AN INVESTIGATION OF THE ROLE OF NUCLEAR ENVELOPE PROTEINS ON ACTIN DYNAMICS

A Thesis

Presented to the Faculty of the Graduate School

of Cornell University

in Partial Fulfillment of the Requirements for the Degree of

M.S.

by

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August 2015

ABSTRACT

Actin is one of the most abundant proteins in eukaryotic cells. Actin structure and actin dynamics play a critical role in a variety of essential biological functions, including cell mobility, intracellular transport and signal transduction. It has been shown that two nuclear envelope proteins - lamin A/C and emerin - may regulate actin dynamics. However, the mechanism behind it remains unclear. Here I tried to elucidate the mechanism of how lamin A/C and emerin regulate actin dynamics from two aspects.

In the first part of my work, I focused on the nuclear actin dynamics. I used a nuclear F-actin probe (Utr230-GFP-3xNLS) and DNase I to detect nuclear F-actin and nuclear G-actin respectively. Lamin A/C deficient cells expressing this probe showed a punctate structure rather than the short filaments in wild-type cells and emerin deficient cells. DNase I staining also showed a more irregular G-actin distribution in lamin A/C deficient cells compared to circular nuclear G-actin patterns in wild-type and emerin deficient cells. These results indicated lamin A/C may play a role in actin dynamics regulation. I proposed that it may regulate actin dynamics through regulating actin binding proteins.

In the second part of this work, I used fluorescence recovery after photobleaching (FRAP) analysis, which is the most widely used method to measure the protein dynamics in live cells, to investigate the cytoplasmic actin dynamics. Unlike previous published data, which showed an enhanced cytoplasmic actin mobility in lamin A/C deficient and emerin deficient cells, my result did not show any difference among them. I proposed that FRAP is not good for cytoplasmic actin dynamics due to the high concentration and mobility of actin.

BIOGRAPHICAL SKETCH

Wei Chen earned his Bachelor of Science in Biotechnology from ShanDong University, Jinan, China in 2012. After graduation, he took a gap year to think about what he will do in the future and then he joined the Master of Science program in Applied Physics at Cornell University.

In college, Wei was always an "outlier" compared to other students. He has a broad interest in biology, math, physics and even economics. He finished course requirements in three years and joined Prof. Sunney Xie's lab in Peking University, where he built a Super-resolution Microscope and use this to solve biological questions.

In Cornell, he joined Prof. Jan Lammerding's lab to work on a project about actin dynamics. At the same time, he expanded his interests in different areas and scientific topics. Now his interests lie in understanding the mechanism behind cell communication and trying to regulate cell behavior. After graduation, he will pursue his PhD in Molecular Engineering at the University of Washington.

This thesis, entitled "Actin Dynamics Regulated by Lamin A/C and Emerin" was supervised by Dr. Jan Lammerding.

I would like to dedicate this thesis to my family, my friends and my advisor, Professor Jan Lammerding.

ACKNOWLEDGEMENTS

Scientific research can be difficult and suffer more failures than successes. I am really lucky to have all my family, friends and my advisor be with me to support my study and give me advice. There are so many people that I want to thank.

I want to start with two whom I owe the most:my parents, for making innumerable sacrifices for my study, for their constant love and support. And I need to thank my advisor Dr. Jan Lammerding for giving me the opportunity to work in his lab and for encouraging me to make the most out of it. I will always keep his advice in mind. Working with him has been one of the great privileges of my life. I also thank my committee member Dr. Warren Zipfel, for his support and advice for my study.

I thank all the lab members for their knowledge and help along with my project. I thank Philipp Isermann and Gregory Fedorchak for their help and advice. I thank Patricia Davidson, Celine Denais, Ashley Kaminski and Alexandra McGregor for their kindness and support.

I gratefully thank all my friends in Cornell and in the U.S.

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CHAPTER 1

INTRODUCTION

In eukaryotic cells, actin is a multifunctional and highly conserved protein that presents in both the cytoplasm and the nucleus. In the cytoplasm, actin dynamically switches between monomer and filaments to execute multiple functions including migration[35], supporting the shape of cell[34] and signal transduction[25, 47, 2]. In the nucleus, actin participates in processes like transcription[32, 31, 55, 15, 22], chromatin remodeling[29, 54, 10, 13] and RNA processing and transport[27, 28]. Two nuclear envelope protein - lamin A/C and emerin - have been shown to regulate actin dynamics[20]. But the mechanism behind it is still unclear. In my project, I am interested in understanding how actin dynamics are regulated by lamin A/C and emerin in the cytoplasm and in the nucleus.

Rho-actin-MKL1-SRF signaling pathway is one of the most important signaling pathway in which actin participate[25, 47, 2]. Previous research in Lammerding lab showed that lamin A/C and emerin can regulate MKL1 activity by regulating actin dynamics[20]. Ho and colleagues found an impaired nuclear translocation of MKL1 and enhanced G-actin proportion and mobility in laminA/C deficient and emerin deficient cells [20]. Considering the fact that emerin can directly bind to the pointed-end of actin filaments and stabilize actin filaments and the fact that introduction of exogenous emerin can completely restore actin mobility and remarkably increase MKL1 nuclear accumulation[20], it was proposed that emerin may play a more important role in regulation of actin dynamics. In my project, I tried to understand whether the altered cytoplasmic actin dynamics are the result of impaired MKL1 signaling or arise from

effects of emerin on the outer nuclear membrane on actin polymerization, either directly or by interaction with other actin-binding proteins. To answer this question, I used FRAP analysis to see if introduction of exogenous MKL1 without RPEL domain, which consistently activate MKL1 pathway, can restore the actin mobility. However, I was unable to observe any difference of actin mobility in different cells. This may be caused by different samples that we chose (more detail will be discussed in 3.2.2).

On the other hand, roles and dynamics of nuclear actin attract lots of attentions. I am interested in whether lamin A/C and emerin can alter nuclear actin structures. I used nuclear F-actin probe - Utr230-GFP-3xNLS - and G-actin probe - DNase I - to label nuclear F-actin and G-actin respectively. Lamin A/C deficient cells with Utr230-GFP-3xNLS showed a punctate structure rather than short filaments in wild-type cells and emerin deficient cells. DNase I staining also showed a different G-actin distribution in lamin A/C deficient cells. Compared to the circular speckles in wild-type cells and emerin deficient cells, lamin A/C deficient cells showed less circular and more irregular speckles in the nucleus. These data showed that lamin A/C may play an important role in nuclear actin dynamics. Since we suspected that Utr230-GFP-3xNLS may induce actin polymerization due to the fact that GFP has a tendency to form dimers, I optimized this probe by replacing GFP with another monomeric fluorescent protein-mNeonGreen. This probe successfully expressed in 293T cells. This probe can further help us improve our understanding of how nuclear actin is regulated.

In the following, I will introduce the necessary background and present and discuss the results which I obtained. I will also mention the difficulties that I encountered and how they may be addressed in the future.

CHAPTER 2

BACKGROUND

2.1 Actin and probes

Actin is a highly conserved globular multi-functional protein that is present in all eukaryotic cells. It exists in two forms: monomeric actin (G-actin) and polymeric actin (F-actin). Actin monomers can bind ATP, which can be further hydrolyzed to ADP to provide the energy for actin polymerization. Actin filaments assemble via non-covalent bonds between monomeric actins and are highly dynamic. Actin filaments are structurally polarized - the faster growing plus (barbed) end the slower minus (pointed) end [40]. A large number of actin-binding proteins regulate actin polymerization by binding to actin monomers and actin filaments(either the barbed end or the pointed end)[40, 34].

In eukaryotic cells, actin plays a critical role in a variety of essential biological functions: it provides a framework for cell shape and polarity[34]; it is essential for cell mobility, cell division and cytokinesis and vesicle movement[35, 7]; it controls cell-cell interactions through adhesion molecules; it also participates in signal transduction, including Rho-GTPases actin MKL1/SRF signaling pathway[19, 2, 47, 25]. Owing to its important roles in biological functions, actin has been studied in detail over the past decades. However, the discovery of nuclear actin opened a new door for this research area.

2.1.1 Nuclear actin: from G-actin to F-actin

Although the presence of nuclear actin was proposed almost 40 years ago, it was not widely accepted until more evidence showed the existence of nuclear actin in the past decade. Nuclear actin has been shown to promote transcription in all three RNA polymerases[32, 31, 55, 15, 22]. It is a component of chromatin remodeling complexes. For example, actin interacts with heterogenous nuclear ribonucleoproteins (hnRNPs) to recruit histone acetyltransferases (HATs) to activate gene expression[29, 54, 10, 13]. It also participates in pre-RNA processing and RNA transport[27, 28]. These results indicate that nuclear actin mainly exists as a monomer in the nucleus and the main function of nuclear actin is to recruit different gene expression complexes and further facilitate gene expression.

Though little direct evidence shows the existence and function of nuclear F-actin, scientists are still interested in whether F-actin exists in nucleus. The first clue of the existence of nuclear F-actin comes from the fact that many nuclear actin related processes, including transcription[51, 24] and nuclear transport of chromosomal loci[12], are interrupted by drugs that inhibit actin polymerization. In addition, actin filament binding protein like myosins also have been shown to participate in multiple nuclear functions, including RNA polymerase I transcription and chromatin remodeling[51, 39]. Further support comes from the discovery of proteins that regulate actin dynamics, including actin nucleation factors ARP2/3 and formins[50, 52], actin depolymerizing/severing protein cofilin, profiling and thymosin $\beta 4$ and actin capping proteins including tropomodulin and gelsolin-like capping proteins[46, 1, 49]. A more direct piece of evidence comes from the observation of actin meshwork structure in the giant

oocyte nucleus of the frog *Xenopus laevis*[4]. This scaffold structure also stabilizes ribonuleoprotein droplets[14]. These results indicate the presence of nuclear F-actin. However, nuclear F-actin in mammalian cells has not been observed until recently with newly developed actin probes[2, 3], which will be discussed in detail in section 2.1.2. Two groups have independently developed probe, Lifeact-GFP-NLS and Utr230-GFP-3NLS respectively, to detect nuclear F-actin. With Lifeact-GFP-NLS, Grosse group found that nuclear actin can directly regulate MKL1/SRF pathway (which is discussed in section 2.2) in nucleus by forming actin filaments[2]. With Utr230-GFP-3NLS, Mullins lab developed nuclear actin probes and found that actin may form a scaffold to help organize nuclear contents[3].

2.1.2 Visualization of nuclear actin

Probes that can specifically bind to actin enable us to monitor the structure of actin and thereby improve our understanding of functions of actin. These probes include phalloidin, DNase I, fluorescent protein tagged actin[53] and actin binding domains (ABDs) fused with fluoresecent proteins. Phalloidin and Dnase I, which specifically label F-actin and G-actin respectively, can only be used in fixed cells, limiting their ability to observe actin dynamics or actin structure in live cells. All the actin fusions (like actin-GFP) are functionally impaired due to the relative large size of fluorescent proteins (GFP (26.9kDa) and actin (42KDa)). Methods that can reliably monitor actin in live cells usually employ the actin binding domains (ABDs) fused with fluorescent protein, such as Lifeact-GFP[38] and Utr230-GFP[3]. Lifeact is a 17 amino-acid long peptide that derived from Abp140, a actin binding protein that binds F-actin in *vivo* and

in *vitro* [38]. Utr230 is a truncation mutant of dystrophin homologue utrophin, which also binds to F-actin with a high affinity[3].

Despite the fact that actin is the most abundant proteins in cells, the concentration of nuclear actin is relative low. Hence, the expression of actin probe in cytoplasm obscures the potential weak signal from nucleus. This problem is solved by fusing nuclear localization sequences (NLS), which theoretically restrict the signal in nulecus. Two groups have used this strategy to independently observe nuclear F-actin in cells.

The Grosse group used Lifeact-GFP-3xNLS to detect nuclear F-actin[2]. With a low expression level in cells, Lifeact-GFP-3xNLS did not show any structures. It shows a homogenous nuclear signal. However, within 30 seconds after serum stimulation, a rapid polymerization of nuclear actin was observed. They verified that this polymerization is regulated by actin regulation protein formins, indicating that nuclear actin is highly dynamic and tightly regulated. Under this situation (serum starvation and stimulation), they also successfully used Phalloidin, for the first time, to detect nuclear actin filaments after serum stimulation (Fig. 2.1). Their research showed the existence of nuclear F-actin and revealed part of the regulation mechanism of nuclear actin polymerization.

The Mullins group screened different actin binding domains and identified that Utr230, a truncation of utrophin, is a good candidate for monitoring nuclear F-actin[3]. They used this probe to monitor nuclear actin and found a punctate structure of nuclear actin filaments with submicron length (Fig. 2.2). In my project, I compared Lifeact-GFP-3xNLS and Utr230-GFP-3xNLS and found that Lifeact-GFP-3xNLS may induce more actin polymerization in nucleus. I chose Utr230-GFP-3xNLS for my experiment. I also optimized Utr230-GFP-3xNLS to

reduce the potential artifact that may induced by this probe. More detail will be discussed in section 3.1.1.

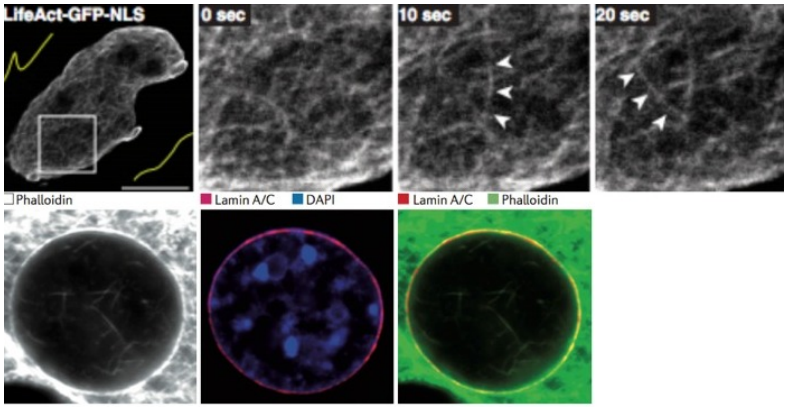

Figure 2.1: **Nuclear actin filaments visualized by Lifeact-GFP-3xNLS and Phalloidin. Top:** Hela cells expressing Lifeact-GFP-3xNLS showed nuclear actin filaments after serum stimulation. **Bottom:** 3T3 cells stained with Phalloidin showed nuclear actin filaments after serum stimulation. Figure panels reproduced from references [2] and [16].

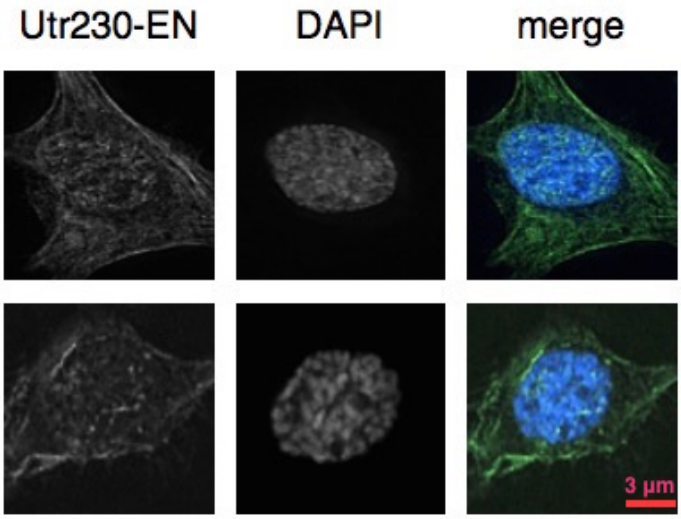

Figure 2.2: **U2OS cells expressing Utr230-EGFP-3xNLS showed short nuclear actin filaments in nucleus.** Figure reproduced from reference[3].

2.2 Rho-Actin MKL1/SRF pathway

Despite the function of mediating cell motility and cell shape, cytoplasmic actin also plays an important role in signal transduction. Actin switches between G-actin and F-actin to execute various functions in response to the environmental stimulation. This polymerization and de-polymerization dynamics are regulated by various actin binding proteins (ABPs). The actin MKL1/SRF pathway provides an example of how the actin dynamics are regulated[25] (Fig. 2.3).

Serum response factor (SRF) is a versatile transcription factor that binds the CArG-box with high affinity[44]. SRF, along with various cofactors, controls genes that are related to a broad range of biological processes, including cardiovascular development, muscle contraction and neuron development [8, 30] . One of the important cofactors of SRF is Megakaryoblastic Leukemia1 (MKL1) from the Myocardin family. The N-terminus of MKL1 contains three G-actin binding domains-RPEL domain[47, 18, 26, 36]. MKL1 is usually in an inactive state in the cytoplasm by reversibly binding to G-actin. In response to the mechanical stimulation or serum stimulation, which activates Rho GTPases, actin is polymerized into F-actins, thereby releasing MKL1 from G-actin and allowing the nuclear translocation of MKL1 [47, 25]. As a result, SRF is activated to express numerous cytoskeleton genes, including actin itself and multiple regulators of actin dynamics, which may stimulate cytoplasmic actin polymerization. Nuclear actin can also modulate this pathway in multiple ways. First, nuclear actin controls the export of MKL1[30]. Second, nuclear actin also can bind to MKL1 so that the binding and release of MKL1 can also control SRF activities directly in nucleus[47, 2].

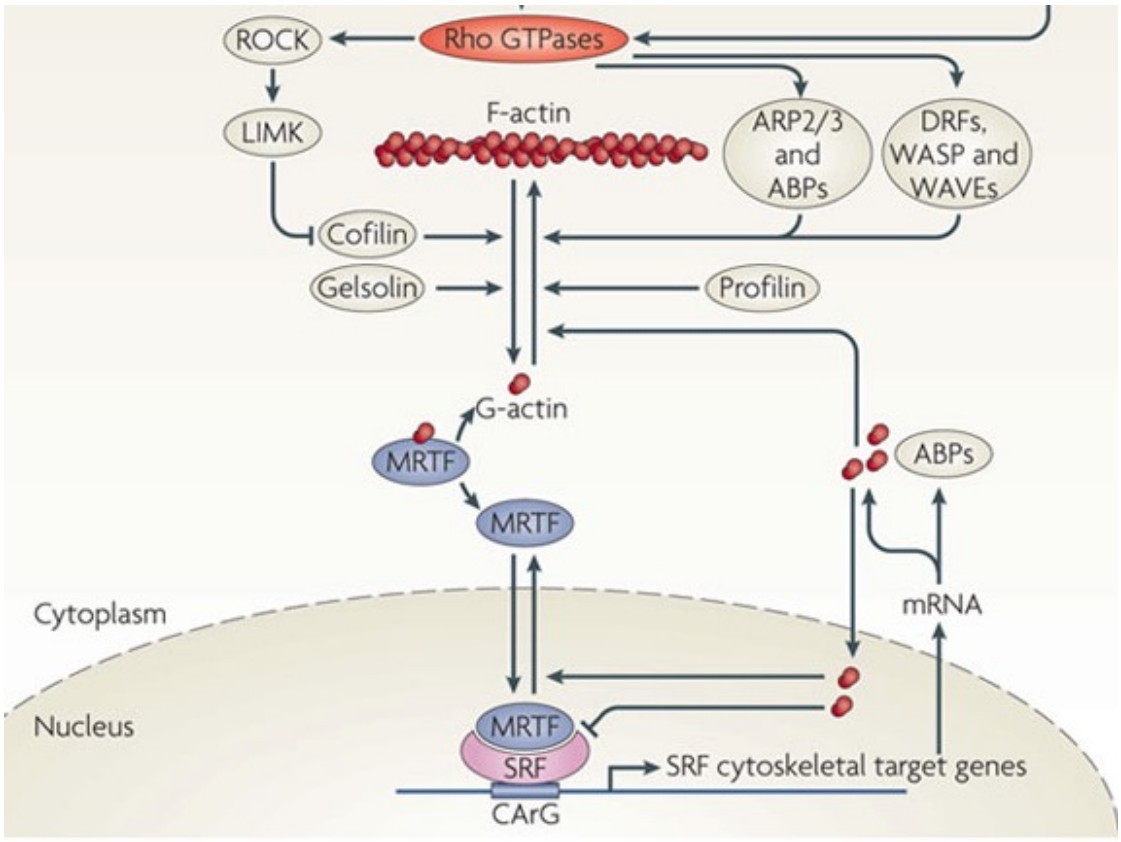

Figure 2.3: **Activation of Actin/MKL1/SRF circuit.** Inactive MKL1 (MRTF in the figure) is bound by G-actin in the cytoplasm and in the nucleus, which inhibits MKL1/SRF regulated gene expression. SRF target genes are expressed in respond to the activation of MKL1/SRF, which includes actin itself and many genes that modulate actin dynamics. These newly produced proteins may stimulate cytoplasmic actin polymerization. Figure reproduced from reference[30].

Previous research done in the Lammerding lab showed that the actin/MKL1/SRF circuit can be regulated by nuclear envelope protein-lamin A/C and emerin[20]. For the next several sections, I will briefly introduce nuclear envelope proteins and their connection to actin/MKL1/SRF ciruits.

2.3 The nuclear envelope

The nuclear envelope (NE) separates cells into nuclear and cytoplasmic regions, spatially and temporally dividing cellular activities in different regions. The NE consists of many complex components, which include the inner and outer nuclear membrane (INM and ONM), nuclear pore complexes and lamina. Nuclear pore complex controls the transport of cargoes including DNA, RNA and proteins. The nucleus is connected to cytoskeleton by the linker of nucleoskeleton and cytoskeleton (LINC) complex across the nuclear evelope, which is also known as the KASH-SUN nuclear envelope bridge[42] due to the names of the components of the complex. The SUN proteins in the INM interact with the KASH proteins in the ONM, creating a 40-50nm perinuclear space between INM and ONM[42]. Recently, many studies revealed that the nuclear envelope is not only a structure that defines nuclear architecture but also plays an important role in mechanotransduction[6]. It has been indicated that the force generated by the cytoskeleton can be transferred to nucleus through the LINC complex, directly regulating the gene expression[30].

2.3.1 Lamins

Lamins are type V intermediate filaments that are associated with INM and help maintain the nuclear structure. Lamins can be grouped into A-type and B-type lamins. In mammals, a single gene, LMNA, encodes all A-type lamins, using alternatively splicing to produce lamin A, lamin C and lamin C2. B-type lamins include lamin B1, B2, B3, which are encoded by two different genes, LMNB1 and LMNB2[6].

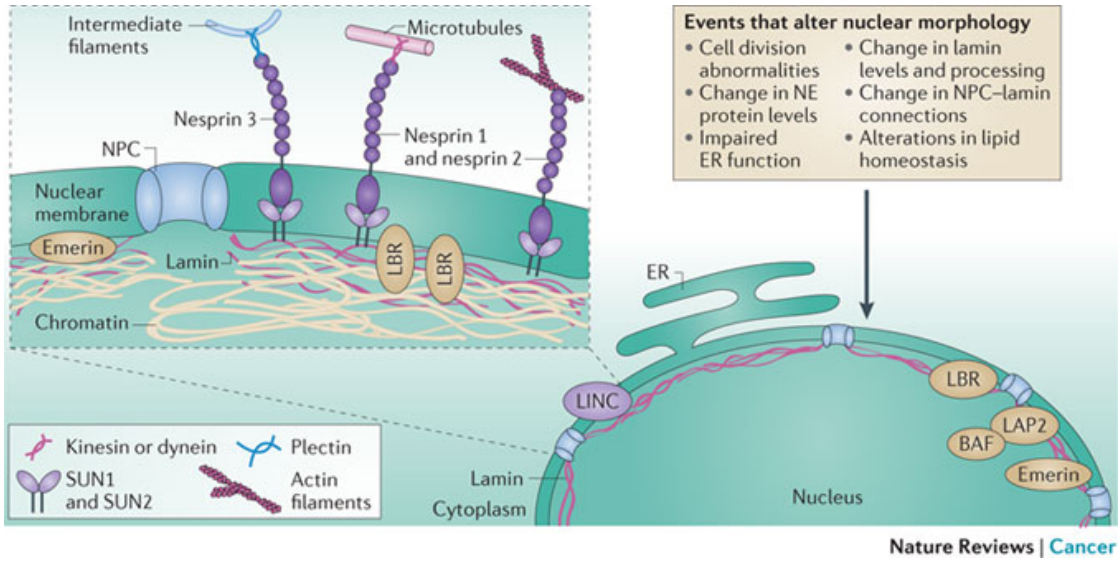

Figure 2.4: **The scheme of nuclear envelope.** Figure reproduced from reference[11].

Lamins are multi-functional proteins due to their variety of interacting partners. They can form complexes with LEM domain proteins, nesprins and SUN proteins. A number of diseases have been shown to be related to mutations in LMNA, which are termed as laminopathies. Mutations in laminA/C can cause Emery-Dreifuss muscular dystrophy (EDMD) by interrupting the localization of emerin on INM. In our lab, we found that in lamin A/C deficient cells, nuclear translocation of MKL1 is impaired due to the reduced import and increased export, as well as increased G-actin proportion and mobility in the cytoplasm[20]. This indicates lamin A/C may regulate actin dynamics.

2.3.2 Emerin

Emerin is an inner nuclear membrane protein that can bind to lamins[20, 9]. Lamin A/C helps emerin to localize at the proper position in the INM[20].

Emerin can be found in almost all tissues and the deletion of its gene is associated with X-linked Emery-Dreifuss muscular dystrophy[17]. Surprisingly, as an INM protein, emerin also binds to the pointed-end of actin filaments to promote actin polymerization and to stabilize actin filaments[21], thus influencing actin dynamics in the cytoplasm or potentially in the nucleus. Emerin deficient cells also showed impaired nuclear translocation of MKL1 and enhanced mobility of G-actin in cytoplasm, as we observed in lamin A/C deficient cells. This result indicates that lamin A/C and emerin both can regulate MKL1/SRF signaling pathway by regulating actin dynamics[20]. In next section, I will introduce this connection in more details.

2.4 Previous work-linking actin MKL1/SRF to nuclear envelope proteins

Previous work done in Lammerding lab investigated whether the loss of lamin A/C and emerin affect the MKL1/SRF pathway. As a result, they found that in lamin A/C deficient and emerin deficient cells, the nuclear translocation of MKL1 is dramatically reduced[20]. This is caused by reduced nuclear import of MKL1 and increased nuclear export of MKL1. However, MKL1 with a mutation on RPEL domain, the actin binding domain, resulted in nuclear accumulation of MKL1, indicating that reduced nuclear translocation of MKL1 is regulated by actin. A larger fraction of highly mobile G-actin as well as slower assembly of stress fibers after disruption actin with cytochalasin D was also observed in lamin A/C deficient cells and emerin deficient cells. Interestingly, exogenous expression of emerin completely restored actin mobility and remarkedly im-

prove MKL1 nuclear accumulation. In contrast, mutant emerin, unable to bind actin, was not able to restore the nuclear translocation of MKL1. These results indicated that emerin may play a critical role in actin dynamics as well as indicating that loss of emerin leads to impaired MKL1 signaling and disturbed actin dynamics.

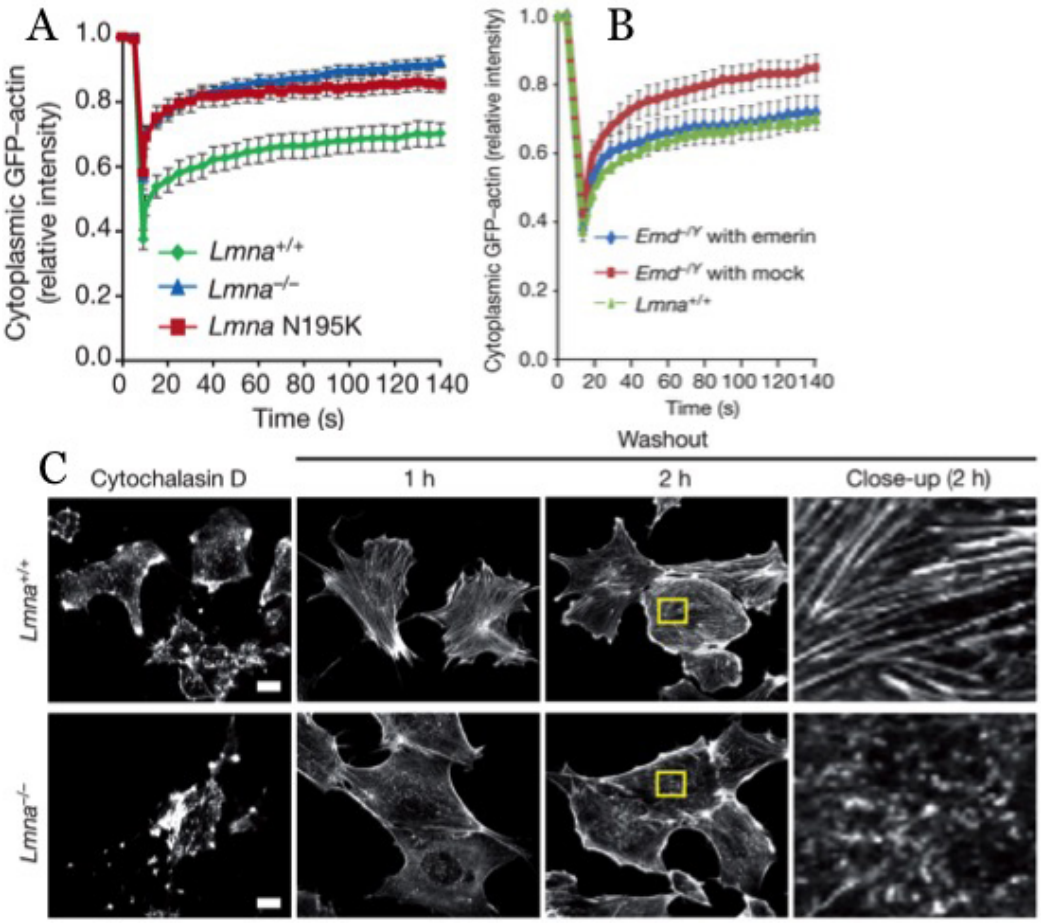

Figure 2.5: **Altered actin dyanamics in lamin A/C deficient cells and emerin deficient cells.** **A,B.** The enhanced actin mobility in lamin A/C deficient cells, lamin A/C mutation cells and emerin deficient cells. This can be restored by exogenous expression of emerin. **C.** Slower stress fiber assembling upon serum stimulation in lamin A/C deficient cells. Figure reproduced from reference[20].

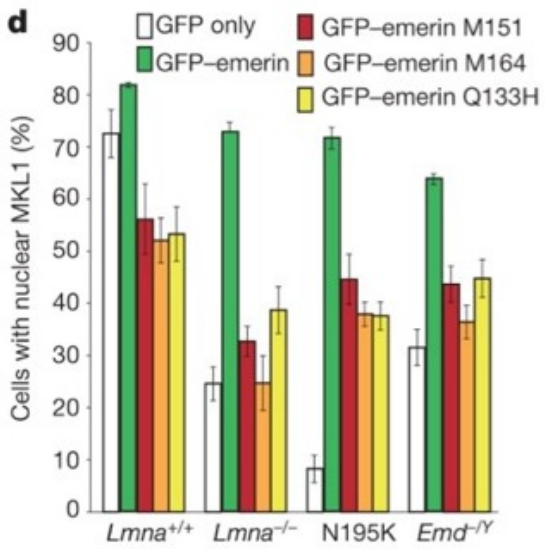

Figure 2.6: **Expression of emerin restored nuclear translocation of MKL1.** Quantification of nuclear MKL1 localization in respond to serum stimulation in wild-type cells, lamin A/C deficient cells, lamin A/C mutant cells and emerin deficeient cells expressing emerin, emerin mutants cannot bind actin and GFP alone. Figure reproduced from reference[20].

Though many pieces of evidence showed that emerin plays a crucial role in actin dynamics regulation, the mechanism behind it remains unknown. Is the altered actin dynamics directly regulated by emerin or it is a downstream consequence of the altered MKL1 signaling? To answer this question, I transfected the constitutively active MKL1-mCherry, which lacks RPEL domain, and the dominant negative MKL1-mCherry, which lacks the TAD domain, in emerin deficient cells to see if they can restore the actin dynamics. Deletion of RPEL domain abrogates MKL1 binding to G-actin, and results in a constant activation of the MKL1/SRF circuit independent of the regulation of actin. TAD domain is the transcription activation domain. Without this, MKL1/SRF complex can bind to transcription site but cannot activate target genes, blocking the transcription site. If the altered actin dynamics are directly regulated by emerin,

neither expression of constitutively active MKL1 nor expression of dominant negative MKL1 in emerin deficient cells is able to restore the actin proportion and mobility to normal level. But if the altered actin dynamic is a result of impaired MKL1 signaling, the constitutively active MKL1 will be able to restore the actin dynamics. Similarly, the dominant negative MKL1 will cause enhanced actin fraction and mobility in cytoplasm in wild-type cells by down-regulating the expression of MKL1 target genes.

To sum up, in my project I am trying to answer three questions. **Firstly**, what structures does actin form in the nucleus? **Secondly**, does deletion of specific nuclear envelope proteins - lamin A/C and emerin - alter the structure and dynamics of nuclear actin? **Lastly**, what is the mechanism of altered actin dynamics? Is it directly regulated by emerin or a consequence of impaired MKL1 signaling?

To answer the first two questions, I used and optimized the nuclear actin probe Utr230-mNeonGreen-3xNLS and tried to find interesting nuclear actin structures with serum starvation and serum stimulation. I also used DNase I to stain the nuclear G-actin to see the G-actin distribution in nucleus. Results of this part will be discussed in section 3.1.3.

To answer the third question, I used constitutively active MKL1 and dominant negative MKL1 to see whether they can restore actin dynamics. Results of this part will be discussed in section 3.2.

CHAPTER 3

EXPERIMENTS AND RESULTS

3.1 Nuclear actin dynamics

3.1.1 Comparison of Lifeact-GFP-3xNLS and Utr230-GFP-3xNLS

Two actin probes are currently available for monitoring nuclear F-actin: Lifeact and Utr230, both tagged with GFP and NLS (Lifeact-GFP-3xNLS and Utr230-GFP-3xNLS). The first step of my project is to compare the reliability of these two probes and choose one for my research. Both Lifeact-GFP-3xNLS and Utr230-GFP-3xNLS were transfected into mouse embryonic fibroblasts cells (MEFs). Cells expressing Lifeact-GFP-3xNLS showed a meshwork-like actin structure in nucleus, with long actin filaments. Cells expressing Utr230 showed less and shorter filaments (Fig. 3.1).

However, in cells that did not express Lifeact-GFP-3xNLS or Utr230-GFP-3xNLS, nuclear actin filamments were not observed by Phalloidin, the most commonly used F-actin labeling drug. This result showed that both Lifeact-GFP-3xNLS and Utr230-GFP-3xNLS may induce actin polymerization in nucleus in our observations. My observation is consistent with previous report about Lifeact-GFP-3xNLS in some other groups. They found that Lifeact-GFP-3xNLS may induce actin polymerization due to its high binding affinity to actin[3]. Compared to Lifeact-GFP-3xNLS, Utr230-GFP-3xNLS has lower binding affinity and less actin filaments in nucleus[3], indicating that Utr230-GFP-

3xNLS is more reliable. Despite the fact that these filaments can only be observed in Utr230-GFP-3xNLS transfected cells, Utr230-GFP-3xNLS is still the best available nuclear actin probe for live cell imaging. In addition, we hypothesized that EGFP, which has a tendency to form dimers, may contribute to actin polymerization. To reduce this artificial polymerization, I decided to optimize Utr230-GFP-3xNLS and this will be discussed in next section.

3.1.2 Utr230-GFP-3xNLS optimization

To minimize the artifacts that may be induced by Utr230-GFP-3xNLS, I decided to optimize it from two aspects. First, it has been reported that EGFP tends to form a dimer[45], which may contribute to artificial actin polymerization induced by the probe. In our construct, EGFP was replaced by a brighter and monomeric green fluorescent protein - mNeonGreen [41]. Second, to find a balance between the detection efficiency and the artifacts that may be induced by the expression of Utr230-GFP-3xNLS, I attempted to construct cell lines that can stably express Utr230-GFP-3xNLS and select for a colony with relative low expression level.

EGFP was replaced by mNeonGreen by PCR and sequence of interest, Utr230-mNeonGreen-3xNLS, was inserted into pCDH lentivirus plasmid by Gibson assembly. This newly constructed probe was tested in 293T cells with transient transfection. I successfully observed signals in the nucleus, dim short actin filaments and punctuate structure (Fig.3.2). However, I was unable to get cell lines that stably expressed this probe. I hypothesized that this probe may affect the vitality of cells. To examine my hypothesis, I used pCDH-

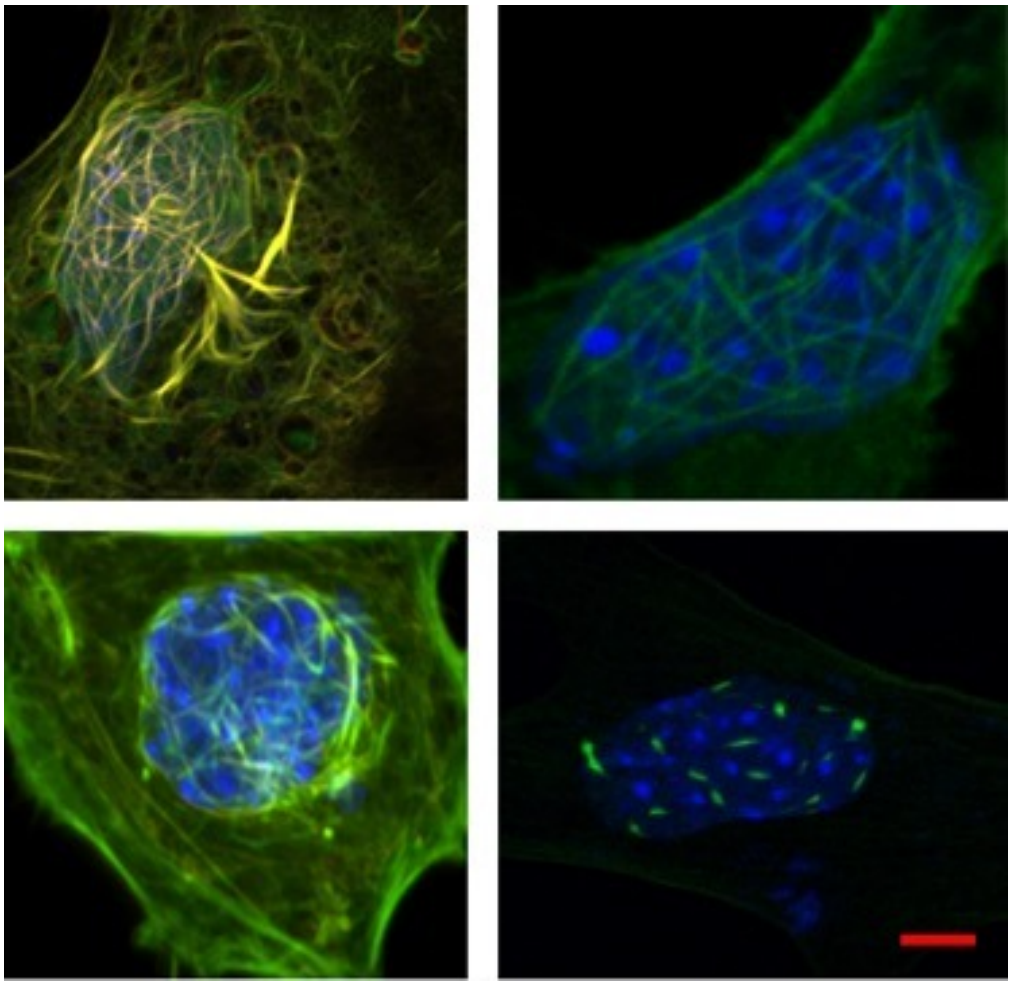

Figure 3.1: **Comparison of Lifeact-GFP-3xNLS and Utr230-GFP-3xNLS in labeling nuclear F-actin. Top:** MEF cells transfected with Lifeact-EGFP-3xNLS. Cells with Lifeact-GFP-3xNLS showed a meshwork-like actin structure with long actin filaments. This structure may be induced by Lifeact-GFP-3xNLS itself. **Bottom:** MEFs cells transfected with Utr230-EGFP-3xNLS. Cells showed a punctate structure of nuclear actin filaments with submicron length. Scale bar size: 3 μm.

NES-mNeonGreen-3xNLS as a positive control to see if this plasmid backbone (pCDH) will affect the cell vitality. After 24 hours of transient transfection, 30 % cells with Utr230-mNeonGreen-3xNLS were dead, while few cells died with the control pCDH plasmid (NES-mNeonGreen-3xNLS) (data not shown), showing that the Utr230 sequence may affect cell vitality. Based on this result, more op-

timizations should be done in the future such as change the concentration of plasmid in transfection.

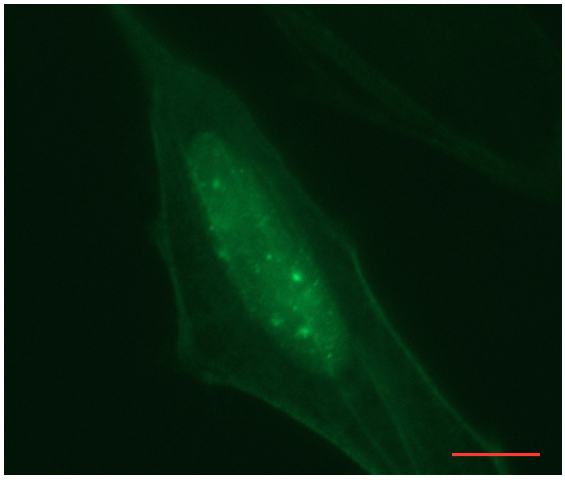

Figure 3.2: **293T cells expressing the Utr230-mNeonGreen-3xNLS (X63).** 293T cells showed dim actin filaments and punctuate structure in the nucleus, which means the probe was successfully constructed. Scale bar size: 5 μm.

3.1.3 Lamin may play a role in actin dynamic regulation.

To investigate the nuclear actin structure and distribution, I used Utr230-GFP-3xNLS and DNase I to label nuclear F-actins and G-actins respectively. Lamin A/C deficient cells showed a more irregular nuclear actin structure for both nuclear F-actin and G-actin, while nuclear actin in wild-type cells and emerin deficient cells did not show much difference (Fig. 3.3 & 3.4).

Nuclear F-actin

Cells expressing Utr230-GFP-3xNLS were fixed and stained with DNA probe Hoechst. Images were taken on Zeiss Confocal microscope. Short filaments

can be observed in both wild-type cells and emerin deficient cells. However, in lamin A/C deficient cells, it showed shorter filaments and punctate structure around the nucleus, indicating that lamin A/C may play a critical role in regulation of nuclear actin dynamics. (Fig. 3.3). I proposed that lamin A/C may regulate the distribution of actin binding proteins that regulate actin polymerization, especially elongation. Further work should be done to verify my hypothesis.

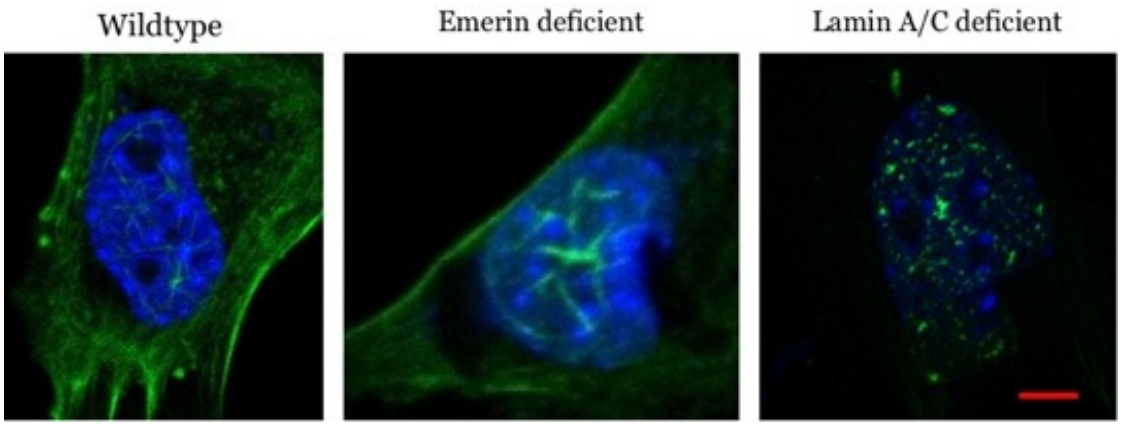

Figure 3.3: **Cells transfected with Utr230-GFP-3xNLS showed different nuclear actin structures.** Short filaments can be observed in nucleus in all cells. Lamin A/C deficient cells showed a shorter filaments and punctate actin structure, showing the potential actin regulation role of lamin A/C. Scale bar size: 3 μm.

Nuclear G-actin

Many researches have shown that serum stimulation after starvation will alter actin dynamics [25, 9, 2, 47]. We treated cells with serum starvation and then serum stimulation, comparing the difference between different cell types. After treatments, cells were fixed and stained with DNase and Hoechst. The staining showed interesting patterns of G-actin in nucleus: actin forms speckles

near nuclear speckles, indicating its role in RNAPI transcription and chromatin remodeling[48]. Compared to the circle speckles in wild type cells and, patterns in lamin A/C deficient cells were more irregular, indicating that lamin A/C may regulate the actin dynamics or distribution in nucleus (Fig. 3.4).

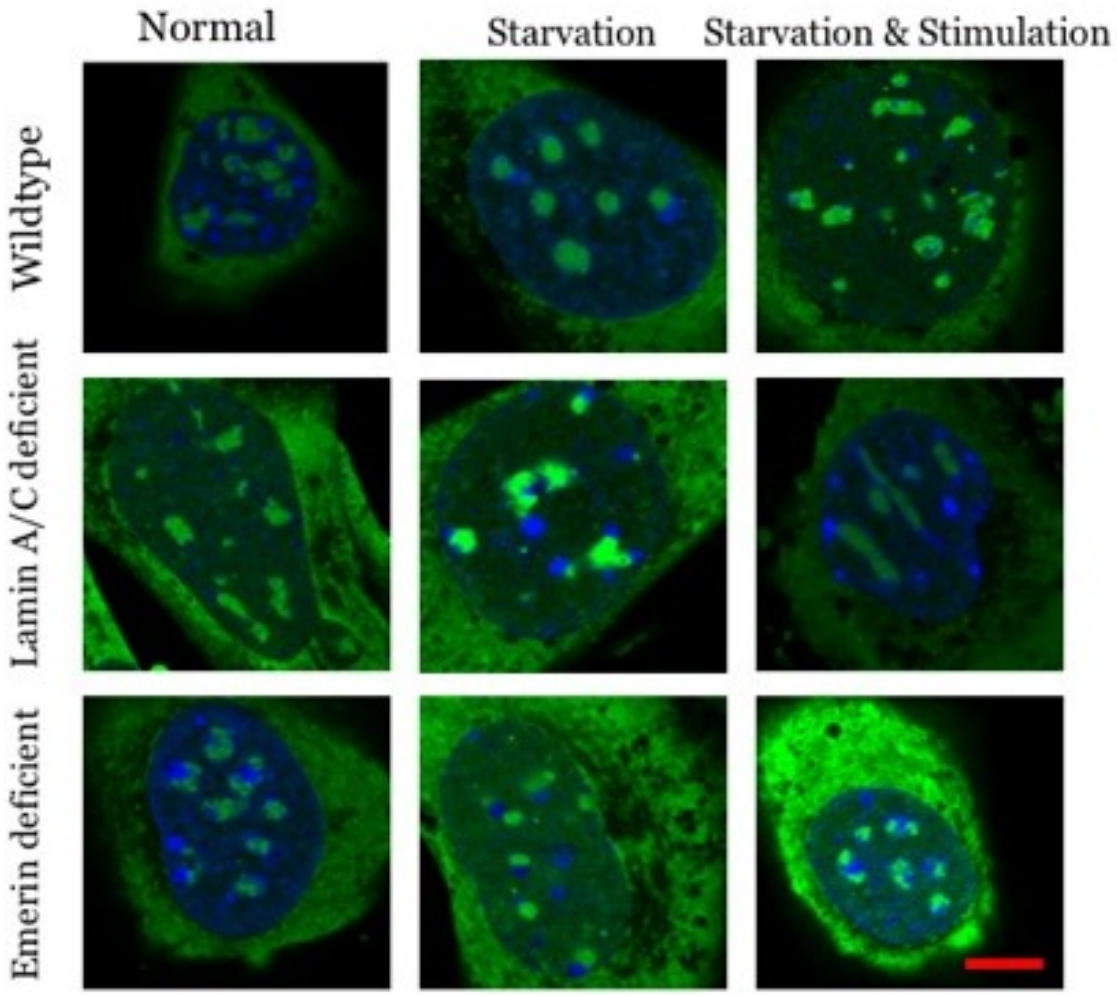

Figure 3.4: **DNase I staining showed a more irregular G-actin distribution in lamin deficient cells.** Cells were stained with DNase I (Green) and Hoechst (Purple). G-actin showed a more irregular pattern in lamin A/C deficient cells after stimulation, indicating lamin A/C may have a role in nuclear actin regulation. Scale bar size: 5 μm.

To further quantitatively compare the difference between patterns in differ-

ent cell types, a MATLAB program was written to analyze the DNaseI staining results. Two criteria - circularity and eccentricity - were used to quantify the structure of the nuclear actin patterns. The circularity is the ratio of the area and the perimeter of the pattern. The value is between 0 and 1, where 1 represents actually a circle and 0 is a line segment. The eccentricity is the ratio of the distance between the foci and its major axis length. The value is between 0 and 1, where 0 represents actually a circle and 1 is a line segment. Length of major axis=2a, minor axis=2b, foci=c. Equations to calculate these two criteria are shown as below:

$$Circularity = \frac{4\pi A}{P^2} \tag{3.1}$$

$$Eccentricity = \frac{c}{a} = \sqrt{1 - \frac{b^2}{a}} \tag{3.2}$$

where P is the perimeters of patterns and A is the area of patterns, $c^2 = a^2 - b^2$.

The statistical analysis is shown in Fig. 3.5 and Fig. 3.6. From Fig. 3.5, we can see that lamin A/C deficient cells showed less circularity after serum starvation and stimulation, while wild type cells and emerin deficient cells did not show any significant difference between before and after starvation and stimulation, indicating the potential regulation role of lamin A/C in actin dynamics. However, the eccentricity analysis did not show any significant difference between different cell types. Since the algorithm is not well established, I proposed that we need to optimize the algorithm for picking up the patterns, which may lead to more accurate measurements.

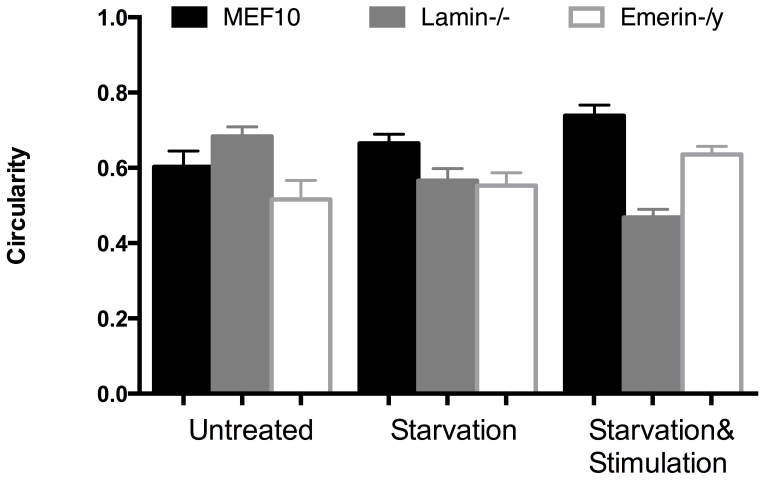

Figure 3.5: **Circularity of nuclear G-actin in different cell types.**

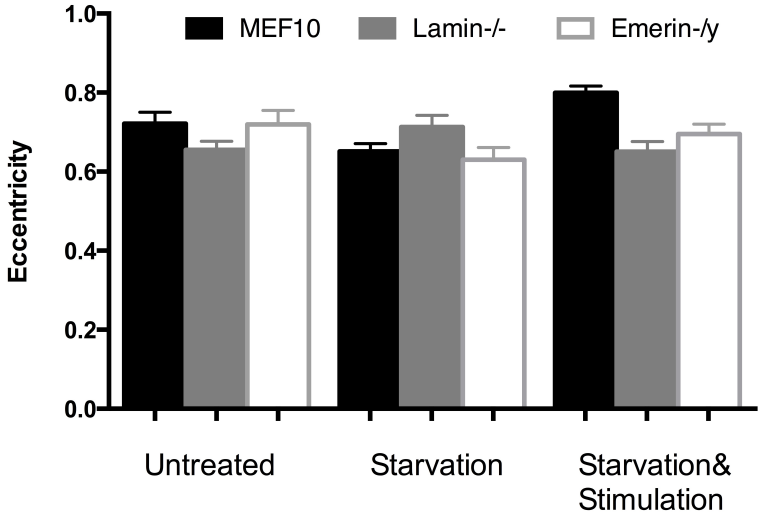

Figure 3.6: **Eccentricity of nuclear G-actin in different cell types.**

3.2 Cytoplasmic actin dynamics

3.2.1 Impaired nuclear translocation of MKL1 in lamin A/C and emerin deficient cells

A previous paper in our lab showed an impaired translocation of MKL1 after serum stimulation in lamin A/C deficient and emerin deficient cells [20]. To repeat this result, I starved three types of cells (lamin A/C deficient, emerin deficient and wild-type cells) for 24h and then stimulated them for 30 minutes before the staining of MKL1 antibody. By comparing the ratio of nuclear MKL1 and cytoplasmic MKL1, we can see whether lamin A/C and emerin can alter the nuclear translocation of MKL1. We found that nuclear translocation of MKL1 in response to serum stimulation is dramatically reduced in laminA/C deficient and emerin deficient mouse embryonic fibroblasts (MEFs) compared to wild-type cells (Fig 3.7, Fig 3.8). The ratio was obtained by dividing the nuclear intensity of MKL1 with the cytoplasmic instensity of MKL1. The nuclear intensity of MKL1 in wild type cells after stimulation was doubled while laminA/C and emerin deficient cells were unable to respond to the serum stimulation. This result was consistent with the results that we obtained before, showing that lamin A/C and emerin can regulate the translocation of MKL1.

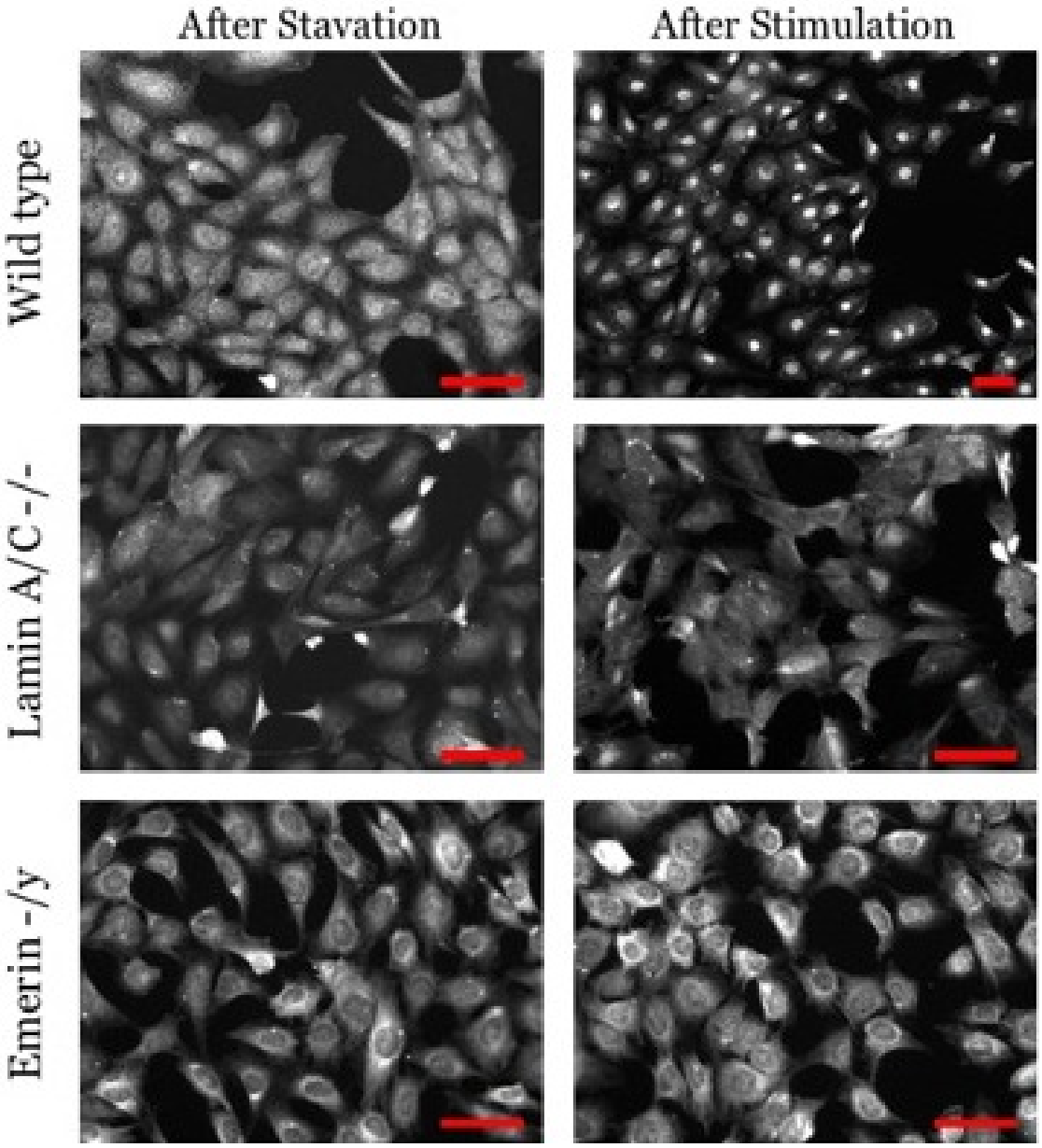

Figure 3.7: **Immunofluorescent staining showed impaired nuclear translocation of MKL1 in lamin-A/C-deficient and emerin deficient cells.** Cells were stained with MKL1 antibody. Wildtype cells showed a significant enhanced nuclear signal of MKL1 after serum starvation and stimulation, while no significant difference was observed in lamin A/C deficient cells and emerin deficient cells, indicating the role of lamin A/C and emerin in MKL1 regulation. Scale bar size: 30 μm.

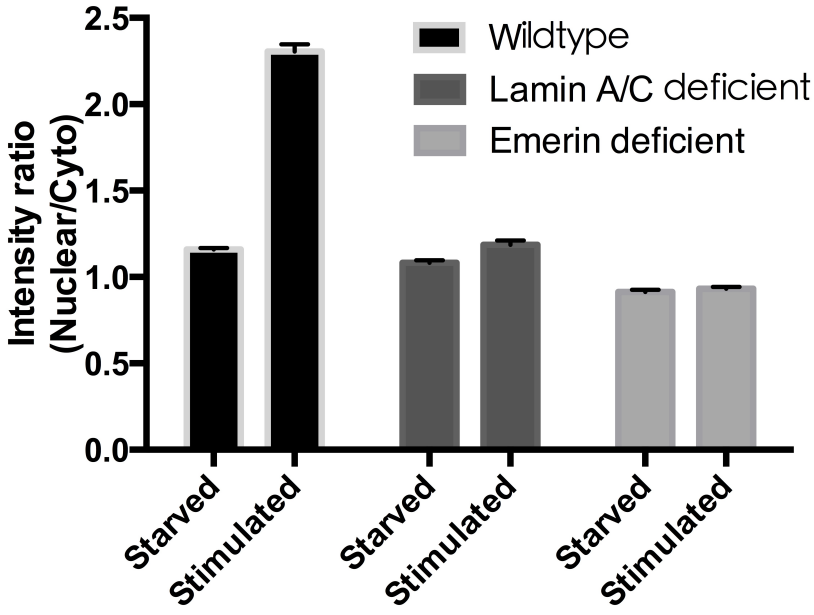

Figure 3.8: **Quantitative analysis of nuclear intensity of MKL1 staining after serum stimulation (n≈ 500 per cell line).** Nuclear intensity of MKL1 was doubled after serum stimulation, while no significant changes were observed in lamin A/C deficient cells and emerin deficient cells.

3.2.2 G-actin dynamics in cytoplasm

Emerin deficient cells were transfected cells with GFP-actin or cotranfected cells with GFP-actin and Δ REPl-MLKL1-mCherry to see whether Δ REPl-MLKL1-mCherry would be able to restore the actin mobility to normal level. However, the result did not show any significant difference between emerin deficient cells and wild-type cells. Several independent experiments had been done to verify this result. Individual experiments were consistent with each other, showing that no significant difference has been observed between cell lines (Fig 3.9a.b.c) and between cells with Δ REPl-MLKL1-mCherry or without Δ REPl-MLKL1-mCherry (Fig 3.9d). By comparing my data and the raw data that we collected

before, I found out that the cells they used had higher expression levels of G-actin and more stress fiber while my cells had low expression level of G-actin and did not have many stress fibers. Since photobleaching and associated fluorescence recovery after photobleach (FRAP) studies can be influenced by a variety of factors, as elaborated on in the discussion, we concluded that FRAP studies of GFP-actin are not the most suitable approach to measure actin dynamics and F-actin/G-actin ratios. Due to time-constraints, unfortunately I was unable to explore additional and alternative approaches.

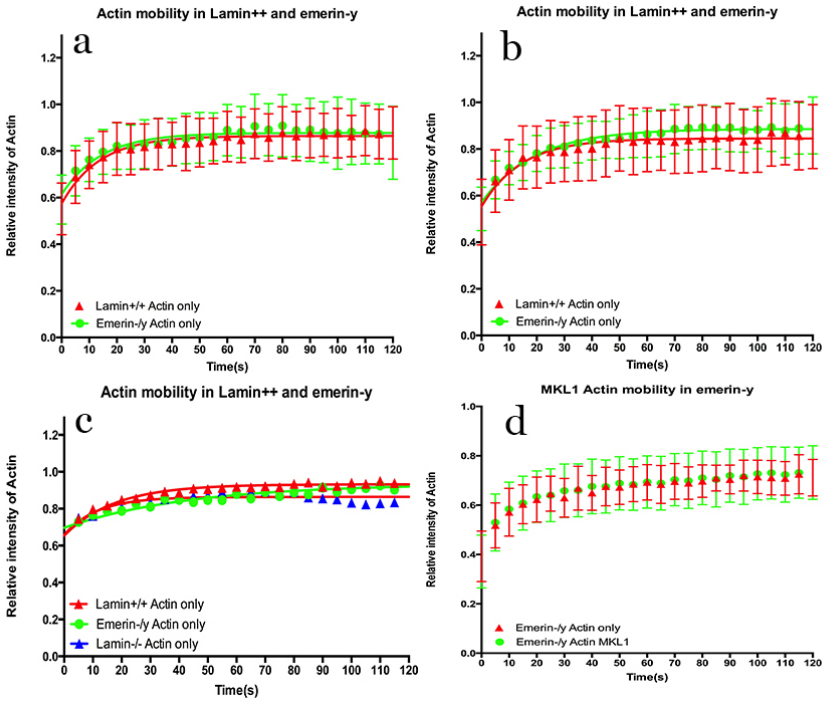

Figure 3.9: **FRAP data did not show significant difference of actin dynamics in different cells**. a~c: Three independent experiments show that there is no significant difference of G-actin dynamic and propotions between wild type cells and laminA/C deficient cells and emerin deficient cells; d. No significant changes has been observed in emerin deficient cells after the transfection with Δ REPl-MLKL1-mCherry

CHAPTER 4

DISCUSSION AND FUTURE WORK

My research focused on the dynamics of actins in the nucleus and in the cytoplasm. Specifically, I am interested in how nuclear envelope proteins (laminA/C and emerin) regulate the dynamics of actin. I developed tools and assay to quantitatively measure the dynamics and structure of actin. Here I will discuss the results that I found, the difficulties that I met and the potential future work that may lead to new discoveries.

4.1 Nuclear actin dynamics

To investigate the structure and dynamics of nuclear actin, I optimized the nuclear actin probe (Utr230-GFP-3xNLS) from Mullins lab. I replaced the EGFP tag with the mNeonGreen, which potentially reduce the artifacts that may be caused by GFP aggregation. This probe enables us to observe the structure of nuclear F-actins. With this probe, I found that the structure of nuclear F-actin in lamin A/C deficient cells is different from those in wild-type cells and emerin deficient cells. Since no evidence showed that lamin A/C directly bind to actin, I proposed that lamin A/C may regulate the localization of actin binding proteins in the nucleus. To verify my hypothesis, we can stain the actin binding proteins, such as mDia[2], profilin[5] and BAF[29, 55], to see if the loss of lamin A/C will alter their locations.

In my fixed cell staining results, I found that actin structure and distribution were more irregular in lamin A/C cells. This result also indicated the potential

role of lamin A/C in actin regulation. Lamin A/C may reguclate the G-actin dynamics by regulating the structure of chromatin since it has been proposed that actin participates in chromatin remodeling[29, 54, 10, 13].

I also tried to construct cell lines that can stably express Utr230-mNeonGreen-3xNLS. As I showed in 3.1.2, 293T cells can express this probe and the signal successfully localized in the nucleus. Many reasons may cause the unsuccessful construction of stable cell lines of lamin A/C deficient cells and emerin deficient cells, including the high passage number of cell lines, the large size of our probe and the potential toxicity of our probe. To exclude these potential reasons, we can borrow a cell line with lower passage number or we can try another plasmid with similar size and see if it will cause cell death.

An alternative solution is using a newly developed nuclear actin probe - nuclear actin chromobody - from Dr. Robert Grosse[33]. This is another powerful tool that enables us to observe the nuclear actin in live cells. In the future, we can test both Utr230-mNeonGreen-3xNLS and nuclear actin chromobody and see if we can observe any interesting nuclear actin structures.

4.2 Cytoplasmic actin dynamics

Based on previous research done in our lab, we proposed that emerin may play an important role in actin regulation. We use FRAP analysis to measure the mobility and proportion of cytoplasmic G-actin. However, my data is consistent with each other but not consistent with previous data. My results did not show any significant difference among wild-type cells, lamin A/C deficient cells and emerin deficient cells(3.2.2). Reasons that causes different results may in-

clude the cells that we choose and photo-bleaching conditions. Photo-bleaching was also a complicated process that was related to the temperature, pH of the buffers, concentration of oxygen in cells and cell conditions etc. Taking all these into considerations, it was possible that we obtained different results. As a result, FRAP may not be a good method to measure the actin dynamics in our project. Regarding the solid evidence shown in previous paper, I may need to use alternative methods to quantitatively compare the difference of actin dynamics in these cell types.

CHAPTER 5

MATERIALS & METHODS

5.1 Plasmids

Lifeact-GFP-3xNLS was a gift from Robert Grosse[2]. Utr230-GFP-3xNLS was a gift from David Mullins[3]. pCDH-Utr230-mNeoGreen-3xNLS construct was built in our lab based on the sequence of Utr230-GFP-3xNLS. MKL1 (ΔRPEL)mCherry, MKL1 (ΔTAD)mCherry were gifts from Maria K. Vartiainen[37]. The MKL1 (ΔRPEL)mCherry does not contain RPEL motifs, abrogating G-actin binding and leading to constitutively nuclear localization independent of actin polymerization. MKL1 (ΔTAD)mCherry does not contain TAD domain that activate the gene expression but still can bind to transcription site.

5.2 Cell lines and cell culture

Cells used in my experiments (lamin A/C deficient (-/-), emerin deficient (-/-) and lamin A/C (+/+) cells) were all MEFs from $Lmna^{+/+}$[43], $Emd^{-/Y}$[23], $Lmna^{-/-}$[43] mice, respectively. They were a gift from C. Stewart.

All cells were cultured in 25T flask containing Dulbeccos Eagles Modified Media (DMEM) with GlutaMax (Gibco, Invitrogen) containing 10% fetal bovine serum (FBS) (PAA), and 1% penicillin & streptomycin at 37 °C in a humidified atmosphere with 5% carbon dioxide and were splited every two days. Before treatment, cells were seeded on glass bottom dish or a coverslip for further treatments (transfection or immunofluorescence staining).

Serum starvation was done by incubating the cells in 0.3% FBS for 18 h. Starved cells were stimulated by DMEM with 20% FBS for 30 minutes.

Transient transfection was carried out using Lipofectamine2000 Reagent (Invitrogen) according to manufacturers protocol. 1 ~ 1.5 μg of plasmid DNA was used per transfeftion reaction in 6-well plate. The ratio of Lipofectamine 2000 and DNA was 3:1 (μl:μg).

5.3 Cloning

5.3.1 PCR

All PCR were run with homemade Phusion DNA Polymerase and Phusion GC 5x buffer(F-519L). Annealing temperature was 68 °C. Three fragments of DNA-Utr230, mNeonGreen and 3xNLS-were amplified by PCR and purified independently. 3′ end of Utr230 and 5′ end of mNeonGreen were overlapped. 3′ end of mNeonGreen and 5′ end of 3xNLS were overlapped. Two rounds of PCR and purification were used to put three fragments into our sequence of interest (Utr230-mNeonGreen-3xNLS).

5.3.2 Double/single enzyme digest

Restriction site was carefully chosen by looking through the sequence. Only NotI-HF (NEBR3189S) was suitable for both pCDH backbone and our sequence of interest (Utr230-mNeonGreen-3xNLS). Purified Utr230-mNeonGreen-3xNLS

fragment and pCDH backbone were digested by NotI-HF for 3 hours at 37 °C. After digestion, products were purified by Gel purification. Ligation was done with NEB Quick *Ligation*^{TM} Kit (NEBM2200L). Both digestion and ligation were conducted by following the manufacturer's protocol.

5.3.3 Gibson Assembly

Gibson Assembly was performed as the manufacturer's protocol described. Primers for Gibson Assembly were designed by NEBBuilder. Utr230-mNeonGreen-3xNLS was amplified with Gibson Assembly primers and purified by PCR purification. pCDH was digested by NotI-HF (NEBR3189S) and purified with Gel purification kit. 60ng of purified pCDH vector and 40ng Utr230-mNeonGreen-3xNLS were used in Gibson Assembly. The ratio of vector and fragment is around 1:10 in this reaction. Add water to 5 μl and mix with 5 μl 2x Master Mix. Incubate samples in a thermocycler at 50 °C for 45 minutes.

5.3.4 Transformation and cell culture

Genome modified competitive *Escherichia coli* was used to amplify the plasmids. 5 μl products of ligation or Gibson Assembly was used for 30 μl competitive cells transformation. After transformation cells were activated by adding LB medium to 200 μl and incubating in shaker with 37 °C, 220 RPM for 1 hour. Activated cells were seeded on LB plates for overnight incubation. Colonies were picked next day for colony PCR. Choose the colonies with correct sequence for expanding and then send them for sequencing.

5.4 Fluorescent staining

Cells were evenly seed on glass bottom dish a day before fixation. Density was about 1/50 of 25 flask in 2 ml medium. Cells were starved for 18 hours in DMEM with 0.3% FBS and then were stimulated with 20% FCS for 30 minutes. Rinse cells with PBS once and then fix instantly with 2% glutaraldehyde in Cytoskeleton buffer (10 mM(pH 6.1) MES, 150 mM NaCl, 5 mM EGTA, 5 mM Glucose, 5 mM $MgCl_2$) for 15 minutes. Quench with freshly prepared $NaBH_4$ buffer for 7 minutes. Wash with PBS for 3 times and 7 minutes each. Block with Blocking buffer (3% BSA and 0.5% Triton-X100 in PBS) for 30min.

5.4.1 Nuclear G-actin staining

Stain with Deoxyribonuclease I (labeled with Alexa Fluor 488 1/300) and Phalloidin (labeled with Alexa Fluor 647, 1/30) in blocking buffer for 1h at room temperature without shaking, since Dnase was extremely sensitive to shaking. Wash with PBS for 3 times and 7 minutes each. Incubate with Hoechst Nuclear stain (1:1000) in PBS- for 15min. Wash with PBS for 2 times and 7 minutes each.

5.4.2 MKL1 staining

Stain with MKL1 (MRTF-A) primary antibody(1/300) for 1 hour at room temperature. Wash with PBS for 3 times and 7 minutes each. Stain with secondary antibody-Goat anti-Mouse IgG congugated with Alexa Fluor 488 (Invitrogen A-11029)- at room temperature for 30 minutes. Wash with PBS for 3 times and 7

minutes each.

5.5 FRAP (Fluorescence recovery after photobleaching) assay

For live cell FRAP imaing, a Zeiss LSM 700 confocal microscope (Carl Zeiss) equipped with a X63 oil imersion objective (Carl Zeiss) was used. Cells were maintained at 37 °C in HEPES-buffered DMEM during the period of imaging. During FRAP experiments, cells were plated on a coverslip and mounted onto a glass slide with a depression containing culture media. For imaging settings, cells were scanned 5 times before photobleaching. Then the region of interest ($\sim$ 2μm) was bleached 80 times at 100% laser intensity of a 488-nm laser and 405-nm laser. Images were then collected at 2s-intervals with an imaging laser power of 10 % of the laser intensity. The FRAP data was collected in Zeiss black software and the intensity information was copied to Excel and analyzed by MATLAB code I wrote.

5.6 MATLAB for image analysis

5.6.1 FRAP data analysis

A MATLAB code was written to analyze the FRAP data. 5 images took before bleach was used to normalize the fluorescent intensity in time series. Continuously photobleaching due to imaging was normalized by average intensity of the whole cell at each frame. Normalized data was binned into a 5s interval. For example, time points between 0 to 2.5 seconds were set to 0s and points

between 2.5 7.5 seconds were set to 5s. (Please check Appendix. A.1 for more details). Analyzed data were plotted in Prism 6.

5.6.2 DNase I staining analysis

A MATLAB code was written to analyze the patterns of DNase I staining of nuclear G-actin. Images were opened in MATLAB and patterns were extracted by setting a threshold to delete all the unwanted regions. Extracted regions were analyzed by the existing function regionprops to measure the parameters that we need - area, perimeter, eccentricity. Statistics of these parameters were performed and plotted in Prism6.

APPENDIX A
CHAPTER OF APPENDIX

A.1 FRAP analysis M file

```
%FRAP_analysis.m
% read the excel file into MATLAB
[FileName,PathName] = uigetfile('*','Select the directory and ...
    datafile');
source=sprintf('%s%s',PathName,FileName);
[status,sheets] = xlsfinfo(source);
filename = FileName(1:(end-5));
suffix = FileName(end-4:end);
filename = strcat(filename,'_after_analysis',suffix);
finals = {'Time','Mean of Bleached region','SEM','# of ...
    samples','Mean of control region','SEM','# of samples'};
for sheet=1:size(sheets,2)
    a=char(sheets(sheet));
    data = xlsread(source,sheet);
    %Start data analysis
    %Be careful: every cell must have 3 columns. Time, Bleached ...
        and Control.
    %Otherwise the data will be messy.
    [r,c]=size(data);
    b=2;%number of frames before bleach
    for i=1:c
        ini=data(b+1,i);%choose the initial time point;
        norm=mean(data(1:b,i));
        for j=(1:r)
            if mod(i,3)==1
                data_1(j,i)=data(j,i)-ini;
            else
                data_1(j,i)=data(j,i)/norm;
            end
        end
    end
    data_1(:,2:3:c)=data_1(:,2:3:c)./data_1(:,3:3:c); ...
        %normalize the bleached region with control region.
    % Bin the time
    dt=5;
    for i=(1:3:c)
        for j=(1:r)
            div=idivide(abs(data_1(j,i)),int32(dt),'fix');
            m=mod(abs(data_1(j,i)),dt);
            if data_1(j,i)<0
                if m-dt/2>0
                    data_1(j,i)=-(dt*(div+1));
                else
                    data_1(j,i)=-(dt*(div));
```

```
                end
            else
                if m-dt/2>0
                    data_1(j,i)=(dt*(div+1));
                else
                    data_1(j,i)=(dt*(div));
                end
            end
        end
    end
    tmax=max(max(data_1(:,1:3:end)));
    tmin=min(min(data_1(:,1:3:end)));
    data_final=zeros(size(tmin:dt:tmax,2),7);
    data_final(:,1)=tmin:dt:tmax;
    index=1;
    for k=1:size(data_final,1)
        log=(data_1==data_final(k,1));
        % bleached region at point t
        shifted_log=log;
        shifted_log(:,2:end)=log(:,1:(end-1));
        shifted_log(:,1)=0;
        data_final(index,2)=mean(data_1(shifted_log)); %mean value
        data_final(index,3)=std(data_1(shifted_log))/ ...
            sqrt(size(data_1(shifted_log),2));    %std
        data_final(index,4)=size(data_1(shifted_log),1);    ...
            %number of points
        % control region at point t
        shifted_log2=log;
        shifted_log2(:,1:2)=0;
        shifted_log2(:,3:end)=log(:,1:(end-2));
        data_final(index,5)=mean(data_1(shifted_log2));
        data_final(index,6)=std(data_1(shifted_log2));
        data_final(index,7)=size(data_1(shifted_log2),1);    ...
            %number of points
        index = index + 1;
    end
    data_plot=vertcat(finals, num2cell(data_final));
    xlswrite(filename,data_plot,a)
    clear data_1 data data_plot data_final
end
```

A.2 Actin analyze M file

```
%Actin_analyze.m
% read the image into MATLAB
[FileName,PathName] = uigetfile('*','Select the directory and ...
    datafile');
```

```
source=sprintf('%s%s',PathName,FileName);
Pic=imread(source);
filename = FileName(1:(end-5));
clear FileName source;
%Start data analysis
threshold=input('Please input the threshold: ')% Manually pick ...
    a threshold based on the value in ImageJ
[r,c] = find(Pic>threshold); %Apply the threshold to the image.
% r,c gives out the positions of pixels whose value are larger ...
    than threshold.
str = num2str(threshold);
filename=[filename,' ','threshold','=',str,'.mat'];
pattern=zeros(size(Pic,1),size(Pic,2));
for i =1:size(r,1)
    pattern(r(i,1),c(i,1))=Pic(r(i,1),c(i,1));
end
clear r c threshold
%figure, imshow(Pic), title('original image');
% Find out the connected pixels to form a pattern.
Connected_pixels = bwconncomp(pattern,8);
A = regionprops(Connected_pixels, 'area'); % Calculate the area ...
    of each pattern
P = regionprops(Connected_pixels, 'Perimeter'); % Calculate the ...
    perimeter of each pattern
E = regionprops(Connected_pixels, 'Eccentricity'); % Calculate ...
    the Eccentricity of each pattern
%convert structure to cell array
A = struct2cell(A);
P = struct2cell(P);
E = struct2cell(E);
% Arbitrarily delete the small pattern. Here I set the ...
    threshold to be 100
for i =1:size(A,2)
    if A{i}< 70
        A{i}=[];
    end
end
full = ¬cellfun(@isempty,A);
A(full==0) = [];%delete the empty cell
P(full==0) = [];
E(full==0) = [];
A=cell2mat(A);% For calculation, convert cell array to array.
P=cell2mat(P);
E=cell2mat(E);
% Calculate the Circularity
C=P.^2./(4*pi.*A);
A=A';
P=P';
C=C';
E=E';
save([PathName, filename]);
```

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