~~ network in prophase. Proper formation of the spindle assembly is a prerequisite for chromosome segregation. Recent studies also claim the existence of a non- microtubule spindle matrix [164, 165]. In our study, we showed the presence of an actin and myosin II isotropic networks that distributes three dimensionally on the spindle matrix. We hypothesize that cytoplasmic actin and myosin II could play an important role in spindle formation.

5. Materials and method

5.1. Kits used

QIAGEN® Plasmid Mini Kit (QIAGEN) for plasmid-miniprep

QIAGEN® Plasmid Maxi Kit (QIAGEN) for plasmid Maxi kit.

5.1.2. Chemicals

5.2. Chemicals

Table

Description	Source
Ammonium hydrogen carbonate	Alfa
Ammonium nitrate	Carl Roth
Ampicillin sodium salt	Carl Roth
L-Arginin	VWR
L-Asparagin-Monohydrate	VWR
L-Aspartic acid	SIGMA
Calcium chloride	Serva
clonNAT	Werner

Chloroform	Roth
Concavalin A	Roth
L-Cysteine	VWR
Demthyl sulfoxide (DMSO)	Sigma
Deoxynucleotide Solution mix	BioLabs
Difco Bacto Agar	Becton Dickinson
Difco Tryptone	Becton Dickinson
Difco Yeast Extract	Becton Dickinson
dNTP Set, 100mM solution	Fermentas
DTT	Fermentas
EDTA (Titriplex)	Merck
EGTA	Roth
Genticin	Roth
Glycerol	Roth

D(+)-Glucose	VWR
L-Glutamine	PAA
Glycerin 86% p.a.	Roth
D(+)-Glucose	VWR
L-Glutamine	PAA
Glycerin 86% p.a.	Roth
HygromycinB	Merck
Kanamycin Sulfate	Invitrogen
L-Serine	VWR
Salmon Sperm DNA	Eppendorf
Sodium azide	Sigma
Sodium bicarbonate solution	Sigma
Sodium carbonate	Sigma
Sodium hydride	Sigma
Sodium sulfate anhydrous	Alfa

L-Threonine	VWR
Tris, HCl	Merck
TritonX 100	Roth
L-Tryptophan	VWR
L-Tyrosine	VWR
L-Valine	VWR

5.3. Other materials

Description	Source
Coverslip	Menzel Gäser
Filter paper 3MM	Whatman
Glassbeads	Sigma
Glassslide	Menzel Gäser
Lense paper	Assistent

Petri dishes	Greiner
Plastic cuvettes	Brand
Pipet Tips	Qiagen

5.4. Buffers and solutions

10x PBS buffer	92 mM Na_2HPO_4 147 mM KH_2PO_4 27 mM KCL 1.39 M NaCl pH = 7.2
10x Pfu buffer	200 mM Tris-HCl 100 mM KCl 100 mM $(NH_4)_2SO_4$ 20 mM $MgSO_4$ 1 % Triton X-100 1 mg/ml BSA pH = 8.8
PEG mix	100mM LiOAc 10mM Tris-HCl pH8 1mM EDTA 40% (v/v) PEG 3350

Potassium phosphate buffer (10x)	1M KH_2PO_4 1M K_2HPO_4 pH = 7.0
RF1	100 mM RbCl 50 mM $MnCl_2$ 30 KOAc 10 mM $CaCl_2$ 15% (w/v) Glycerol pH = 5.8
RF2	10 mM MOPS 10 mM RbCl 75 mM CaCl2
Rhodamine-Phalloidin staining solution	6.6 µM Rhodamine Phalloidin In Methanol
SORB	100 mM LiOAc 10 mM Tris – HCl 1 mM EDTA 1 M Sorbitol
50x TAE buffer	2M Tris – Base 2M Acetic Acid 50 mM EDTA pH = 8.0

10x TBE buffer	440 mM Tris Base 440 mM Boric Acid 10 mM EDTA pH = 8.0
10x TBS buffer	50 mM Tris – HCl 150 mM NaCl pH = 7.5
10x TE buffer	10 mM Tris-Base 1 mM EDTA pH = 8.0
10x Thermopol buffer	500 mM KCl 15 mM $MgCl_2$ 100 mM Tris-HCl pH = 8.3
1M Tris buffer	619 mM Tris – HCl 381 mM Tris Base pH = 8.0

5.5. Media

***E. coli* media**	
LB-medium (liquid)	1% (w/v) acto ptone 0.5% (w/v) Bacto Yeast-Extrakt 0.5% (w/v) NaCl
LB plates	1% (w/v) Bacto Tryptone 0.5% (w/v) Bacto Yeast-Extrakt 0.5% (w/v) NaCl 0.8% (w/v) Bacto agar
YT-medium (liquid)	0.8% (w/v) Bacto Tryptone 0.5% (w/v) Bacto Yeast-Extrakt 0.5% (w/v) NaCl
YT plates	0.8% (w/v) Bacto Tryptone 0.5% (w/v) Bacto Yeast-Extrakt 0.5% (w/v) NaCl 0.8% (w/v) Bacto agar

5.6. Cell culture

MDCK, HeLa, HaCat cells were culture in DMEM medium with 10% of fetal bovin serum and100µg/ ml penicillin/ streptomycin at 5% CO_2 and 37^0C. Getting low confluent cells, we plated less than 0.5×10^5 cells/ml in 35mm glass bottom dish, after 24 hours we use the cells for imaging. Getting high confluent cells, we plated more than 4×10^5 cells/ml in 35mm glass bottom dish, 3-4 days cell become high confluent (mono layer).

5.7. Transfections and stable cell line

Lifeact plasmid, mRFP actin, MLC II GFP and MHC II GFP were transfected into the MDCK cells using Lipofectamin 2000 kit (Invitrogen). We made stable cell line of Lifeact, MLC II GFP, and MHC II GFP by selecting the cells using 500µg/ml G418. After the transfection we grow the cells four weeks in growth medium contain G418 for selecting the transfected cells. Then we sorted fluorescent cells with help of fluorescent cell sorter.

5.8. Drug treatment

Cells were treated with 50µm of blebbistatin (Invitrogen) for 30 min for inhibit the myosin II activity. We use 2µm of LaT A (Cal bio chem) to inhibit actin.

5.9. Cell staining

Remove the growth medium and wash the cells with phosphate saline buffer three times. We used PTEM to fixed cells (50 mM PIPES, pH 6.8, 10mM EGTA, 1 mM MgCl2, 0.2% Triton X-100, 4% formaldehyde) and incubate 30 minute for fixing cells. Rinse the cells three time using phosphate buffer saline (PBS), We incubate 15 minute in PBS containing 0.5% of the Triton X-100 for permeabilization of the cells. Actin in the cells was stained with 1:200 phalloidin (Invitrogen).

5.10. Laser ablation

We used 12 picosecond-pulsed 405 nm UV laser for the laser ablation. UV laser is coupled to the Olympus FV1000 confocal. 100X objective was use for the ablation and same objective was used for the image acquisition after the ablation.

5.11. Fluorescence recovery after photobleaching

(FRAP) We used 405 nm UV laser to make FRAP. FRAP device was coupled to the UltraVIEW Vox spinning disk microscope. We use velocity software to analyze the half time after the FRAP.

5.12. Live cell imaging

Fluorescent Time laps movie obtained through the imick Microscope attached a spinning disk unit; we capture the image through Andor EMCCD. We used 1.45 NA Olamps objective for imaging and Andor software used for

controlling the microscope stage and EMCCD. Metamorph software was used for the image processing and all the images were further processed by Photoshop and Illustrator.

5.13. EM fixation

1. Preliminary fixation.

Cells were washed with PBS three times. Then added 1 % Paraformaldehyde and 0.02 % glutaraldehyde for 5 min. Cells were washed with PBS three times at room temperature. Cell were kept on the top of dry ice, then add -15^0 C cold MeOH to the cells for 20 min. Then cells were kept at the room temperature for 20 min. After cells were washed with PBS for three times.

2. Washing. 0.1 M Sodium Cacodylate buffer pH 7.4 (Electron Microscopy Sciences, cat.#11653), 3x 5 min at 4°.

3. Osmication. 0,25% OSO4 (2% Osmium Tetroxide, Electron Microscopy Sciences, cat.#19152) in 0.1M Sodium Cacodylate (Sodium Cacodylate 0,2M, Electron Microscopy Sciences, cat.#11653), pH 7.4 for 15 min. at 4°.

4. Washing. 3 times wash in dest. water

5. Dehydration.

- 50% Ethanol for 5 minutes.
- 70% Ethanol for 5 minutes .
- 100% Ethanol for 5 minutes .
- 100% Ethanol for 5 minutes .
- 100% Ethanol/Epon* for 24 h .
- 100% Epon* for 2 h.

6. Embedding in freshEpon*.

7. Polymerization for 48h at 60°.

8. Cutting of 1µm thick sections and localization of cells.

9. Ultra thin serial sections (70 nm thickness), with Ultramicrotome EM UC6 (Leica).

10. Counterstaining of grids in Ultra stainer (Leica) with 0.5% Uranyl acetate (Ultrostain 1, Leica) and 3% Lead citrate(Ultrostain 2, Leica).

11. Image acquisition with JEOL JEM-1230 transmission electron microscope with 80 kV and with Gatan Orius SC1000 digital Camera and with software Gatan Digital Micrograph™.

6. Future perspectives

6.1. Dynamic Microvilli

We identified dynamic microvilli on the apical region of the non confluent epithelial cells like MDCK, HeLa, and HaCaT. In confluent layers, dynamic microvilli are not present but are replaced by the commonly known microvilli actin structures. Conversely, to our knowledge single non confluent epithelial cells have not been studied yet in much detail, except at the basolateral side. Nevertheless, the understanding of intracellular regulatory processes is of high interest to get a closer insight into collective cell tissue behavior. In this context, the identification of dynamic microvilli in single non confluent epithelial cells is a promising step towards the understanding of cell morphogenesis, cellular mechanical force distributions, and cell migration. Interestingly, our studies of dynamic microvilli directly lead us to the identification of an underlying branched network of non-muscle-myosin II (NMMII). So far, isotropic myosin II network structures with prominent myosin filaments, and a structural independence from related actin structures, have to our knowledge, only been reported during epithelial tissue dynamics in Drosophila [166]. The observation that myosin motor proteins can generate a network structure without intercalation between actin filaments gives rise to the question how usually actin-dependent molecules can form network? This question remains to be elucidated in the context of molecular binding mechanisms and structural arrangement.

Apart from the yet unknown myosin network-generating mechanism, we were able to show the dynamic interdependence of the dynamic microvilli network and its underlying myosin motors. Using various drugs (LatA, Blebbistatin)

and laser ablation experiments, we demonstrated dynamics of blocking, depolymerization, or rupture of the two networks. In particular, the rupture of the dynamic microvilli network after Lat A treatment and the local distortion of the myosin network after laser ablation cutting demonstrate that the novel network structures generate a cell cortical tension. We observed slow and uniform recovery of myosin II structures within 1 min following ablation, suggesting that myosin II is capable of efficiently assembling into new filaments and to seal the laser-induced cortical lesion. How the myosin II filament seal the laser-induced cortical lesion and molecules involve in this process need to be explored further. To date, cortical tension has mostly been discussed in the context of tissue morphogenesis [167, 168]. Moreover, the question of force distributions during tissue generation, when epithelial cells are not yet polarized and arranged in the typical hexamer shape, has not yet been addressed in detail. Additionally, epithelial cell can migrate, e.g. during wound healing, or undergo epithelial mesenchymal transition (EMT). For these cases, single epithelial cells have to maintain their cell shape or undergo cell morphogenesis. In this context, the novel dynamic microvilli with its underlying myosin network are presumably responsible to fulfill the tasks. The identification of a maintained cortical tension induced by these networks is a first step to clarify the cell morphogenetic regulations. However, additional experiments addressing the force distribution aspects, such as shear flow or local perturbation experiments have to be conducted to gain deeper insight into the mechanical stabilization and morphological transition processes. Moreover, dynamic microvilli and myosin II network is predominant network in apical region of the epithelial cells and these networks can influence the dynamics of trans membrane proteins on the apical cell membrane.

6.2. Cytoplasmic actin and myosin II network

Tensegrity cell model (TCM) is considered as architecture of life [169]. The major assumptions of the TCMare, 1) mechanical coupling between the cell, the cytoskeleton, and the nucleus, 2) Live cell have an internal pre-stress. Previous studies suggested that actin fibers can connect cell membrane and the nucleus. Our data suggested that actin bundles in the cells help to connect the nucleus of one cell to the nuclei of adjacent. These connections may not only help to transfer mechanical stimulus from the cell membrane to the nucleus within one cell but also to nucleus of other cells within the epithelial sheet. In conclusion, nucleus within the epithelial sheet is physically connected through cytoplasmic actin. This is in line with the first assumption of TCM. We also showed the existence of cytoplasmic network of actin and myosin II and this network can exert force on the nucleus. These data is supporting the second assumption of the TCM. Taken together, our data is strongly suggesting the existence of the TCM.

Cytoplasmic actin and myosin II can play a significant role in the epithelial sheet migration. Our studies showed that actin bundle from the neighboring cells are connected to the nucleus of the respective cells. This could help the nucleus in epithelial sheet to move in the same direction. We also showed that actin bundle is physically attached to the nucleus. It is possible that protein complexes help to attach the actin bundle on the nuclear membrane and these protein complexes could have significant role in maintaining the nuclear shape and mechanotransduction. We can call this complex as Nuclear Focal Adhesion (NFA). Our data suggest that some of the cytoplasmic actin bundle is in proximity to the Nuclear Pore Complex (NPC) (Fig. 3-8). Cytoplasmic actin bundles could attach to the NPC and NPC can

attain different conformation based on the cytoplasmic actomyosin contraction. Conformational changes of NPC play a significant role in the protein diffusion through NPC.

Previous studies suggested that cortical actin and myosin help to position the chromosomes in metaphase. In our study we showed that cytoplasmic actin and myosin II plays an important role in maintaining the chromosome position in metaphase. Chromosome positioning and chromosome segregation mainly depend on the spindle morphology. Spindle morphology is determined by the concentrated action of microtubule dynamics, different motor proteins (kinesin 5, kinesin 10 , kinesin 14 and dynein), and different spindle associate proteins [147]. Mitotic spindle consist of array of microtubules, microtubule associated proteins, and different motor proteins that are self organized to position and segregate the chromosomes. How this self organization is achieved still need to be explore further, but it is clear that different mechanical force plays an important role. Although, studies showed that different proteins can colocalize with spindle, but it is not clear whether these proteins can exert a mechanical force on the spindle. Recent studies suggest that kinesins can interact with both actin and microtubules [170, 171]. Based on these studies, we hypothesize that cytoplasmic actin and myosin can exert mechanical force on the spindle assembly. This mechanical force can play a major role in spindle assembly as well as the chromosome segregation. In future, we will try to address the interaction between cytoplasmic actin network and spindle with the help of *in vivo* and *invitro* experiments. We strongly believe that kinesin motors help to couple the cytoplasmic actomyosin network and spindle assembly.

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Contribution

Figure 2-5c,d, 2-7b,c and 2-9c,d analysis performed by Dr. Philipp M.Diesinger.

Figure 2-9h taken by Christoph Klingner

Figure 2-11e-I, 3-6c taken by Dr. Roland Wedlich-Söldner

Curriculum Vitae

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Publication

Paper:

1) Anoop V. Cherian and Kaustubh R. Rau., *Pulsed laser-induced damage in rat corneas: time-resolved imaging of physical effects and acute biological response*. J.Biomed.Opt.13, (2008).

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2) Anoop V. Cherian, Christoph Klingner, Philipp M. Diesinger, Nicola Maghelli, Thomas Keil, Roland Aufschnaiter, Gisela Beck, Sim Weng Jin, Jean Paul Thiery, Mark Bathe Iva M. Tolić-Nørrelykke and Roland Wedlich-Soldner[1] . *Acto-myosin network drive cell cortex organization in epithelial cells*. (Manuscript under preparation)

3) Anoop V. Cherian , Marianne Braun, Alvaro Hernán Crevenna Escobar , Christoph Klingner , Roland Wedlich-Söldner. *Cytoplasmic acto-myosin assemblies determine nuclear shape*. (Manuscript under preparation)

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network maintaining chromosome positioning during mitosis. (Manuscript under preparation)

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1) Anoop V. Cherian, Roland Wedlich-Soldner, *Actin worms and myosin network – A new perspective on the cell cortex*. Cell Dynamics & Disease, Münster Graduate School Meeting, April 28th- 30th, 2010. (Oral presentation)

2) Anoop V. Cherian, Michael Sixth and Roland Wedlich-Soldner, *Actin " worms ", a novel three dimensional actin network*. International Workshop on Blebs and Cell Cortex Mechanics in Cell Movement. Held on October 6-8, 2008 in Max-Planck Institute for Physics and Complex system. (Oral presentation)
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Poster:

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