1-1-2017

# Identifying The Function Of The Calpain Small Subunit In The Mechanics Of Cell Migration

Bingqing Hao
*Wayne State University,*

# IDENTIFYING THE FUNCTION OF THE CALPAIN SMALL SUBUNIT IN THE MECHANICS OF CELL MIGRATION

by

**BINGQING HAO**

**DISSERTATION**

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

**DOCTOR OF PHILOSOPHY**

2017

MAJOR: BIOLOGICAL SCIENCES

Approved By:

_____

Advisor Date

_____

_____

_____

## DEDICATION

I would like to dedicate this thesis to my dissertation advisor, Karen A. Beningo; my parents, Peizhi Hao and Fengzhi Jiao; and all my loved family members. The years' of study and the thesis dissertation would not have been possible without my advisor's guidance in research and constant encouragement to move forward, my parents' unconditional love, and strong support from all my friends. All of you are the power escorting my road ahead.

## ACKNOWLEDGEMENTS

This thesis becomes a reality with the kind support and help from many individuals. I would like to extend my sincere gratitudes to all of them.

First and foremost I would like to express my gratitude to my dissertation advisor, Dr. Karen A. Beningo. The realization of this thesis would not have been possible without her constant encouragement, guidance and patience.

I would like to thank all my dissertation committee members, Dr. Miriam L. Greenberg, Dr. Raymond R. Mattingly, and Dr. David Njus for their continued support, helpful questions and suggestions for my thesis and experiments, and even lab resources including equipment and reagents.

I would like to also thank Dr. Athar Ansari and Dr. Xiangdong Zhang for allowing me to have access to their equipment.

I would like to thank all members of the Beningo Lab, both past and present, for being such wonderful colleagues and friends by giving technique suggestions, sharing scientific thoughts and working together as a team. I would like to thank Vishnu for getting me started with my project. I would also like to thank Indrajyoti, Shalini, Tara, Shawna, Snehal, Liz and Imjoo with whom I worked together in the laboratory. I would like to specially thank Alex for the editing work for my thesis. I learned a lot from each of them not only as team members but also as friends.

I would like to thank all professors I have taken classes with for sharing their knowledge. I would like to thank all past and present Biological Science Department staff for their help and support for both of my study and teaching experience.

I would like to thank the Graduate School for finalcial support in the form of Graduate Teaching Assistanship, Graduate Research Assistantship, Graduate Enhancement Research and Travel Awards, and. Dissertation Fellowship.

I would like to thank the funding support by NIH R01GM084248 to K.A.B to make this project possible.

I would like to also thank all my friends for always being on my side.

Last but not the least, I would like to thank my dear parents for unconditioned love, understanding, and support for both of my study and life. They have made so much sacrifice for me during these years so that I can pursue my dream. I know no matter where I am, they will always have my back.

## TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

# CHAPTER 1 INTRODUCTION

Cell migration plays a pivotal role in many physiological events including morphogenesis, wound healing, and immune response. Dysfunctional cell migration underlies multiple disease states, such as chronic inflammation, vascular disease, and tumor metastasis (Franco and Huttenlocher, 2005). Not surprisingly, given its physiological importance, cell migration is also crucial to technological applications including tissue engineering (Lauffenburger and Horwitz, 1996). As early as 1675, migrating cells were observed by van Leeuwenhoek with his hand-made microscopes. However, the extensive study on the mechanism of cell migration did not start until 1970s (Ananthakrishnan and Ehrlicher, 2007; Mogilner and Oster, 2003). Significant progress on the molecular mechanisms regulating cell migration has been made within the last decade. The bulk of this progress can be attributed to rapid technological advances in microscopy and to the advent of omics.

The community has come to learn that cells migrate in response to signals from the external environment. These signals can be transmitted in a chemical or physical form and are detected by receptor proteins on the cell membrane and transmitted intracellularly through signaling cascades (Alberts et al., 2002). The effects of biochemical signals on cell migration have been heavily studied (Keller, 2005; Parent and Devreotes, 1999). Nevertheless, the understanding of the effects of the physical factors on cell migration has been expanded extensively only in the past 20 years. As with the understanding of migration mechanisms this can be attributed to technical advances, principally the

application of multidisciplinary approaches in nanotechnology, biophysics, and modern cell biology. It is already well established that the mechanical properties of a cell and chemical signals co-contribute to the regulation of cell migration. Physical influences on migration, can be intracellular or extracellular, and can include dimension, fluid shear stress, hydrostatic pressure, compression stress, environmental stiffness, and topography to name just a few (Friedl and Wolf, 2010; Georges and Janmey, 2005).

Classically, focal adhesions (FAs) are critical membrane sites where both inside-out and outside-in signaling occurs and believed to be the nexus of mechanical communication. FAs are large aggregates of proteins that most often accumulate around the transmembrane receptors of the integrin family. Integrin receptors span the plasma membrane connecting extracellular matrix (ECM) components with the intracellular actin cytoskeleton (Burridge & Chrzanowska-Wodnicka, 1996). Thus integrins are known to serve as both a linkage to the cytoskeleton and signal transducers in multiple signaling pathways both biochemically and biophysically, and play key roles in development, immune responses, leukocyte traffic, and cancer (Hynes, 2002). Numerous proteins that associate with FAs are also involved in regulating multiple signaling pathways (Clark and Brugge, 1995; Burridge and Chrzanowska-Wodnicka, 1996), including regulating cell migration (Browning et al., 2014; Fang et al., 2014; Fogh et al, 2014; Hopkinson et al., 2014). Members of the calpain family of proteases are known to localize to FAs, and are implicated in the turnover of FA component proteins (Bhatt et al., 2002; Dourdin et al., 2001; Franco et al., 2004b; Goll et al., 2003). Furthermore, previous studies indicate that

calpains are involved in the regulation of cell migration (Bhatt, 2002; Dourdin et al., 2001; Huttenlocher, 1997; Mamoune, 2003; Potter, 1998). In this chapter, we will discuss the mechanical aspects of the cellular microenvironment that affect cell migration and the functions of calpains on cell motility.

## Integrated Mechanical Events in Cell Migration

### *Focal Adhesions*

Focal adhesions are not present in all cell types, in fact, some cell linages, such as leukocytes, migrate effectively without any detectable focal adhesions (Burridge & Guilluy, 2015). However, for those cells that rely on focal adhesions for migration, adhesion strength and traction forces must be coupled dynamically to ensure the effective migration of these migratory cells (Burridge & Guilluy, 2015). The number of focal adhesion component proteins is massive. Over 150 proteins are identified in integrin-mediated adhesions including adaptor proteins, structural proteins, cytoskeletal proteins, actin-binding proteins, serine/threonine protein kinases, serine/threonine protein phosphatases, tyrosine phosphatases, proteases, tyrosine kinases, modulators of small GTPases, to name just a fraction (Zaidel-Bar et al., 2007).

Focal adhesions undergo cycles of assembly and disassembly during cell migration. During migration, nascent adhesions (smaller than ~0.25 μm) assemble near the cell periphery within the lamellipodium (thin, sheet-like membrane protrusions at the leading edge of a motile cell) in an actin polymerization-dependent manner (Stricker et al., 2011). As the leading edge moves forward, a subpopulation of the nascent adhesions

disassembles leaving the remainder of them to mature into focal complexes (~0.5 μm) and finally into focal adhesions (1–5 μm) (Gardel et al., 2010). A subset of focal adhesions may further mature into stable fibrillar adhesions or disassemble (Gardel et al., 2010; Laukaitis et al. 2001; Webb et al. 2004; Zaidel-Bar et al. 2003).

Accompanying the gross morphological change of adhesion maturation, the molecular composition of adhesions also undergoes change. Studies indicate that early and mature focal adhesions are different in composition. For example, short-lived focal complexes that form along the leading lamella, contain $\beta_3$-integrin, paxillin, vinculin, α-actinin, and Arp2/3, while proteins in focal adhesions at the cell periphery are highly tyrosine phosphorylated and usually contain $\alpha_v\beta_3$ integrin. The proteins found in the fibrillary adhesions, located centrally in the cell, contain $\alpha_5\beta_1$ integrin and no phosphotyrosine (Zaidel-Bar et al., 2003). After integrins are activated, the adapter protein paxillin is recruited to the protrusive regions of the cell to form the nascent adhesions (Laukaitis et al., 2001). When nascent adhesions continue to grow into the cell center, α-actinin is recruited to focal adhesions and associates with actin cytoskeleton (Choi et al., 2008; Laukaitis et al., 2001; Pasapera et al., 2010). This adhesion maturation requires myosin II. Vinculin and zyxin recruitment to focal adhesions are dependent on the elongation of adhesion-associated actin bundling promoted by the actin crosslinking property of myosin II (Choi et al., 2008; Pasapera et al., 2010). Focal adhesion kinase (FAK) phosphorylation of the adaptor protein paxillin mediates the myosin II-dependent recruitment of vinculin to focal adhesions (Pasapera et al., 2010). In addition, tyrosine

phosphorylation of early FA proteins, including FAK, paxillin, etc., can act as scaffolds for phosphotyrosine (PY)-binding SH2 domain-containing proteins to bind (Pasapera et al., 2010).

As indicated above, posttranslational modifications such as tyrosine phosphorylation and dephosphorylation of the various focal adhesion proteins play critical roles in maintaining focal adhesion dynamics and functions (Pasapera et al., 2010). Other types of posttranslational modification of focal adhesion components include dimerization, protease proteolysis, etc. The site-specific dimerization of FAK is required for activation of FAK's kinase-dependent functions (Brami-Cherrier et al., 2014). Many critical focal adhesion proteins are modified by proteolysis mediated by calpain proteases, including FAK (Carragher et al., 1999), paxillin (Carragher et al., 1999), Rho A (Kulkarni et al., 2002), and talin (Franco et al., 2004b).

***Traction Forces***

While migrating, cells physically interact with the ECM through focal adhesions. Integrins, the key components of focal adhesions, are involved in bi-directional transmission of mechanical forces and mechanosensing (Na et al., 2008). Traction force is the force generated by the acto-myosin cytoskeleton and transmitted onto the ECM through focal adhesions. The process of traction force generation and regulation has been extensively studied and many mechanistic questions remain unanswered although some fundamental observations have been made (Bershadsky et al., 2003; Burridge and Chrzanowska-Wodnicka, 1996; Dumbauld et al., 2010; Wang, 2009). Evidence supports

a model that activation of Rho by growth factors, peptides, or adhesion, stimulates contractility by elevating MLC phosphorylation. This activates myosin function promoting myosin filament assembly and generating force that aligns the actin filaments and bundles them into stress fibers. The tension transmitted to the integrins results in their clustering and further stimulates FAK activity leading to the assembly of focal adhesions (Burridge and Chrzanowska-Wodnicka, 1996; Dumbauld et al., 2010; Hotchin & Hall, 1995).

***Mechanosensing***

Mechanical perturbations from the environmental factors continuously act at the interface between cells and between cells and ECM. Mechanosensing is the ability of a cell to sense the mechanical properties of the extracellular environment in terms of changes in the compliance of the substrate, localized forces, topography, and so on (Bershadsky et al., 2003; Lo et al., 2000; Kshitiz et al., 2012). Cells sense these mechanical factors and react via local structural changes in adhesions and the cytoskeleton, cell motility, proliferation, and survival (Bershadsky et al., 2003). The detailed mechanism for mechanosensing is being intensely studied but is not very well understood yet.

***Modes of Cell Migration***

There are different modes of cell migration of individual cells based on the cell type and the environment, these are referred to as amoeboid and mesenchymal cell migration. Amoeboid cell migration commonly refers to the migration of fast moving cells (about 20

μm/min) that do not have a highly organized cytoskeleton and tend to adhere weakly (Friedl and Wolf, 2010; Krakhmal et al., 2015; Panková et al., 2010). In amoeboid cell migration the cell is rounded or ellipsoid in shape when migrating. This mode is often used by leukocytes, neutrophils, circulating stem cells and certain types of tumor cells. The cells utilizing amoeboid migration either move by plasma membrane blebbing without adhering or pulling on substrates, or generating weak adhesive interaction with the substrates through actin-rich filopodia at the leading edge (Friedl and Wolf, 2010). Amoeboid migration is usually accompanied by fast deformability in cell shapes and adaption of cell shapes to the structure of the surrounding ECM and a lack of ECM proteolysis (Krakhmal et al., 2015).

Cells using mesenchymal cell migration are usually slow moving (about 0.1–2 μm/min) and have an elongated spindle-like shape (Panková et al., 2010). This type of migration is often referred as “fibroblast-like” migration and has been observed in endotheliocytes, smooth muscle cells, keratinocytes, fibroblasts, some types of tumor cells, and so on (Krakhmal et al., 2015). Cells using mesenchymal migration commonly have elaborate cytoskeletal structures and adhesions, and the low migration speed is likely limited by variables including ligand levels, integrin level, integrin-ligand binding affinities, etc (Palecek et al., 1997; Panková et al., 2010). Existence of proteolysis is required to remodel surrounding ECM and generate trails for cells to transmigrate during mesenchymal cell migration (Friedl and Wolf, 2010).

The amoeboid and mesenchymal types of cell migration are mutually switchable (referred as mesenchymal-amoeboid transition / MAT, or amoeboid-mesenchymal transition / AMT) (Krakhmal et al., 2015; Panková et al., 2010). The mechanisms of MAT or AMT remain unclear. AMT was described in macrophage development process. Freely moving monocytes using amoeboid mode of migration develop into resident macrophages at peripheral tissue that perform mesenchymal type of migration (Friedl, 2004). Inhibiting Rho or ROCK function in A375m2 and LS174T cells resulted in a morphological switch from blebbing amoeboid-like phenotype to mesenchymal-like phenotype (Sahai and Marshall, 2003). Inhibition of the Cdc42 regulator DOCK10 or its downstream effectors N-WASP and PAK2 also result in AMT transition (Gadea et al., 2008). On the other hand tumor cells can switch to a rounded mode of motility when elongated motility is inhibited by inhibiting extracellular proteases (Sahai and Marshall, 2003). The factors described to result in the MAT transition include inhibition of pericellular proteolysis, reduction in the activity of integrin receptors and integrin-ECM interactions by antagonists, and strengthening of RHO/ROCK signal pathways (Friedl, 2004; Krakhmal et al., 2015).

Most migratory cell types migrating on two dimensional (2D) subtrates or three dimensional (3D) matrices employ a mesenchymal mode of migration (Friedl, 2004). This is a highly orchestrated process and generally involves four stages: cytoplasmic protrusion of the leading cell edge, adhesion formation, generation of traction stresses through the adhesions, and detachment of the rear adhesions (Chang et al., 2013; Ridley

et al., 2003). Thus this mode of migration requires a highly spatially and temporally regulated dynamic interaction between the cell and substrates (2D and 3D) (Friedl, 2004). Mesenchymal migration begins with the cell assuming a polarized morphology, a distinction between cell front and rear (Lauffenburger and Horwitz, 1996). An early event in polarization involves filamentous F-actin redistribution to concentrate at a particular region, followed by redistribution of other molecules including integrin adhesion receptors, chemotactic peptide receptors, and integrin-cytoskeleton linkages to name a few (Lauffenburger and Horwitz, 1996; Schmidt et al., 1993; Sullivan, 1984). Polarization results in extension of membrane protrusions in the direction of movement, referred as lamellipodium and filopodium (Condeelis, 1993). The overall rate of cell migration in the absence of stimulus gradients is dependent on the linear migration speed and directional persistence time (Lauffenburger and Horwitz, 1996).

The lamellipodia and filopodia mainly contain actin and actin-associated proteins, and are devoid of cytoplasmic organelles (Lauffenburger and Horwitz, 1996). In lamellipodia, actin filaments are cross-linked into a lattice-like meshwork, and in filopodia, they are cross-linked into bundles. Actin polymerization is sufficient for extension of the structures and thought to push the membrane outward (Condeelis, 1993). The key to rapid growth and shrinkage involves uncapping the existing filaments, severing of them, and formation of new actin trimeric nucleation sites for actin polymerization (Lauffenburger and Horwitz, 1996).

Integrins are transported preferentially to the cell front where nascent adhesions preferentially form (Schmidt et al., 1993). These adhesive structures grow in size and intensity as the cell migrates, and become linked to the cytoskeleton. They persist and remain fixed to the substrate until they reach the cell rear (Schmidt et al., 1993).

In addition to the protrusive force generated by actin polymerization to extend membrane processes, lamellipodia or filopodia, contractile force is also generated during migration in order to move the cell body forward (Lauffenburger and Horwitz, 1996; Schmidt et al., 1993). Contractile force is produced by actomyosin machinery (Kim, 2015). The traction force is a readout of the contractile force, but they are not identical. Traction force can be lost by cell deformation and by disruption of cell-substratum attachments, activities where contractile forces are still active (Lauffenburger and Horwitz, 1996; Schmidt et al., 1993). In a migrating cell, the nascent focal adhesions formed at the leading edge can generate higher magnitudes of traction force whereas more mature larger focal adhesions found in the center and tail exert weaker forces (Beningo et al., 2001). Detachment of the cell rear occurs through weakened integrin-cytoskeleton interactions or ripping of the cell membrane leaving integrin containing fragments behind (Lauffenburger and Horwitz, 1996; Schmidt et al., 1993). Cytoskeleton contractility contributes to the detachment of the cell rear and peptides that inhibit the actin-myosin interactions inhibit the breakdown of focal adhesion complexes (Crowley and Horwitz, 1995; Lauffenburger and Horwitz, 1996).

Clearly a number of signaling pathways contribute to the finely orchestrated detachment of the cell rear (Crowley and Horwitz, 1995; Lauffenburger and Horwitz, 1996). Tyrosine phosphorylation is implicated in destabilization of focal adhesions since addition of a constitutively active recombinant tyrosine phosphase inhibits both the phosphorylation and focal adhesion destabilization (Crowley and Horwitz, 1995). The protease calpain was observed to play a role in the regulation of cell migration through the control of rear-end detachment of focal adhesions (Glading et al., 2002). Characterization of MEKK1-null MEFs demonstrates that MEKK1 regulates the ERK1/2 pathway for control of calpain-catalyzed rear-end detachment (Cuevas et al., 2003). In summary, efficient cell migration via the mesenchymal mode of migration is a highly coordinated process both temporally and spatially and can be regulated at multiple stages involving different levels of sophistication, even through the direct proteolysis of adhesion proteins.

**Structure and Properties of Calpain Proteases**

Members of the calpain family are cytoplasmic cysteine proteases that require calcium for their activation. In the human calpain gene superfamily, there are 15 known calpain catalytic genes (*CAPN1-3* and *CAPN5-16*) (Maki et al., 2012; Ono and Sorimachi, 2011), two calpain regulatory small subunits genes *CAPNS1* and *CAPNS2*, and an endogenous inhibitor called calpastatin, which inhibits the proteolytic activity of calpains in a highly specific manner (Goll et al., 2003; Maki M et al., 2012; Ono and Sorimachi, 2011; Suzuki et al., 2004) (Table 1.1). Two of the most heavily studied calpain

holoenzymes, μ-calpain and m-calpain, are each composed of a common 28 kDa small subunit, known as calpain 4 (CAPNS1 or CAPN4, encoded by *CAPNS1* or *CAPN4* gene), which heterodimerizes with 80 kDa large subunits known as calpain 1 or calpain 2, respectively (CAPN1 and CAPN2, encoded by *CAPN1 and CAPN2* genes respectively). CAPN1, CAPN2, and CAPNS1 are considered conventional calpains. Calpains with domain structures similar to CAPN1 or CAPN2 are defined as classical calpains, these include calpain 1, 2, 3, 8, 9, 11, 12, 13, and 14. These calpains contain a C2-domain-like (C2L) and $Ca^{2+}$-binding penta-EF-hand (PEF) domains plus a cysteine protease (CysPc) domain. Calpains in which C2L and/or PEF are missing are classified as non-classical calpains. These include calpain 5, 6, 7, 10, 15, and 16 (Maki M et al., 2012; Ono and Sorimachi, 2011). Out of all the calpain family, calpain 3, 6, 8, 9, 11, 12 are tissue specific, while the rest are expressed ubiquitously (Ono and Sorimachi, 2011).

The structures of conventional calpains are shown in Figure 1.1 (Franco and Huttenlocher, 2005). A conventional calpain large subunit is composed of four domains (domain I-IV), while the small subunit contains domain V and VI. Domain I is an N-terminus single α-helix, which binds to domain VI of the small subunit (Franco and Huttenlocher, 2005). This direct interaction is important for stabilizing of the confirmation of domain II (Suzuki et al., 2004). Domain II is the protease domain and is further divided into IIa and IIb. The catalytic triad site Cys105 on IIa is too far away from the other two sites His262 and Asn286 on IIb, suggesting an inactive conformation that requires modification upon activation. Domain III contains eight β-strands arranged in a

**TABLE 1.1 Calpain family members are listed with their tissue specificity, domains and motifs, and their classification.**

| Calpains | Tissue Specificity | Domains and Motifs | Classical/ Non-classical |
|---|---|---|---|
| **CAPN1** | Ubiquitous | CysPc, C2L, PEF | Classical |
| **CAPN2** | None in erythrocytes | CysPc, C2L, PEF | Classical |
| **CAPN3** | Skeletal muscle | CysPc, C2L, PEF | Classical |
| **CAPN5** | Ubiquitous | CysPc, C2L, C2 | Non-classical |
| **CAPN6** | Embryonic muscles, placenta | CysPc, C2L, C2 | Non-classical |
| **CAPN7** | Ubiquitous | $MIT_{(2)}$ , CysPc, $C2L_{(2)}$ | Non-classical |
| **CAPN8** | Gastrointestinal tracts | CysPc, C2L, PEF | Classical |
| **CAPN9** | Gastrointestinal tracts | CysPc, C2L, PEF | Classical |
| **CAPN10** | Ubiquitous | CysPc, $C2L_{(2)}$ | Non-classical |
| **CAPN11** | Testis | CysPc, C2L, PEF | Classical |
| **CAPN12** | Hair follicles | CysPc, C2L, PEF | Classical |
| **CAPN13** | Ubiquitous | CysPc, C2L, PEF | Classical |
| **CAPN14** | Ubiquitous | CysPc, C2L, PEF | Classical |
| **CAPN15** | Ubiquitous | ZnF, CysPc, SOH | Non-classical |
| **CAPN16** | Ubiquitous | CysPc variant, IQ | Non-classical |

| | | | |
|---|---|---|---|
| **CAPNS1** | Ubiquitous | PEF | N/A |
| **CAPNS1** | Ubiquitous | PEF | N/A |
| **Calpastatin** | Ubiquitous | Four repetitive inhibitory units: domain 1, 2, 3, 4 | N/A |

MIT(2) and C2L(2) indicate two repeated domains. ZnF, zinc finger; IQ, calmodulin-binding motif; SOH, SOL homology domain; N/A, not applicable. Classical or non-classical indicates that the protein has or does not have tandem domains of CysPc-C2L-PEF. Calpastatin contains four repetitive inhibitory units referred to as domain 1, 2, 3, and 4. All four domains contain the consensus sequence GxxE/DxTIPPxYR.

structure that is similar to C2 domains (C2-domain-like domain, C2L), which binds $Ca^{2+}$ and phospholipids (Franco and Huttenlocher, 2005). It has been suggested that this domain interacts with the plasma membranes (Gil-Parrado et al., 2003). Both domain IV in the large subunits and domain VI in the small subunits contain five consecutive EF-hand motifs, which also bind calcium. However, the fifth EF-hand of domain IV and VI do not bind calcium, but interact with each other to form the heterodimeric holoenzymes. One exception is calpain 3 that forms homodimers and binds $Ca^{2+}$ at the fifth EF-hand motif instead of interacting with another PEF domain (Goll et al., 2003; Partha et al., 2014). Domain V at the N-terminus of the small subunit is a glycine-rich domain and is thus highly flexible. The structure of this domain remains unresolved by crystallography (Goll et al., 2003).

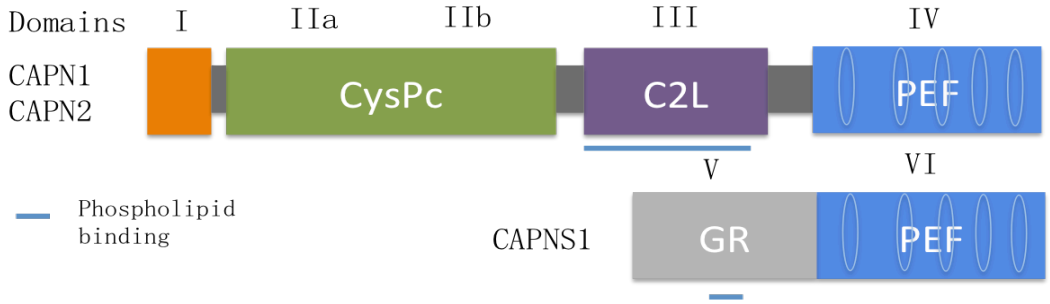



**Figure 1.1: Schematic representation of the domain structure of conventional calpains.** The holoenzymes of μ-calpain and m-calpain, each include the common 28 kDa small subunit, which heterodimerizes with 80 kDa large subunits calpain 1 and calpain 2, respectively. The 80 kDa large subunits calpain 1 or 2 are composed of four domains, domain I-IV, and the 28 kDa small subunit is composed of domain V and VI. Domain II is the protease domain, which is further divided into domain IIa and domain IIb. Domain III is a C2-like domain, which is known to interact with $Ca^{2+}$ and phospholipids. Domain IV and domain VI both contain five consecutive EF-hand motifs and interact with each other through the fifth EF-hand motif. EF-hand motifs also bind calcium. Domain V in the small subunit is a glycine-rich domain and is thus highly flexible. Both calpain 1 and 2 large subunits contain multiple phosphorylation sites.

**Calpains and Diseases**

Given the involvement of calpains in multiple signaling pathways that regulate cell proliferation, apoptosis, cell differentiation, and cell migration, aberrant regulation of calpains is associated with numerous human diseases such as Parkinson's disease (PD), Alzheimer's disease (AD), and diabetes. Calpains are involved in the degeneration and dysfunction of retinal neurons in acute ocular hypertensive rats, possibly by causing the loss of cone-ON bipolar and amacrine cells and activation of Muller cells (Suzuki et al., 2014). Aggregated αSynuclein is contained in Lewy bodies, a pathological hallmark of PD, and the role of calpain cleavage of αSyn was studied. The decreased number of αSyn-positive aggregates caused by reduced calpain activity, and the increased truncation of αSyn resulting from loss of calpastatin implicate calpains, especially calpain 1, in

disease-associated aggregation of αSyn and the pathogenesis of PD (Diepenbroek et al., 2014). In other PD studies, inhibiting calpain activity reduces MPTP-induced PD symptoms (Lazzara et al., 2015; Samantaray et al., 2015). In a study of AD, activated calpains are found to cleave DARPP-32 that regulates CREB phosphorylation in AD affected brains, resulting in a lower level of CREB phosphorylation (Cho et al., 2015). Moreover, in another AD study, the truncation of Dyrk1A by calpain 1 may contribute to Tau pathology by promotion of exon 10 exclusion and hyperphosphorylation of Tau, which is pivotal in pathogenesis of AD (Jin et al., 2015). *CAPN10* has been identified to be a type 2 diabetic gene through positional cloning (Horikawa et al., 2000), and is also found to be related to atherosclerosis independent of diabetes-related phenotypes (Goodarzi et al., 2005). In Duchenne muscular dystrophy, m-calpain is identified to target Tmod isoforms as proteolytic substrate, resulting in increased thin filament lengths (Gokhin et al., 2014). The crosstalk between calpain activation and TGF-β1 promotes collagen-I synthesis in primary human lung fibroblasts and in pulmonary fibrosis (Li et al., 2015). Multiple coding mutations in *CAPN5* are discovered to cause autosomal dominant neovascular inflammatory vitreoretinopathy (ADNIV), a blinding autoimmune eye disease (Bassuk et al., 2015; Wert et al, 2015).

Among the numerous diseases affecting different signaling pathways by calpains, many pathological conditions are related to their influence on cell migration. A study on wound healing indicated that calpain inhibition inhibits myofibroblast differentiation and alters fibroblast contractile properties (Nassar et al., 2012). Lissencephaly is a

neurological disorder caused by defective neuronal migration and *LIS1* is the gene mutated in patients with this disease. In a study of lissencephaly, it was found that calpain inhibition improves neuronal migration of *Lis1+/-* cerebellar granular neurons and rescues the *in vivo* disease phenotypes in a mouse lissencephaly model (Yamada et al., 2009). Many studies have also implicated calpains in the regulation of cancer cell motility. The calpain/calpastatin system has an impact on growth and metastatic dissemination of melanoma cells (Raimbourg et al., 2013). Calpain 4 significantly correlates with invasiveness of non-resectable hepatocellular carcinoma (HCC), and promotes proliferation, migration, and invasion of HCC cells through the FAK-Src signaling pathway (Bai et al., 2009; Dai et al., 2014). Furthermore, calpain 4 promotes human nasopharyngeal carcinoma metastasis via nuclear factor-κB-induced matrix metalloproteinase 2 expression (Zheng et al., 2014). Numerous other pathological studies are reviewed by Franco & Huttenlocher (2005), Goll et al. (2003), and Hua & Nair (2014).

**Calpain Substrates**

Calpains mediate proteolysis of more than 100 substrates in a limited fashion, the two exceptions are casein and myelin that are proteolyzed exhaustively by calpains (Franco and Huttenlocher, 2005; Sorimachi et al., 2012). The substrates of calpains function in numerous pathways as transcription factors, transmembrane receptors, signaling enzymes, and cytoskeletal proteins (Franco and Huttenlocher, 2005). However, a significant number of the calpain substrates are related to cell motility (Table 1.2)

(Franco and Huttenlocher, 2005; Glading et al., 2002). Many of the proteins resulting from the limited proteolysis by calpains have different functions from those of their intact forms.

Among the substrates of calpains, no consensus sequence has been identified (Goll et al., 2003). Instead, substrate recognition is more likely to be controlled by the substrates folded conformation into recognition patches, PEST score, and a particular sequence immediately surrounding the site of proteolysis favoring cleavage. Therefore, this subsite recognition by the calpains implicates large areas of the polypeptide substrate (Franco and Huttenlocher, 2005; Goll et al., 2003; Tompa et al., 2004). Furthermore, binding of calmodulin and phosphorylation of the protein substrate can sometimes change the rates of calpain digestion, or even alter the sites of calpain cleavage that may be used as a posttranslational modification strategy of the substrates (Goll et al., 2003).

**Calpains in Cell Migration**

Calpains' multiple substrates (Table 1.2) function in a wide range of signaling pathways, hence, calpain-mediated proteolysis affects many physiological processes that are not limited to apoptosis, proliferation, endocytosis, and cell migration (Goll et al., 2003). However, calpains' function in cell motility has been well studied for their impact on cell spreading, protrusions, focal adhesion dynamics, and organization of stress fibers (Franco and Huttenlocher, 2005). Pharmacological inhibition of calpains results in stabilization of adhesion complexes, reduced rate of detachment of the rear of the cell, and thus reduced integrin-mediated cell migration (Huttenlocher et al., 1997). Inhibition

**TABLE 1.2 Calpain substrates that are related to cell migration are listed in the table together with their localization within the cell**. The references for each substrate are listed.

| Calpain Substrate | Cellular Location | References |
|---|---|---|
| α -actinin | Adhesion complex | Selliah et al., 1996 |
| β integrins | Adhesion complex | Potts et al., 1994; Pfaff et al., 1999 |
| β -catenin | Adhesion complex | Rios-Doria et al., 2004 |
| Cadherins | Cell-cell adhesion | Kudo-Sakamoto et al., 2014 |
| Cortactin | Cell periphery and perinuclear region | Perrin et al., 2006 |
| EGFR | Plasma membrane | Gates and King, 1983 |
| Ezrin | Adhesion complex | Yao et al., 1993 |
| FAK | Adhesion complex | Carragher et al., 1999 |
| Filamin | Adhesion complex | Guyon et al., 2003 |
| MAP2 | Pan-cellular | Fischer et al., 1991 |
| MARCKS | Focal adhesions | Dedieu et al., 2003 |
| MLCK | Pan-cellular | Kambayashi et al., 1986 |
| Paxillin | Adhesion complex | Carragher et al., 1999 |
| PKC | Pan-cellular | Saido et al., 1991 |
| PTP-1B | Cytosolic face of the | Frangioni et al., 1993 |

| | | |
|---|---|---|
| | endoplasmic reticulum/cytosol | |
| RhoA | Pan-cellular | Kulkarni et al., 2002 |
| Spectrin | Adhesion complex | Franco et al., 2004a |
| Src | Adhesion complex | Oda et al., 1993 |
| Talin | Adhesion complex | Carragher et al., 1999; Yan et al., 2001 |
| Tau | Pan-cellular | Litersky & Johnson, 1992 |
| Vinculin | Adhesion complex/Secreted | Serrano and Devine, 2004; |

of the calpain small subunit also results in reduced cell migration (Dourdin et al., 2001). Inhibiting calpains reduces the ability to spread in multiple cell types including vascular smooth muscle cells, myoblasts, and NIH-3T3 fibroblast cells (Dedieu et al., 2004; Paulhe et al., 2001; Potter et al., 1998). *Capn4*$^{-/-}$ mouse embryonic fibroblasts (MEFs) exhibit less spreading compared to wildtype cells (Dourdin et al., 2001). However, inhibiting only calpain 1 does not affect cell spreading in several different fibroblast cell lines (Franco et al., 2004a). Conversely, inhibiting calpains in human neutrophils even leads to an increase in cell spreading (Lokuta et al., 2003). *Capn4*$^{-/-}$ MEF cells display prominent thin membrane projections compared to wildtype MEFs (Dourdin et al., 2001). Cells overexpressing the endogenous calpain inhibitor calpastatin have abnormal

filopodia and lamellipodia (Potter et al., 1998). Overexpression of calpastatin in myoblasts results in the accumulation of MARCKS (myristoylated alanine-rich C kinase substrate) and cells exhibit a major defect in new adhesion formation (Dedieu et al., 2003 & 2004). *Capn4*$^{-/-}$ MEF cells display a stabilization of prominent vinculin containing focal complexes located at the cell periphery (Dourdin et al., 2001). Calpain-mediated proteolysis of talin is critical for focal adhesion disassembly and turnover of other adhesion proteins also depend on the proteolysis of talin by calpains, including paxillin, vinculin, and zyxin (Franco et al., 2004b). Moreover, central stress fibers are absent from *Capn4*$^{-/-}$ MEF cells and the actin cytoskeleton is highly disorganized (Dourdin et al., 2001). In the myoblasts that overexpress calpastatin, a similar condition occurs as they present a disorganized actin cytoskeleton with an absence of central stress fibers (Dedieu et al., 2004).

Previous studies from our lab have focused on functions of calpains in the mechanical aspects of cell migration (Table 1.3). The functions of the catalytic large subunits and small regulatory subunit were tested with respect to traction force and mechanosensing. Many cellular conditions were used, including silencing of CAPN1, CAPN2 or CAPNS1 individually with siRNA, knockout MEFs, or cells with overexpression of calpastatin to simultaneously inhibit CAPN1 and CAPN2 protease activity. It was discovered that when compared to wildtype MEFs, *Capn4*$^{-/-}$ cells displayed reduced traction force and this was not observed when the large catalytic subunits were silenced respectively or when calpastatin was overexpressed. Our data also

demonstrated that stress fibers were fewer and less prominent by immunofluorescence in *Capn4*$^{-/-}$ cells. Fewer stress fibers colocalized with vinculin-containing adhesions, and adhesion strength was also reduced in *Capn4*$^{-/-}$ cells but not in *Capn1*- and *Capn2*-knockdown cells (Undyala et al., 2008). Interestingly, mechanosensing of localized tension was deficient in cells lacking the large subunits, or calpain 4, or when the holoenzyme activity was inhibited by calpastatin; in addition these cells failed to engage dorsal integrins (Undyala et al., 2008). An unpublished result from this study also indicated that the ability of MEFs to sense the homeostatic tension (substrate rigidity) was not affected by inhibiting the calpain large or small subunits suggesting that sensing localized tension requires different sets of elements to function than sensing homeostatic tension. These results together lead to the conclusion that the regulatory small subunit calpain 4 must modulate the production of traction forces independently of the catalytic activity of the calpain holoenzymes, but function together with the large subunits to regulate the mechanosensing to localized tension.

Further studies into the mechanism of calpain 4 mediated regulation of traction force identified a surprising protein, galectin-3, a lectin-binding protein. Galectin-3 was identified through 2D gel electrophoresis by comparing tyrosine phosphorylation profiles of *Capn4*$^{-/-}$ MEFs with wildtype MEFs and MEFs deficient in calpain large subunits. Subsequently it was found that calpain 4 was required for the secretion of galectin-3, and that failure to be secreted was due to a lack of tyrosine-phophorylation of galectin-3 (Menon et al., 2011). Galectin-3 is an atypical member of the galectin family of proteins

and can be found in the cytoplasm, nucleus, and media of many cell types (Nakahara and Raz, 2006). The addition of recombinant galectin-3 externally to the media rescued multiple defects of the *Capn4*$^{-/-}$ MEF cells including traction force, focal adhesion turnover and maturation defects, and poor adhesion strength. Meanwhile, extracellular galectin-3 did not affect mechanosensing of either the localized or homeostatic tension (Menon, 2012). Furthermore, silencing of galectin-3 in MEF cells did not alter the level of Y397 FAK phosphorylation, suggesting that galectin-3 mediated enhancement of adhesion strength and focal adhesion turnover may not be modulated through the FAK pathway.

**TABLE 1.3 The functions of calpain 1, 2, and 4 on the various mechanical aspects of cell migration are summarized in the table**.

| Assay | MEF | *capn4 -/-* MEF | Capn1 KD MEF | Capn2 KD MEF | Calpastatin overexpression |
|---|---|---|---|---|---|
| Traction Stress | + | - | + | + | + |
| Reponse to Localized Tension | + | - | - | - | - |
| Response to Homeostatic Tension | + | + | + | + | n.a. |
| Dorsal Integrin Engagement | + | - | - | - | - |
| Substrate Adhesion | + | - | + | + | n.a. |
| Adhesion-Stress Fiber | + | - | + | + | n.a. |

n.a. indicates not applicable.

In gaining understanding of the interplay of traction force and mechanosensing, this study continued with chapter 2 and chapter 3 to investigate the function of calpain 4 in cell migration by answering two questions: whether domains of calpain 4 independently regulate traction force production and mechanosensing; what are the binding proteins of calpain 4 that possibly function in the signaling pathway of traction force production. Elucidating these questions helps to expand our understanding in the mechanical aspects of cell migration.

# CHAPTER 2 TRACTION FORCE AND MECHANOSENSING CAN BE FUNCTIONALLY DISTINGUISHED THROUGH THE USE OF SPECIFIC DOMAINS OF THE CALPAIN SMALL SUBUNIT

## ABSTRACT

Cell migration is a fundamental process pertaining to many critical physiological events. The ability to form and release adhesion structures is necessary for cell migration. The calpain family of cysteine proteases are known to target adhesion proteins as their substrates and modulate adhesion dynamics. The two best studied calpains, calpain 1 and calpain 2 form catalytically active holoenzymes through heterodimerization with a common non-catalytic regulatory small subunit known as calpain 4. In previous studies, we determined that calpains are important in the production of traction forces and in the sensing of mechanical localized stimulation from the external environment. We found that perturbation of either Calpain 1 or 2 had no effect on the generation of traction forces. However, traction forces were defective when calpain 4 was silenced. On the other hand, silencing of calpain 1, 2, or 4 resulted in deficient sensing of external mechanical stimuli. These results together suggest that calpain 4 functions independently of the catalytic large subunits in the generation of traction forces but functions together with either catalytic subunit in sensing external mechanical stimuli. The small subunit calpain 4 contains 268 a.a. and is composed of 2 domains, the N-terminal domain V and C-terminal domain VI. Domain VI is a calmodulin-like domain containing five consecutive EF-hand motifs, of which the fifth one heterodimerizes with a large subunit. Moreover, domain V contains the common sequence GTAMRILGGVI that suggests cell membrane interactions. Given

these attributes of domain V and VI of calpain 4, we speculated that an individual domain might provide the functional properties for either traction or sensing. Therefore, each domain was cloned and expressed individually in *Capn4*$^{-/-}$ cells and assayed for traction and sensing. Results revealed that overexpression of domain V was sufficient to rescue the traction force defect in *Capn4*$^{-/-}$ cells while overexpression of domain VI did not rescue the traction force. Consistent with our hypothesis, overexpression of domain VI rescued the sensing defect in *Capn4*$^{-/-}$ cells while overexpression of domain V had no effect. These results suggest that individual domains of calpain 4 do indeed function independently to regulate either traction force or the sensing of external stimuli. We speculate that membrane association of calpain 4 is required for the regulation of traction force and its association with a catalytic subunit is necessary for mechanosensing.

## Introduction

Cell migration has been implicated in many critical biological processes, including embryonic development, wound healing, immunological responses, and cancer metastasis. A coordinated series of events are required for cell migration, including: protrusion at the cell front, adhesion of the protruded area to the substrate, pulling of the cell body, and retraction at the cell rear (Friedl and Alexander, 2011; Ridley, 2003). Migration integrates both biochemical and mechanical signals for regulation of the process. At the nexus of this regulation are cell-matrix adhesions that are used to transmit the traction forces exerted onto the substrate by cells and to sense the mechanical signals from the external

environment (Flevaris, et al., 2007; Hynes, 2002; Lauffenburger and Horwitz, 1996; Ridley, 2003).

Focal adhesions, a mature form of cell-matrix adhesions, are complex dynamic assemblies of adaptor proteins and integrin transmembrane receptors that couple the extracellular matrix (ECM) to the actin cytoskeleton. Members of the calpain family of calcium dependent cysteine proteases have long been implicated in the turnover of focal adhesion component proteins (Bhatt et al., 2002; Dourdin et al., 2001; Franco et al., 2004b; Goll et al., 2003). The two isoforms μ-calpain and m-calpain are the most well characterized members of this family. These two holoenzymes have a common 28 kDa small subunit, known as calpain 4 (CAPNS1 OR CAPN4, encoded by *CAPN4* gene), which heterodimerizes with distinct 80 kDa large subunits known as calpain 1 and calpain 2 (CAPN1 AND CAPN2, encoded by *CAPN1 and CAPN2* genes respectively). Structurally, the protease domains are only located within the large subunits but are absent in the small subunit. There are two terminal domains that make up the small subunit, also known as the regulatory subunit: the $NH_2$-terminal domain V, and the COOH-terminal domain VI (Goll et al., 2003). Domain V is Gly rich and contains a potential phospholipid binding region GTAMRILGGVI (Crawford, 1990; Daman, 2001; Imajoh et al., 1986). Domain VI contains five EF-hand motifs with the fifth EF hand interacting with the corresponding fifth EF hand from domain IV of the large subunit for assembly of the holoenzyme (Franco and Huttenlocher, 2005).

The role of calpains in cell migration has been widely investigated. Inhibition of calpains resulted in reduced cell migration, delayed retraction of the cell's rear, inhibition of focal adhesion disassembly and translocation, stabilization of adhesion complexes, impaired cell spreading, and modulation of cancer cell invasion (Bhatt, 2002; Dedieu et al., 2004; Huttenlocher, 1997; Mamoune, 2003; Potter, 1998). However, in other cases, inhibiting only calpain 1 did not affect cell spreading in several different fibroblast cell lines (Franco et al., 2004a). Sometimes, inhibiting calpains even led to an increase in cell spreading instead in human neutrophils (Lokuta et al., 2003). Silencing *CAPN2* in NIH 3T3 cells resulted in decreased talin proteolysis and involved calpain 2 in the modulation of dynamics of talin-containing adhesion (Franco et al., 2004b).

Although much attention has been given to studying the functions of calpain holoenzymes in cell migration, the calpain small subunit has been largely uninvestigated as it was presumed to be associated with a regulatory role specific to the activities of the holoenzymes (Goll et al., 2003). *Capn4*$^{-/-}$ embryonic fibroblasts display a reduced rate of cell migration, abnormal organization of focal adhesions with a loss of centralized focal adhesions, and delayed retraction of membrane projections, suggestive of a deficiency in focal adhesion maturation and turnover (Dourdin, 2001). Our lab explored the function of calpains in the generation of traction forces and mechanosensing, and discovered that the production of traction forces were inhibited by the disruption of *CAPN4* expression, but not by the inhibition of the large subunits or the overexpression of calpastatin. On the other hand, inhibiting either large subunit or interrupting the small subunit led to defects

in the mechansensing to the localized force and substrate topography. Meanwhile, *Capn4*$^{-/-}$ cells have abnormal stress fibers and a reduced number of stress-fiber-associated, vinculin-containing adhesions (Undyala, 2008). These results implicate the calpain small subunit alone in the regulation of traction forces but both large and small subunits in mechanosensing.

Here we have performed a domain function study of the CAPN4 subunit. We speculated that specific domains of the CAPN4 subunit could function in either mechanosensing or the production of traction. To this end, we overexpressed either domain V or domain VI in *Capn4*$^{-/-}$ cells. We discovered that not only did the overexpression of domain V rescue the deficient traction force and abnormal focal adhesion organization observed in *Capn4*$^{-/-}$ cells, but it also promoted cell migration. On the other hand, overexpression of domain VI restored both the ability to sense the localized mechanical force (mechanosensing) and the protease activity that is lost in *Capn4*$^{-/-}$ cells. These results suggest that the calpain small subunit has a protease-independent activity that functions in promoting the production of traction force through domain V, while domain VI is involved in a mechanosensing function that requires protease activity.

# EXPERIMENTAL PROCEDURES

## Cell Culture

MEFs expressing a defective small calpain subunit have been described previously (Arthur et al., 2000; Dourdin et al., 2001), and are referred to as *Capn4*$^{-/-}$ cells in this study. MEFs and *Capn4*$^{-/-}$ cells were used in this study. MEFs were purchased from ATCC. Cells were maintained in Dulbecco's Modified Eagle's Medium-high glucose (Sigma) supplemented with 10% fetal bovine serum (FBS) (Hyclone) and 1% penicillin/streptomycin/glutamine (Gibco) and incubated at 37 ˚C under 5% $CO_2$ in a humidified cell culture incubator. Cells were passed by trypsinization using 0.1% trypsin-EDTA (0.25% trypsin-EDTA diluted with HBSS, Gibco). Trypsinization was terminated by adding complete media. The passage number of either cell type never exceeded eight passages.

## Cloning of Domain V and VI of CAPN4 and DNA Constructs

The pAcGFP1-N1 (Clontech) was transformed into E. coli and collected by minipreping with an E.Z.N.A Plasmid Mini Kit (Omega). Sequences of domain V, VI or full length *Capn4* were amplified by PCR from a pEGFP-CAPN4 plasmid under the following conditions: 30 cycles of 98˚C for 10 sec followed by 68˚C for 1 min using PrimeSTAR HS DNA Polymerase with GC buffer (Takara, R044A). The primers used were as follows: full length *CAPN4* was amplified with the forward primer 5'-ACCGCTCGAGATGTTCTTGGTGAATTCGTT-3' and the reverse primer

5'-ATCGGGATCCGCGGAATACATAGTCAGCTGCA-3'; domain V was amplified with the forward primer 5'-ACCGCTCGAGATGTTCTTGGTGAATTCGTT-3' and the reverse primer 5'-TACGGGATCCGCGAACTGACGGACTTCTTCA-3'; and domain VI was amplified with the forward primer 5'-ACCGCTCGAGATGAGGAAACTTTTTGTCCAG-3' and the reverse primer 5'-ATCGGGATCCGCGGAATACATAGTCAGCTGCA-3'. PCR products were resolved on 1% agarose gels and visualized by ethidium bromide (1% solution, Fisher) staining. The resolved bands were then purified using a Qiaquick gel extraction kit (Qiagen, 28706). Purified PCR products and pAcGFP1-N1 were incubated with XhoI and BamHI (New England Biolabs) at 37˚C for 4 hrs in 1X buffer 3 supplemented with 1% BSA. The double digested PCR products and plasmid were again purified with the Qiaquick gel extraction kit. To insert either domain V or VI into pAcGFP1-N1, ligation of double digested fragment of either domain with double digested pAcGFP1-N1 was performed with the LigaFAst Rapid DNA Ligation System (Promega, M8226) following the manufacturer's suggested protocol. These constructs were transformed into E. coli to collect plasmids, and successful insertions were confirmed by sequencing (Applied Genomics Technology Center, Wayne State University).

**Nucleofection of *Capn4*$^{-/-}$ Cells and Overexpression of Domains**

Nucleofection was performed using the Amaxa MEF2 Nucleofector Kit (Lonza) following the manufacturer's suggested protocol. Briefly, *Capn4*$^{-/-}$ cells were trypsinized with 0.1% Trypsin-EDTA and collected by centrifuging at 2000 rpm for 5 min. Collected

cells were then resuspended in an appropriate volume of the mixture of the included MEF 2 nucleofector solution and supplement 1 followed by adding up to 5 μg of the prepared plasmid. The total volume of the MEF 2 Nucleofector solution and supplement 1 mixture and the plasmid added up to 100 μl, which was mixed well and transferred to an electroporation cuvette. The cuvette was then inserted into the Nucleofector II system (Amaxa) and the program MEF A-023 was run. 500μl of RPMI-1640 medium (Sigma) was immediately added into the nucleofection cuvette before it was removed after the program was run to minimize cell damage. Nucleofected cells were then seeded according to the requirement of the following procedures.

**Protein Extraction and Western Blotting**

Proteins were extracted from each cell line with triple detergent lysis buffer (TDLB): pH 8, 50 mM Tris HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, into which Protease Inhibitor Cocktail (Sigma) and HaltTM Phosphatase Inhibitor Cocktail (Thermo) were added. An 80% confluent 100-mm culture dish (Nunc$^{TM}$) was placed on ice and washed twice with ice-cold phosphate-buffered saline (PBS) followed by 25 min of incubation with 300 μl TDLB on ice. Lysed cells were collected by an ice-cold cell lifter and centrifuged at 13,000 rpm for 10 min to get rid of cell debris. Protein concentration was measured by Lowry method with the Bio-Rad DC protein assay kit. 20 μg of proteins from each cell line were loaded into a 4-20% gradient Tris–HEPES–SDS precast polyacrylamide gel system (Pierce) and resolved at 100 V for 1 hour. Proteins were then transferred onto an Immuno-Blot PVDF Membrane (Bio-Rad)

using a Trans-blot SD Semi-dry Transfer Cell (Bio-Rad) at 20 V for 30 min. Following transfer, the membrane was blocked for 1 hour using 5% milk in Tris Buffered Saline – 0.1% Tween (0.1% TBS/T) and then probed with the primary antibody. Primary antibody for GFP (sc-8334, Santa Cruz) was diluted at 1:500 in 5% milk in 0.1% TBS/T and incubated at 4˚C overnight with mild agitation. After washing 20 min for 3 times with 0.1% TBS/T, the secondary antibody HRP-linked Rabbit IgG (NA934, Amersham) was diluted at 1:10,000 in 5% milk in 0.1% TBS/T and incubated for 1 hour at room temperature. After washing 20 min for 3x, the membrane was detected using ECL Plus Western Blotting Detection Reagents (Amersham).

**Preparation of Polyacrylamide Substrates**

A series of polyacrylamide substrates of different stiffnesses were prepared as described previously (Beningo et al., 2002). Briefly, a flexible 75 μm x 22 mm polyacrylamide substrate was made in a cell culture chamberdish in which 0.2 μm fluorescent microbeads were embedded. The acrylamide (acryl, Bio-rad) concentration was fixed at 5% while N,N-methylene-bis-acrylamide (bis, Bio-rad) varied from 0.04% to 0.1% to attain different stiffnesses of the substrates. Traction force microscopy (TFM) was performed with the 5%/0.08% Acry/Bis substrates and the mechanosensing assay to applied forces was performed with 5%/0.1% Acryl/Bis substrates. The substrates were then coated with 5 $\mu g/cm^2$ fibronectin (Sigma) at 4˚C overnight. Cells were seeded onto the substrates overnight prior to TFM or mechanosensing.

**Traction Force Microscopy (TFM)**

Cells were seeded onto flexible polyacrylamide substrates of 5%/0.08% Acryl/Bis coated with fibronectin, which was prepared as described above. After the chamber dishes were kept in the incubator under regular cell culture conditions overnight, images for cells were collected as described previously (Beningo et al., 2002). Briefly, three images were taken for a single cell under 40X objective lens: a bright field image of the cell, an image for the fluorescent beads with the cell on the substrate, and another image for the fluorescent beads after the cell was removed by a pointed microneedle. Bead displacement with or without the cell and the cell and nuclear boundaries calculated by DIM software (Yu-li Wang) were used to generate and render traction stress values by using a custom made algorithm provided to our lab by Dr. Micah Dembo (Boston University) as described previously (Dembo and Wang, 1999; Marganski et al., 2003). Images of 12-18 cells for each cell line were collected.

**Mechanosensing Assay to Applied Mechanical Stimulation**

Flexible polyacrylamide substrates of 5/0.1% Acryl/Bis coated with fibronectin were prepared as described above. Cells were seeded onto the substrates and allowed to adhere overnight under regular cell culture conditions. As described previously (Lo et al., 2000), a cell was monitored for 10 min for its migration trajectory before a blunted microneedle was pressed onto the substrate in front of the direction the cell was migrating to generate a pushing force onto the cell. The pushing force will release the tension on the substrate. Images were taken every 3 min for 1 hour. If a cell responds to the pushing force by

avoiding it, a "1" is recorded; if a cell continues to migrate on the same trajectory, a "0" is recorded. For each cell line, 12-18 cells were observed.

**Immunofluorescence**

After being flamed, no. 1.5 glass coverslips (Fisher) were coated with 5 μg/cm$^2$ fibronectin (Sigma) at 4˚C overnight and blocked with 1% BSA in PBS at 4˚C overnight. Cells were seeded onto the coated glass coverlips and allowed to attach overnight under regular cell culture conditions. The cells were then fixed and permeabilized with the following steps: first incubate for 10 min with 2.5% paraformaldehyde in 1X PBS at 37˚C; then incubate with 2.5% paraformaldehyde in 1X PBS with 0.1% Triton X-100 at 37˚C; followed by incubation of 5 min with 0.5 mg/ml $NaBH_4$ solution. After fixation and permeabilization, cells were blocked with 5% BSA in PBS for 1 hour at room temperature, and then incubated with anti-vinculin antibody (Sigma, V4505) at a 1:200 dilution for 3 hours at room temperature. Following 3 washes of 15 min, Alexa Fluor® 546 anti-mouse secondary antibody was added at a 1:500 dilution in 5% BSA for an incubation of 1 hour at room temperature. After the final washes (3 x 15 min each), mounting media (pH 7.8, 0.1% PPD, 1X PBS, 50% glycerol, 30% Q-$H_2O$) was added. Images were taken with appropriate filters for both GFP and RFP signals. The number and size of vinculin containing plaques were measured using the NIH Image J (NIH).

**Calpain Activity Assay**

Calpain activity was quantified using a calpain activity fluorometric assay kit (Biovision) following the manufacturer's instructions, except using a modified lysis

buffer. Briefly, cells were lysed with TDLB as described above, into which Protease Inhibitor Cocktail (Sigma) and HaltTM Phosphatase Inhibitor Cocktail (Thermo) were added. The protein concentration was calculated by Lowry method with the Bio-Rad DC protein assay kit. 50 μg of cell extracts was mixed and incubated with the reaction buffer and calpain substrate Ac-LLY-AFC provided by the kit for 1 hour at 37˚C in the dark. The samples were then transferred to a 96-well plate, and the reactions were measured at 400/505 nm with a Spectramax Gemini Fluorescence Luminescence Microplate Reader (Molecular Devices, Sunnyvale, CA).

**Cell Migration Assay**

Glass coverslips were coated with 5μg/cm$^2$ fibronectin (Sigma) at 4˚C overnight, then cells were seeded and allowed to attach overnight under regular cell culture conditions. The migration trajectory of a single cell was observed for 2 hours at 2 min intervals with a 40X objective lens. All the images were analyzed with the custom built dynamic image analysis system software (DIM, Y-L. Wang) to calculate the linear speed and persistence of 10-15 cells of each cell line.

**Microscopy**

Images for all experiments described above were acquired with an Olympus IX81 ZDC inverted microscope fitted with a custom-built stage incubator to maintain cells at 37˚C under 5% $CO_2$ for live cell imaging and a SPOT Boost EM-CCD-BT2000 back-thinned camera (Diagnostic Instruments Inc., Sterling Heights, MI). The camera was driven by the IPLab software (BD Biosciences).

# RESULTS

## Plasmid Construction and Overexpression of CAPN4 Domains in *Capn4*$^{-/-}$ Cells

Calpain 4 regulates the generation of traction forces in MEF cells in addition to the canonical regulatory function for the holoenzyme (Undyala et al., 2008). Our previous study showed that the generation of traction forces was attenuated by the disruption of *CAPN4* expression but not by the knock-down of *CAPN1* or *CAPN2* or even the overexpression of calpastatin, the endogenous calpain inhibitor. However, the ability of the cells to sense locally applied tension sensing required the function of both large and small subunits of the holoenzyme (Undyala et al., 2008). To further evaluate the functions of domain V (DV) and VI (DVI) on the generation of traction forces and mechanosensing, each domain or the full-length *CAPN4* were cloned and overexpressed in *Capn4*$^{-/-}$ cells (Figure 2.1 *A*). The overexpression of each plasmid was confirmed by immunoblots. Successful overexpression of the *CAPN4* domains in *Capn4*$^{-/-}$ cells makes it possible to test the impact of either *CAPN4* domain on the cell's ability to generate traction forces and sense the external stimulus.

## Overexpression of DV Rescues the Defect of Traction Force Generation in *Capn4*$^{-/-}$ Cells

Previous studies of *Capn4*$^{-/-}$ revealed a defect in traction forces, however the inhibition of *CAPN1* or *CAPN2* or the overexpression of calpastatin did not affect the production of traction forces (Undyala et al., 2008). To understand the function of each domain of the calpain small subunit on traction force in migrating fibroblasts, *Capn4*$^{-/-}$

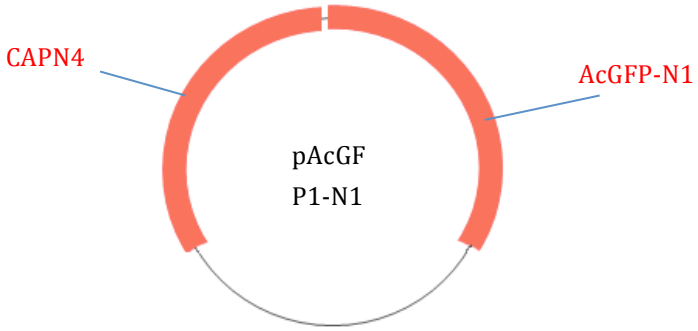



**FIGURE 2.1: Over-expression of domain V or VI of *CAPN4* in *Capn4*$^{-/-}$ cells.** A schematic diagram illustrating the insertion of either DV or DVI of *CAPN4*, or full-length *CAPN4* into the plasmid pAcGFP1-N1 (Clontech).

cells expressing either the DV or DVI plasmid were plated on flexible polyacrylamide substrates covalently coated with fibronectin and traction was measured by followed by traction force microscopy (TFM) (Dembo & Wang, 1999). Traction forces were calculated based on the magnitude of bead displacement within the substrate with or without the attached cell, and then vector maps were generated (Figure 2.2 *A*). The magnitude of the traction forces produced in *Capn4*$^{-/-}$ cells expressing either DV, DVI, or full-length *CAPN4* gene were compared with wildtype MEFs and *Capn4*$^{-/-}$ cells.

Compared to wildtype MEF cells (avg. 2.69kPa), *Capn4*$^{-/-}$ cells produced significantly less traction force (avg. 1.99 kPa, $p$=0.03) (Fig. 2.2, *B*), which is consistent with the previous study (Undyala et al., 2008). Moreover, the expression of the full-length *CAPN4* restored the traction forces in *Capn4*$^{-/-}$ cells to wildtype levels (avg. 2.72 kPa, $p$=0.03), and the empty plasmid had no effect (avg. 1.89 kPa). Surprisingly, expression of DV in *Capn4*$^{-/-}$ cells rescued the traction force production to a similar magnitude as in MEF cells (avg. 2.80 kPa, $p$=0.02), while *Capn4*$^{-/-}$cells expressing DVI

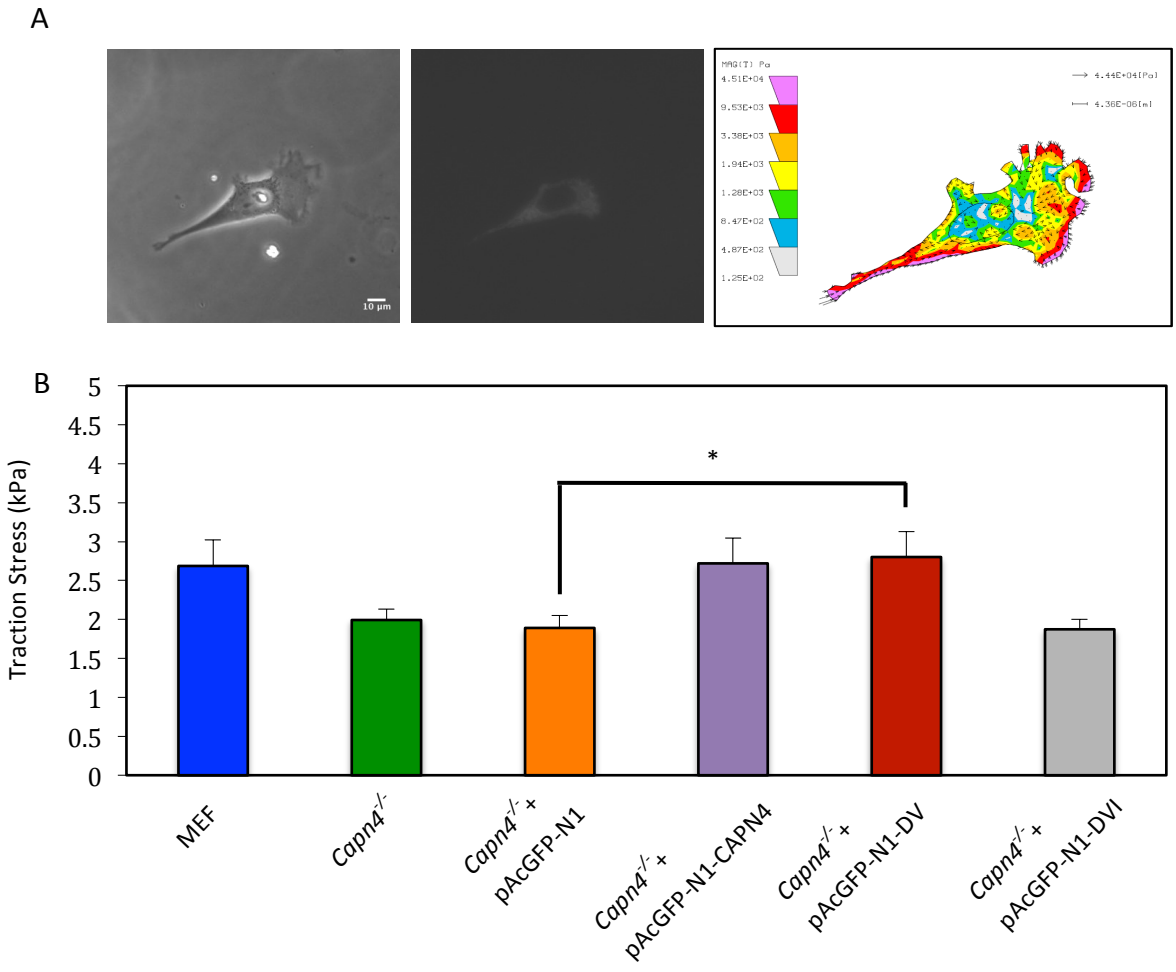



**FIGURE 2.2: Overexpression of DV rescues the defect of traction force in *Capn4*$^{-/-}$ cells.** *A*, Cells were seeded onto flexible polyacrylamide substrates coated with fibronectin and allowed to attach overnight. Images of the embedded fluorescent microbeads with or without the cell applying traction forces onto the substrate were taken for a single cell. These bead displacements with or without the cell attached to the substrate and the cell and nuclear boundary information were used to generate traction stress values by using a custom made algorithm. The vector plot on the right indicates the magnitude and directon of traction stress exerted by a single cell. In these vector maps, arrowheads indicate direction and magnitude of forces. Red and pink highlight areas of strongest force and blue and gray indicate regions of weaker force as indicated on the color bar (Mag. bar = 10µm). B, The bar graph indicates the average traction stress exerted by these cell lines: MEFs, *Capn4*$^{-/-}$ cells, *Capn4*$^{-/-}$ cells expressing the empty plasmid pAcGFP1-N1, *Capn4*$^{-/-}$ cells expressing full-length *CAPN4* gene, *Capn4*$^{-/-}$ cells expressing DV, and *Capn4*$^{-/-}$ cells expressing DVI. Statistical analysis was performed by student's t-test. * indicates $p<0.05$.

only produced traction forces at a similar magnitude to *Capn4*$^{-/-}$ cells (avg. 1.87 kPa). These results suggest that expression of DV in *Capn4*$^{-/-}$ cells is sufficient to rescue the traction force production defect, and that generation of these forces is mainly mediated through DV of calpain 4 but not DVI.

**The Deficient Mechanosensing in *Capn4*$^{-/-}$ Cells is Rescued by Overexpressing DVI**

Cells sense the mechanical signals from the extracellular environment including the substrate stiffness, topography, and localized mechanical stimuli. These signals are coupled to mechanosensitive changes in the cytoskeletal networks, interaction with the extracellular matrix (ECM), and production of cellular force (Engler et al., 2006; Guilak et al., 2009; Liedtke & Kim, 2005; Menon and Beningo, 2011). In previous research, our lab tested various calpain deficient cells for their ability to respond to localized mechanical stimuli in an assay where cells were seeded onto polyacrylamide substrates and a blunted microneedle was used to push on the substrate against the direction the cell was migrating. A wildtype MEF cell responds to the localized pushing force by avoiding it. However, *Capn4*$^{-/-}$ cells were deficient in sensing the applied force. *CAPN1*, *CAPN2*, or *CAPN4* deficient cells were found to be unresponsive to the localized pushing force (Undyala et al., 2008).

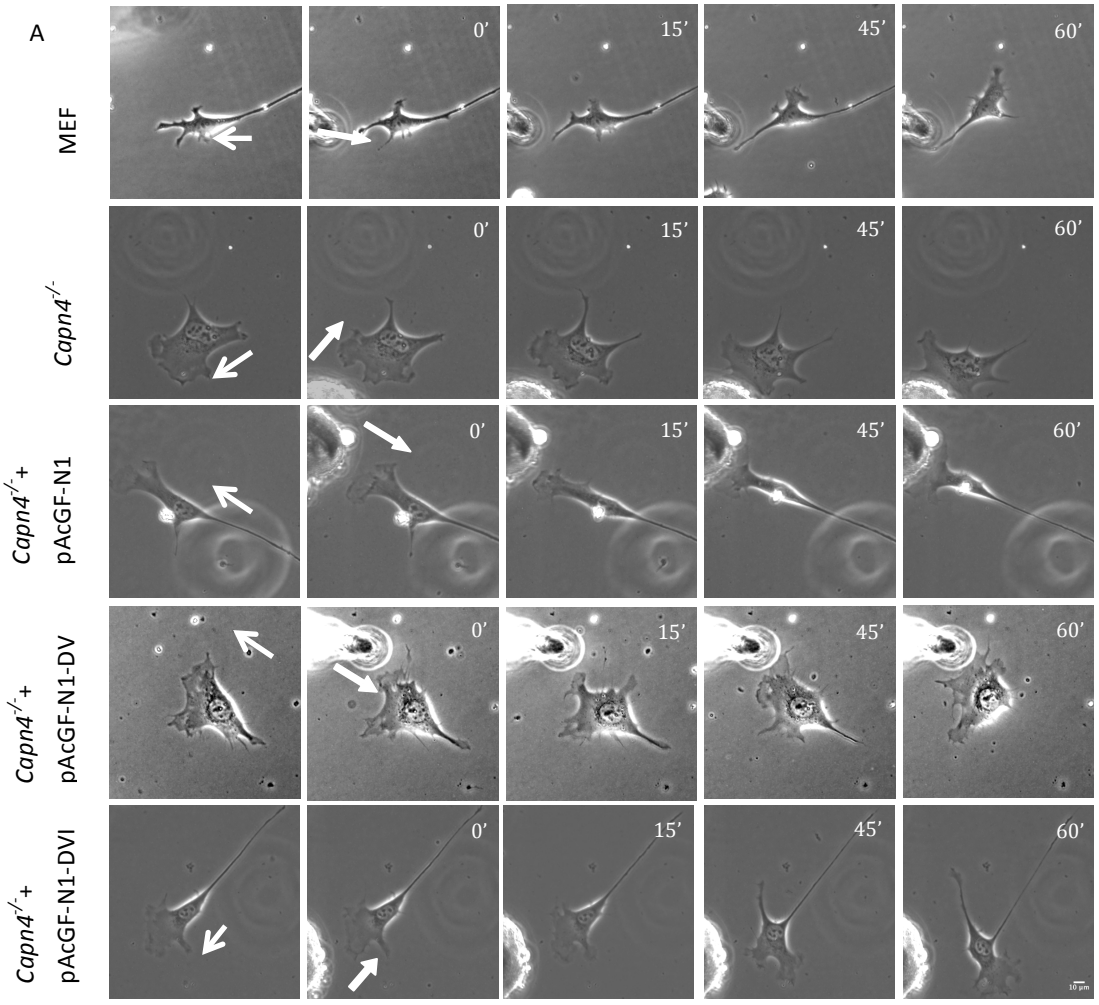
Many studies have suggested that there is a feedback loop that directly couples the mechanical sensing process with traction force (Azatov et al., 2016; Gupta et al., 2016; Mui et al., 2016). Given the fact that DV of calpain 4 rescues the defect of traction force production in *Capn4*$^{-/-}$ cells, we anticipated that this domain will also participate in the

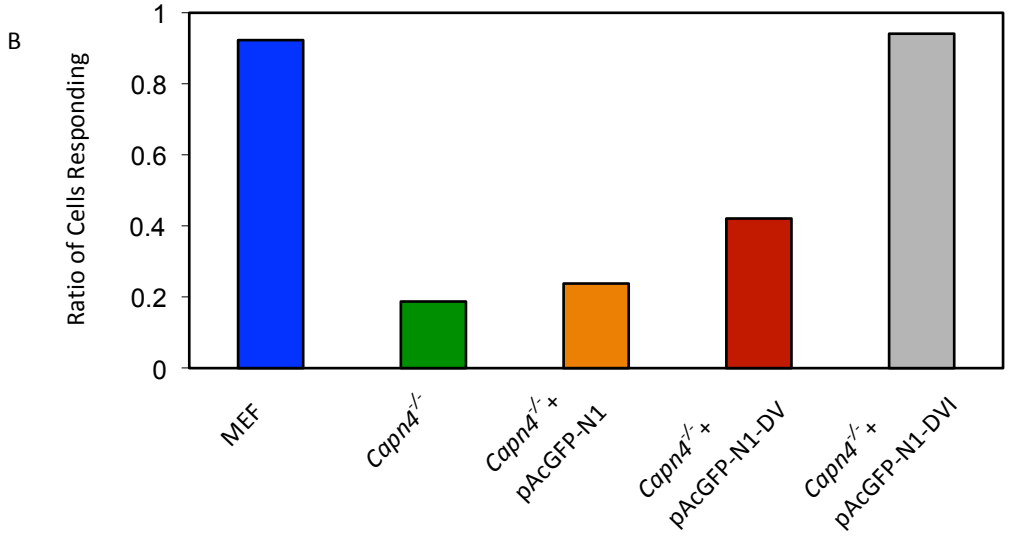
process of mechanosensing. We tested *Capn4*$^{-/-}$ cells expressing DV or DVI, wildtype MEFs and *Capn4*$^{-/-}$ cells for response to the application of a localized stimulus. Data were recorded as either "1" for responding or "0" for non-responding. Contrary to expectations, we discovered that expression of DVI restored the mechanosensing defect in *Capn4*$^{-/-}$ cells to the level of MEF cells, while expression of DV was unable to restore the defect (Figure 2.3 *A, B*). Unlike *Capn4*$^{-/-}$ cells expressing DVI, *Capn4*$^{-/-}$ cells and control *Capn4*$^{-/-}$ cells expressing an empty GFP plasmid were unable to sense the localized pushing force (Figure 2.3 *A, B*). These results suggest that instead of DV, the function of sensing the localized stimulus is mediated through DVI of the calpain small subunit.

**Overexpression of DV Promotes the Maturation of Focal Adhesions**

Traction forces are exerted onto the substrate through focal adhesions, which connect the actin cytoskeleton to the extracellular matrix (ECM) (Chrzanowska-Wodnicka and Burridge, 1996; Hotulainen and Lappalainen, 2006; Schoenwaelder and Burridge, 1999). *Capn4*$^{-/-}$ cells were previously found to have distinct morphology, including a loss of central focal adhesions, stabilization of focal complexes at the cell periphery, and fewer and less prominent actin stress fibers compared to wildtype MEFs. The same phenomena were not observed in *CAPN1*- and *CAPN2*- knockdown cells (Dourdin et al., 2001; Undyala et al., 2008).

Focal adhesions are dynamic structures. Nascent focal adhesions originate in lamellipodium. While the sizes of many focal adhesions continue to increase as they



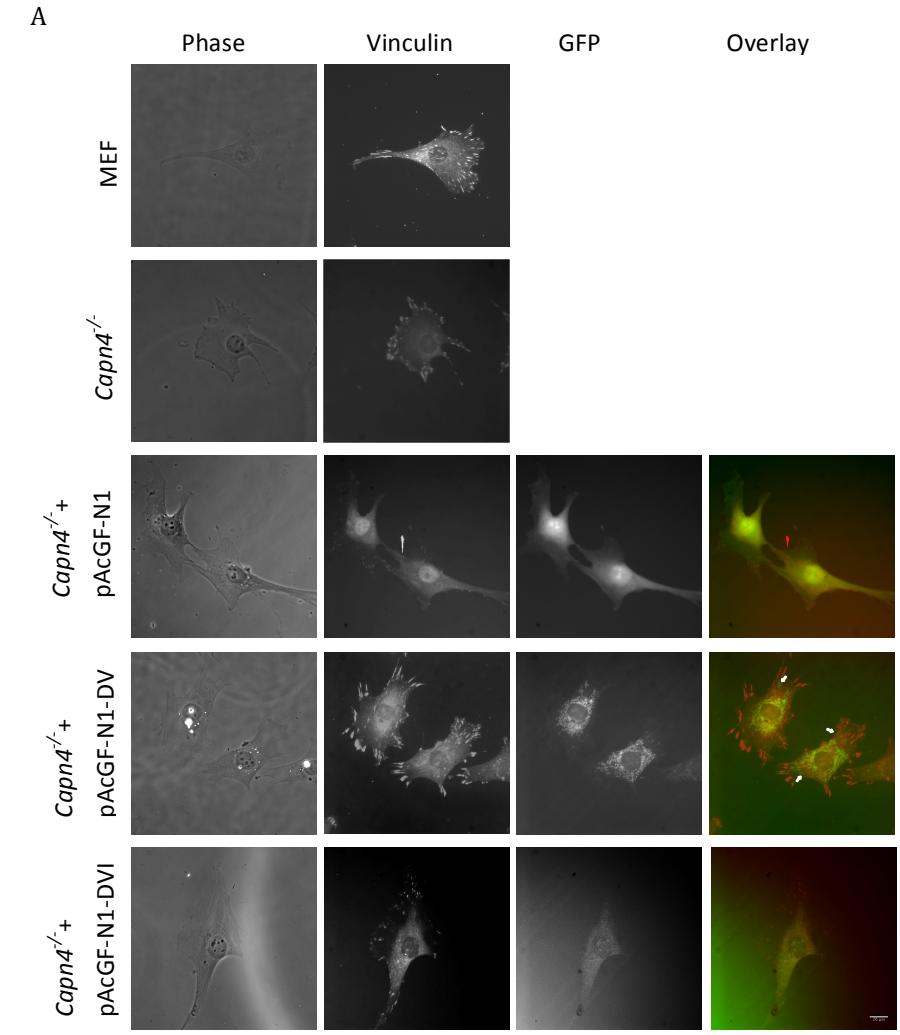




| Cell Type | MEF | *Capn4*$^{-/-}$ | *Capn4*$^{-/-}$ +pAcGFP-N1 | *Capn4*$^{-/-}$+pAcGFP-N1-DV | *Capn4*$^{-/-}$+pAcGFP-N1-DVI |
|---|---|---|---|---|---|
| Response | + | - | - | - | + |
| # of cells | 12 of 13 | 3 of 16 | 5 of 21 | 8 of 19 | 16 of 17 |

**FIGURE 2.3: Overexpression of DVI in *Capn4*$^{-/-}$ cells restores the ability to sense the localized stimulus.** *A*, Representative time-lapse images show the responses of cells to the applied localized stimulus. The included cells lines are: a MEF cell (top row), *Capn4*$^{-/-}$ cells (the second row); *Capn4*$^{-/-}$ cells expressing pAcGFP-N1 (the third row); *Capn4*$^{-/-}$ cells expressing DV (the fourth row), and *Capn4*$^{-/-}$ cells expressing DVI (the bottom row). The thin arrow denotes the original migration direction of the cell, and the thick arrow denotes the direction of the pushing force by the blunted needle (Mag. bar = 10μm). *B*. The bar graph indicates the percentage of cells responding to the localized stimulus by a blunted needle. The observed cell lines are: MEFs, *Capn4*$^{-/-}$cells, *Capn4*$^{-/-}$ cells expressing pAcGFP1-N1, *Capn4*$^{-/-}$ cells expressing full-length *CAPN4*, *Capn4*$^{-/-}$ cells expressing DV, and *Capn4*$^{-/-}$ cells expressing DVI. *C*. The table summarizes the responses of cells for each cell line. "+" represents a positive reaction and "-" represents a negative reaction. The numbers of the representative cells for each cell line are also listed in this table. As expected, *Capn4*$^{-/-}$ cells displayed deficient mechanosensing compared to MEFs. In comparison to *Capn4*$^{-/-}$ cells expressing DV that are deficient in mechanosensing, *Capn4*$^{-/-}$ cells expressing DVI sensed the stimulus from the external environment as well as MEFs.

mature into the center of a cell and become larger plaques, others may simply disassemble (Alexandrova et al., 2008; Beningo et al., 2001; Mathew et al., 2011; Papusheva and Heisenberg, 2010). The lack of centralized focal adhesions suggests a perturbation in the focal adhesion maturation process in *Capn4*$^{-/-}$ cells.

To determine whether expressing DV in *Capn4*$^{-/-}$ cells changes the focal adhesion organization, *Capn4*$^{-/-}$ cells expressing either DV or DVI, MEFs, and *Capn4*$^{-/-}$ cells were seeded onto fibronectin-coated glass coverslips, fixed with paraformaldehyde and probed with anti-vinculin antibody. As expected, MEFs displayed normal focal adhesion organization localized to both the cell center and periphery in contrast to *Capn4*$^{-/-}$ cells where a loss of centralized focal adhesions and prominent focal adhesions located at the cell periphery were observed. Furthermore, expression of DV in *Capn4*$^{-/-}$ cells rescued the abnormal organization of focal adhesions with many found in the center of cells, but


A
Phase
Vinculin
GFP
Overlay
MEF
Capn4-/-
Capn4-/- + pAcGF-N1
Capn4-/- + pAcGF-N1-DV
Capn4-/- + pAcGF-N1-DVI

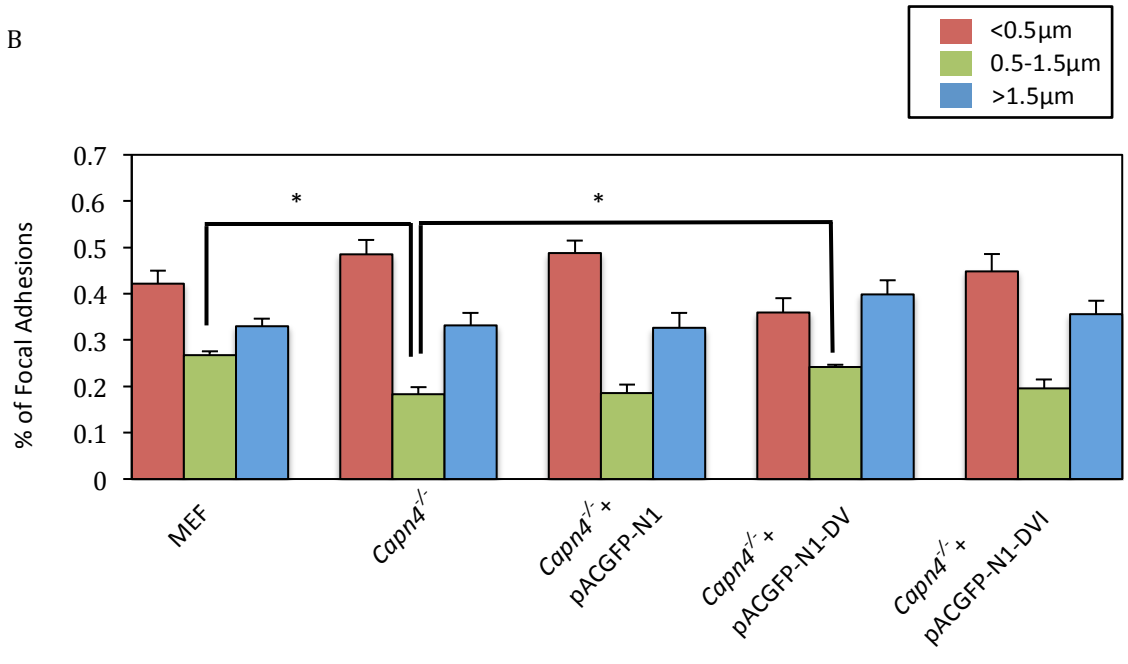

B
<0.5µm
0.5-1.5µm
>1.5µm
% of Focal Adhesions
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
*
MEF
Capn4-/-
Capn4-/- + pACGFP-N1
Capn4-/- + pACGFP-N1-DV
Capn4-/- + pACGFP-N1-DVI

**FIGURE 2.4: Overexpression of DV rescues the abnormal focal adhesion organization in *Capn4*$^{-/-}$ cells and promotes maturation of focal adhesions**. *A*. Representative images show the immunofluorescence of focal adhesions with anti-vinculin antibody. Focal adhesions fail to mature into the cell body in *Capn4*$^{-/-}$ cells expressing DVI or an empty AcGFP-N1 plasmid compared to MEFs, while maturation of focal adhesions is rescued in *Capn4*$^{-/-}$ cells expressing DV (Mag. bar = 20μm). *B*. A Bar graph illustrates the percentage of average number of adhesions in terms of varying sizes in each of the cell lines. The numbers of focal adhesions were collected from 6 cells for each cell line. The number of nascent adhesions (0.5-1.5 sq.μm) is significantly reduced in *Capn4*$^{-/-}$ cells compared to wildtype MEFs, and overexpressing domain V increased this category significantly. Meanwhile, the overexpression of domain VI in *Capn4*$^{-/-}$ cells didn't change this category significantly. The number of focal adhesions smaller than 0.5 sq.μm decreased significantly than *Capn4*$^{-/-}$ cells when domain V was expressed. When measuring focal adhesions larger than 1.5 sq.μm, no significant difference was observed between any two cell-lines. Statistical analysis was performed by student's t-test (* denotes $p<0.05$).

this was not observed in *Capn4*$^{-/-}$ cells expressing DVI (Figure 2.4 *A*). Also, quantification of the size and number of focal adhesions in each cell line displayed a significant decrease ($p=0.0007$) in the number of adhesions with sizes ranging from 0.5 to 1.5 sq.μm (nascent adhesions) in *Capn4*$^{-/-}$ cells. Yet, expressing DV in *Capn4*$^{-/-}$ cells increased the number of nascent adhesions significantly ($p=0.005$), although the numbers were not completely restored to the level of MEF cells (Figure 2.4 *B*). However, *Capn4*$^{-/-}$ cells expressing DVI showed no significant increase in the number of nascent adhesions compared to *Capn4*$^{-/-}$ cells. For focal adhesions with a size smaller than 0.5 sq.μm, the only significant difference was between *Capn4*$^{-/-}$ cells and *Capn4*$^{-/-}$ cells expressing DV (Figure 2.4 *B*). When measuring focal adhesions larger than 1.5 sq.μm, no significant difference is observed between any two cell-lines, although *Capn4*$^{-/-}$ cells expressing DV do have elevated quantities of focal adhesions larger than 1.5 sq.μm. Altogether, these

results suggest that in addition to restoring the production of traction forces in *Capn4*$^{-/-}$ cells, expression of DV, but not DVI, rescues the abnormal focal adhesion organization defects observed in *Capn4*$^{-/-}$ cells, and contributes to aid in their maturation.

**Overexpression of DV in *Capn4*$^{-/-}$ Cells Promotes Cell Migration**

The speed and persistence of cell migration is affected by both biochemical and biophysical factors including dimension, stiffness, cell-cell and cell-matrix adhesion, traction forces, cytoskeletal polarity, and the capacity to degrade ECM by proteolytic enzymes, to name just a few. (Friedl and Wolf, 2010; Plotnikov et al., 2012; Wolf et al., 2013). It was previously observed that *Capn4*$^{-/-}$ cells have reduced migration rates (Dourdin et al., 2001; Undyala et al., 2008). To determine whether migration persistence and speed are affected by expressing either domain, *Capn4*$^{-/-}$ cells expressing either domain, *Capn4*$^{-/-}$ cells, and MEFs were seeded onto fibronectin-coated glass coverslips and imaged for 2 hours. Cell migration rates and persistence were calculated based on the locomotion of the nuclei. As expected, *Capn4*$^{-/-}$ cells migrated at a lower linear speed (0.50 μm/min) than MEFs (0.76 μm/min). Unlike *Capn4*$^{-/-}$ cells, *Capn4*$^{-/-}$ cells expressing DV migrated significantly faster (0.65 μm/min) than *Capn4*$^{-/-}$ cells in comparison to *Capn4*$^{-/-}$ cells expressing DVI (0.47 μm/min) (Figure 2.5 *A* $p<0.01$). No significant difference was found in persistence between these two cell lines (Figure 2.5 *B*). Together, these findings demonstrate that expressing DV in *Capn4*$^{-/-}$ cells rescues the defect in migration speed, which is consistent with the observation that it also rescues the focal adhesion organization and traction force.

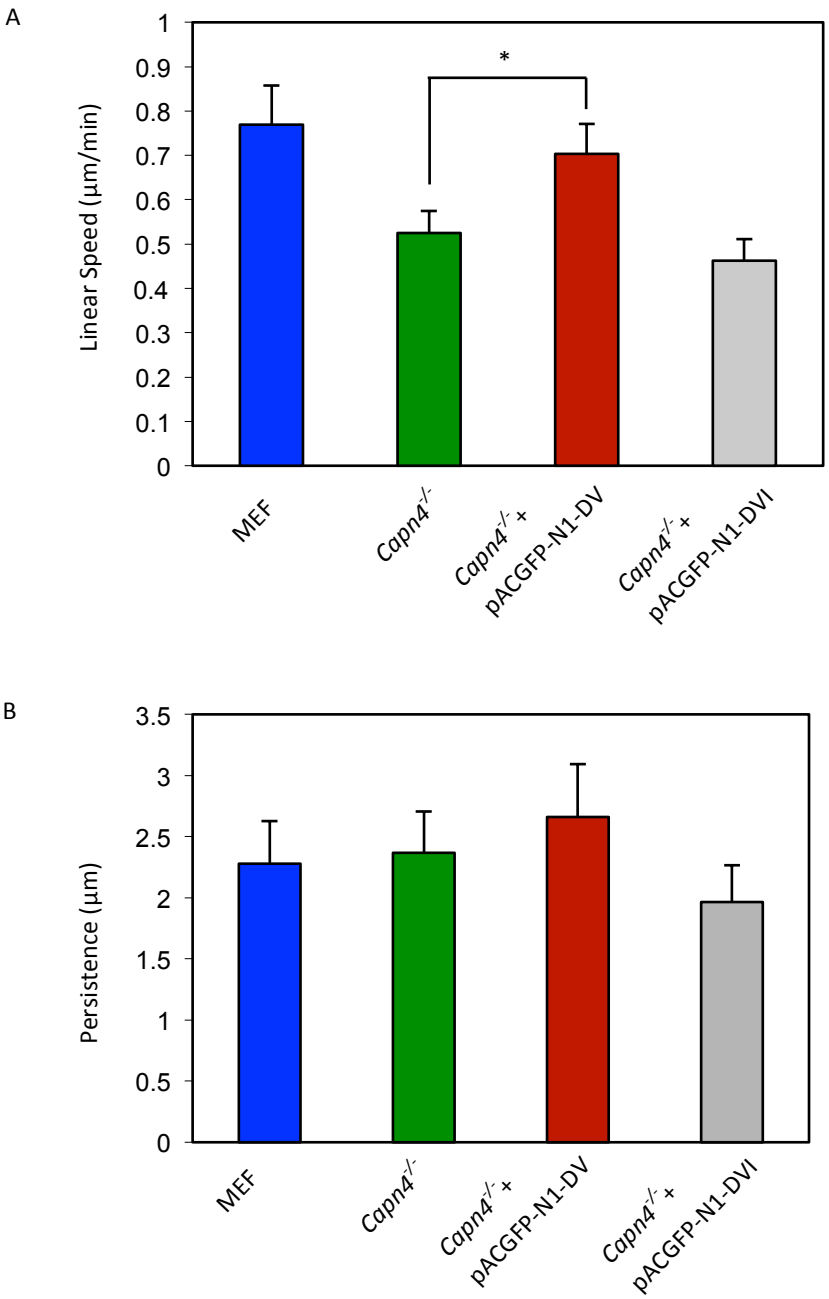



**FIGURE 2.5: Overexpression of DV promotes cell migration speed in *Capn4*$^{-/-}$ cells but not the persistence**. *A*. The bar graph represents the average of migration speed of MEFs, *Capn4*$^{-/-}$ cells, *Capn4*$^{-/-}$ cells expressing DV, and *Capn4*$^{-/-}$ cells expressing DVI. MEF cells migrated significantly faster than *Capn4*$^{-/-}$ cells. Expressing DV but not DVI in *Capn4*$^{-/-}$ cells increases the migration speed significantly compared to control *Capn4*$^{-/-}$ cells. *B*. The persistence of migration in each cell line was also calculated. No significant difference in persistence was observed between any two cell lines. Statistical analysis was performed by student's t-test (* denotes $p<0.05$).

**Overexpression of DVI Restores the Proteolytic Activity in *Capn4*$^{-/-}$ Cells**

It was previously reported that knocking-out the calpain small subunit diminishes the proteolytic activity of the holoenzyme (Dourdin et al., 2001; Undyala et al., 2008). Since domain VI dimerizes with the calpain large subunit through its fifth EF-hand motif (Goll, 2003), we asked whether restoring DVI to *Capn4*$^{-/-}$ cells would restore the proteolytic activity of the holoenzyme. A calpain activity fluorometric assay kit was used to measure the proteolytic activity of calpain in lysates from *Capn4*$^{-/-}$ cells expressing DV, DVI, full length *CAPN4*, *Capn4*$^{-/-}$ cells, and MEFs. As shown in figure 2.6, *Capn4*$^{-/-}$ cells present significantly lower levels of calpain proteolytic activity in comparison with MEFs. However, in *Capn4*$^{-/-}$ cells expressing DVI and full length *CAPN4*, this loss of proteolytic activity was restored. The same phenomena was not found in *Capn4*$^{-/-}$ cells expressing DV. This activity assay revealed that the presence of DVI is critical in the generation of holoenzyme proteolytic activity. The dimerization between the calpain small and large subunit mediated by domain VI is likely responsible in regulating the holoenzyme.

## DISCUSSION

The proteolytic function of calpain holoenzymes play critical roles in normal cellular function, including cytoskeletal remodeling, cell differentiation, apoptosis, and signal transduction (Carafoli and Molinari, 1998; Sato and Kawashima, 2001). Domain II on the large subunit contains the active site and is the only cysteine protease domain of the holoenzyme. For many years, the calpain small subunit's function has been believed to be limited to supporting the proteolytic process of calpain holoenzymes (Goll et al., 2003).

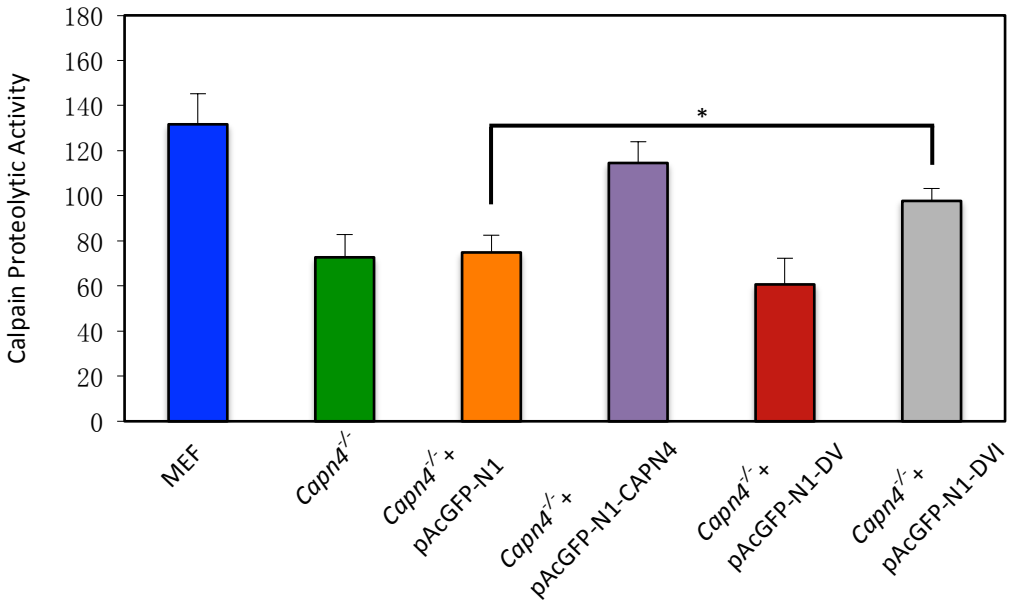



**FIGURE 2.6: The bar graph indicates the relative fluorescence units representing calpain activity levels in each cell line obtained using the Biovision assay kit.** The calpain activity was significantly reduced in *Capn4*$^{-/-}$ cells compared to MEFs. Expressing DVI in *Capn4*$^{-/-}$ cells significantly elevated the calpain activity compared to control *Capn4*$^{-/-}$ cells while expressing DV did not have the same effect. Statistical analysis was performed by student's t-test (* denotes $p<0.05$).

Previous research indicated that in *Capn4* deficient fibroblasts, the production of traction forces is impaired (Undyala et al, 2008). One would expect *Capn4* deficient fibroblasts to generate consistent phenotypes similar to the ablation of *CAPN1* and *CAPN2* genes based on the canonical concept of the calpain small subunit's function. However, our study attributes the reduction of traction forces in *Capn4*$^{-/-}$ cells solely to the calpain small subunit. We found that *CAPN4* disruption reduces both traction force production and mechanosensing, whereas inhibition of *CAPN1* and/or *CAPN2* impairs only mechanosensing but not traction force production (Undyala et al., 2008). These findings suggest a novel protease independent function for the calpain small subunit. To

understand the mechanism that regulates traction force through the calpain small subunit, we evaluated the magnitude of traction force and mechanosensing when each domain of the calpain small subunit was overexpressed in *Capn4*$^{-/-}$ fibroblasts. The most intriguing finding is that only the overexpression of domain V was sufficient to rescue the deficient traction force in *Capn4*$^{-/-}$ cells, and that the overexpression of domain VI, but not domain V, restored the ability to sense the applied force.

Domain V of the calpain small subunit is Gly rich with two regions of 11 and 20 Gly residues and contains a common motif (GTAMRILGGVI) at the C-terminus. Numerous studies have suggested a phospholipid binding property for this common motif (Brandenburg et al., 2002; Daman et al., 2001), although the presence of this binding and attributed function are controversial (Goll et al., 2003). It has been suggested that the binding between domain V and phospholipids brings the holoenzyme close to the cell membrane in order to decrease the $Ca^{2+}$ requirement for m-calpain activation (Johnson and Guttmann, 1997). Another possibility is that this interaction is also important for domain V to position close to adhesion structures and initiate a protease independent pathway to regulate traction force. The calpain holoenzyme undergoes a fast autolysis process during which 91 $NH_2$-terminal amino acids are removed sequentially to produce 26-27kDa, then 22-23kDa, and finally, 18kDa autolytic fragments (Goll et al., 2003). Whether autolysis still occurs and if the rescue of the traction force requires the presence of the entire domain V, or just the fragments released by autolysis, is unclear.

While overexpression of domain V rescues the traction force defect in *Capn4*$^{-/-}$ cells, it also rescues the abnormal focal adhesion arrangement and maturation observed in these cells. More focal adhesions mature into the center of cells, and a higher percentage of focal adhesions fall into the category of nascent focal adhesions (0.5-1.5 sq.μm). This is consistent with previous observations that traction forces modulate lamellipodial extension, maturation of focal adhesions, and translocation of focal adhesions toward interior regions of the cell (Ridley et al., 2003), and that nascent adhesions generate stronger forces (Beningo et al., 2001). Multiple parameters are known to modulate the speed and persistence of cell migration, such as adhesiveness, strength of traction stress, and the capacity to degrade ECM by proteolytic enzymes (Friedl and Wolf, 2010; Plotnikov et al., 2012; Wolf et al., 2013). In concert with elevated level of traction forces and rescue of focal adhesion arrangements, the migration speed was greater in *Capn4*$^{-/-}$ cells overexpressing doman V in our study when cultured on fibronectin coated glass.

Calpain 4 has been found to regulate the secretion of galectin-3 by indirectly mediating tyrosine phosphorylation (Menon et al., 2011). A possible mechanism for this is that calpain 4 mediates the secretion of galectin-3 indirectly through the binding of domain V with other interacting proteins or the cell membrane. Galectin-3 in the extracellular environment leads to clustering and activation of integrins (Goetz et al., 2008). Activated integrins then activate more downstream signaling proteins that ultimately lead to increased levels of traction forces, cell migration speed, and adhesion maturation. Studies are currently underway in our laboratory to address this hypotheis.

Domain VI of the calpain small subunit is a calmodulin-like domain and contains five EF-hand motifs, the fifth of which heterodimerizes with the large subunits to form holoenzymes (Goll et al., 2003). It is already known that sensing the applied force requires functional calpain 1, 2, and 4 (Undyala et al., 2008). In concert, our results indicate that expressing domain VI restores the ability for *Capn4*$^{-/-}$ cells to sense the applied pushing force onto the substrate. Given the evidence that the large and small subunits remain associated when calpain is active (Johnson and Guttmann, 1997), this binding between the large and small subunit might play an important role in regulating mechanosensing. Moreover, since expression of domain VI also restores the calpain protease activity in *Capn4*$^{-/-}$ cells, as shown by our study, it is possible that mechanosensing is related to the holoenzyme's protease function. Previous research identified an interaction between αPIX and calpain 4 (Rosenberger and Kutsche, 2005). αPIX interacts with the C-terminus of calpain 4 at the triple domain of SH3-DH-PH found within domain VI, and the integrity of the triple domain is necessary for efficient interaction between two proteins. This interaction is required for a cell to spread since the impairment of cell spreading resulting from inhibition of m-calpain in CHO-K1 cells can be rescued by overexpression of αPIX wildtype or GEF activity-deficient mutant, but not by the αPIX mutant in which domain DH is missing. These results also suggest that αPIX acts downstream of calpain to regulate cell spreading (Rosenberger et al., 2005). Based on these findings, αPIX is highly likely to be implicated in the mechanosensing pathway. Upon engagement to the ECM proteins, integrins are activated and cluster to form

complexes. At the same time, structural and signaling molecules are recruited intracellularly to these early integrin clusters in which β1 integrin, ILK, calpain proteases, β-parvin, α-actinin, and αPIX are present but without paxillin and vinculin. These clusters might then allow mechanosensing to occur and may or may not require the GEF exchange activity of αPIX (Bialkowska et al., 2000; Rosenberger et al., 2005; Schoenwaelder and Burridge, 1999). These results are also consistent with our finding that mechanosensing in *Capn4*$^{-/-}$ cells could not be restored when only domain V of calpain 4 is overexpressed. There may be other unidentified proteins containing the triple domain that interact with calpain 4, either directly or indirectly, to mediate mechanosensing signal transduction.

In summary, we have found that the calpain small subunit not only plays a role in traction force production in addition to its regulatory function for the holoenzyme activity, but also that this function is only mediated through domain V. Meanwhile, it was also discovered that mechanosensing to localized forces is mediated through domain VI, but not domain V. This functional segregation is the first observation that both the traction force production and mechanosensing to localized mechanical forces are regulated through different domains of the same protein. This study provides new insight into the mechanism involving the calpain small subunit that regulates the generation of traction forces and the coordinate series of events that occur during cell migration.

## ACKNOWLEDGEMENTS

This work was supported by NIHR01GM084248 to K.A.B.

# CHAPTER 3 DEFINING A MECHANISM FOR THE CALPAIN 4 MEDIATED REGULATION OF TRACTION FORCE THROUGH IDENTIFICATION OF DIRECT BINDING PARTNERS OF CALPAIN 4

## ABSTRACT

Traction forces and mechanosensing are two biophysical processes required for normal cell migration. Previous research showed that the calpain small subunit, calpain 4, regulates traction force production independently of the catalytic large subunits of the calpain 1 and 2. Moreover, we found that calpain 4 regulates both traction force and mechanosensing separately through two different protein domains. To further understand how calpain 4 regulates traction force, we sought to identify its binding partners and further participantes in this pathway. In this study, we have identified basigin as a direct binding partner of calpain 4. Furthermore we found that traction force was deficient when basigin expression was inhibited in MEFs. This defect was accompanied by substrate adhesiveness that was significantly weaker in strength. Despite these shortcomings, mechanosensing to the localized stimuli and homeostatic tension were not affected in MEFs with reduced expression of basigin. Together, these findings implicate basigin in the calpain 4 mediated pathway responsible for the regulation of cellular traction force. This pathway was previously found to be independent of the catalytic large subunits.

## INTRODUCTION

Cell migration is necessary for many normal and abnormal physiological processes, including embryonic development, wound healing, immunological responses, and cancer metastasis. In addition, cell migration is also crucial to technological applications such as

tissue engineering (Lauffenburger and Horwitz, 1996, Friedl and Alexande, 2011; Walters and Gentleman, 2015; Whelan et al., 2014). Although numerous studies have been done to extend our understanding about how the complex process of cell migration is regulated, the mechanism still remains unclear.

Focal adhesions function dynamically in cell migration, specifically in biophysical terms of transmitting both traction forces and mechanosensing between the actin cytoskeleton and extracellular matrix (ECM) (Flevaris, et al., 2007; Fouchard et al., 2014; Goldmann, 2014; Pasapera et al., 2015; Ridley, 2003). Calpains have been long implicated in the study of cell migration since calpain proteases are actually located within focal adhesions and play important roles in the turnover of several focal adhesion components (Beckerle et al., 1987; Bhatt et al., 2002; Dourdin et al., 2001; Goll et al., 2003). The two best characterized calpains, μ-calpain and m-calpain, both contain a distinct 80 kDa catalytic large subunit (calpain 1/CAPN1 and calpain 2/CAPN2, encoded by *CAPN1 and CAPN2* genes respectively) and a common 28kDa small subunit (calpain 4/CAPNS1/CAPN4, encoded by *CAPN4* gene) (Goll et al., 2003). Inhibiting calpains through overexpressing endogenous inhibitor calpastatin and pharmacological inhibitors leads to an inhibition of both adhesive complex disassembly and actinin localization to focal contacts (Bhatt et al., 2002).

Calpains are known to be regulated post-translationally through phosphorylation events, an endogenous inhibitor, and interactions with a regulatory small subunit. The protein phosphatase 2A (PP2A) is identified as a calpain phosphatase of μ-calpain and

m-calpain and can directly dephosphorylate both heavy chains. The dephosphorylation by PP2A inactivates μ-calpain and m-calpain and results in suppression of migration of lung cancer cells (Xu and Deng, 2006). The small subunit was previously considered to mainly serve a regulatory function for calpain holoenzymes (Goll et al., 2003). However, a finding that *Capn4*$^{-/-}$ embryonic fibroblasts present abnormal organization of focal adhesions, reduced rates of cell migration, and delayed retraction of membrane projections implicate the small subunit in the regulation of cell migration (Dourdin et al, 2001). In addition, a study from our lab indicated that traction force was attenuated by the knockout of the calpain small subunit but not by the large subunits, while all subunits are required for mechanosensing. This study implicated only the small subunit as an independent entity in the regulation of traction force (Undyala et al, 2008).

To gain understanding of how the calpain small subunit regulates the production of traction force we screened for its direct binding partners. In this study we used the whole gene of calpain 4 as bait in a yeast two-hybrid assay. From a screen of the entire mouse embryonic genome we identified the protein basigin as a direct binding partner for calpain 4. Basigin (Bsg), also known as CD147 or EMMPRIN, is a heavily glycosylated transmembrane protein belonging to the immunoglobulin (Ig) superfamily (Muramatsu and Miyauchi, 2003; Gabison et al., 2005). Basigin has been found to play roles in a variety of biological processes, and in the progression of cancers. Mice deficient in the basigin gene showed abnormal embryogenesis, spermatogenesis and fertilization (Chen et al., 2011; Igakura et al., 1998; Saxena et al., 2002). Knock-out mice of *Bsg* gene showed

abnormalities in vision and insensitivity to irritating odor (Igakura et al., 1996; Hori et al., 2000). Basigin is also implicated in the study of pathogen infections as it was found to stimulate an early step of HIV-1 (human immunodeficiency virus) replication in a CyA (cyclophilin A)-dependent manner (Pushkarsky et al., 2001). The basigin cytoplasmic domain, but not the signaling from basigin was essential for stimulation of HIV-1 infection (Pushkarsky et al., 2007). In a study of measles virus, it was found that the infection could be triggered via basigin and virion-associated cyclophilin B independently of measles virus hemagglutinin (Watanabe et al., 2010). Moreover, basigin is commonly over-expressed in many tumors (Liu et al., 2010; Xiong et al., 2014; Yang et al., 2013), and is implicated in almost all types of cancer (Xiong et al., 2014). On the surface of tumor cells, basigin was found to stimulate the production of matrix metalloproteinases (MMPs) in adjacent fibroblasts, resulting in enhanced tumor invasion (Biswas et al., 1995; Kanekura et al., 2002).

Basigin is known to interact either indirectly or directly with numerous proteins, including MCT1, MCT2, integrin-β1, cyclophilin, and ubiquitin C (Li et al., 2012; Mannowetz et al., 2012; Wanaguru et al., 2013; Xiong et al., 2014). Many of the known interacting proteins are related to cell migration. Basigin's functions in tumorigenesis and the interactions with proteins involved in cell migration render it a reasonable target candidate for elucidating how calpain 4 regulates the production of traction force.

In this study, basigin was identified as one of the binding partners for calpain 4 via the yeast two-hybrid assay. Furthermore we discovered that upon knockdown of basigin,

traction force was significantly reduced and these cells were defective in substrate adhesion. Surpringly, the ability to sense the application of a localized pushing force or homeostatic tension, was not affected in these basigin-inhibited MEFs. These results implicate basigin in the same pathway that calpain 4 functions to regulate the production of traction force, a pathway that it is independent of the catalytic activity of the holoenzyme.

## EXPERIMENTAL PROCEDURES

### Cell Culture

Mouse embryonic fibroblasts (MEFs) with a disrupted small calpain subunit gene have been previously described (Arthur et al., 2000; Dourdin et al., 2001), and are referred to as *Capn4*$^{-/-}$ cells in this study. MEFs, *Capn4*$^{-/-}$ cells and 293T cells were used in this study. MEFs were purchased from ATCC. 293T cells were kindly provided by Dr. Xiangdong Zhang (Wayne State University). MEFs and *Capn4*$^{-/-}$ cells were maintained in Dulbecco's Modified Eagle's Medium-high glucose (Sigma) supplemented with 10% fetal bovine serum (FBS) (Hyclone) and 1% penicillin/streptomycin/glutamine (PSG, Gibco) and incubated at 37˚C under 5% $CO_2$ in a humidified cell culture incubator. These cells were split by trypsinizing cells with 0.1% trypsin-EDTA (0.25% trypsin-EDTA diluted with HBSS,Gibco), diluted and passed into new culture dishes. Trypsinization was terminated by adding complete media. 293T cells were maintained and split similarly with 1% Pen/Strp (Gibco) replacing 1% PSG. The passage number of all cell types never exceeded eight passages.

**Cloning of *CAPN4* and Yeast Two Hybrid Assay**

Full length *CAPN4* was amplified by PCR from a pEGFP-CAPN4 plasmid under the following conditions: 30 cycles of 98˚C for 10 sec followed by 68˚C for 1 min using PrimeSTAR HS DNA Polymerase with GC buffer (Takara, R044A). The primers used for the purpose of inserting *CAPN4* into the two-hybrid plasmids pCWX200 and pLexA both supplied by ProteinLinks Ind. (Passadena, CA) were: the forward primer, 5'-ATCGGGATCCTTATGTTCTTGGTGAATTCGTTCTTGAAGG-3', and the reverse primer, 5'- ACCGCTCGAGTCAGGAATACATAGTCAGCTGCAGCCAC-3'. PCR products were resolved on 1% agarose gels and visualized by ethidium bromide (1% solution, Fisher) staining. The resolved bands were then purified using a Qiaquick gel extraction kit (Qiagen, 28706). Purified PCR products, pCWX200 and pLexA were incubated with XhoI and BamHI (New England Biolabs) under 37˚C for 4 hrs in 1X buffer 3 supplemented with 1% BSA. The double digested PCR products and plasmids were again purified with the Qiaquick gel extraction kit. To insert *CAPN4* into pCWX200 and pLexA, double digested *CAPN4* PCR product was ligated with double digested pCWX200 or pLexA using the LigaFAst Rapid DNA Ligation System (Promega, M8226). The constructs were transformed into E. coli to collect plasmids, and successful insertions were confirmed by DNA sequencing (Applied Genomics Technology Center, Wayne State University). These bait plasmids were then sent to ProteinLinks Inc. (Pasadena, CA) for yeast two-hybrid sceening.

**Small Interfering RNA (siRNA) Nucleofection**

Wildtype MEFs were used for selectively silencing *Bsg* via siRNA. The knock-down was generated through transient transfection with either control siRNA oligonucleotides or siRNA oligonucleotides targeting the *Bsg* gene using the siGENOME SMARTpool system (Dhamacon). The siRNA oligonucleotides targeting *Bsg* gene were: GAUUGGUUCUGGUUUAAGA, CAUCAGCAACCUUGACGUA, GCAAGUCCGAUGCAUCCUA, GGACAAGAAUGUACGCCAG. Nucleofection was performed using the Amaxa MEF2 Nucleofector Kit (Lonza) following the manufacturer's suggested protocol. Specifics include the use of MEF 2 nucleofector solution and supplement 1 followed by adding up to 5 ug of control siRNA or siRNA targeting *Bsg* gene and the nucleofector program MEF A-023. Nucleofected cells were then seeded according to the requirements of the need for the procedure. Inhibition of basigin expression reached a maximum at 36 hrs post-nucleofection.

**Protein Extraction and Western Blotting**

Proteins were extracted from each cell line with triple detergent lysis buffer (TDLB): pH 8.0, 50 mM Tris HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, into which Protease Inhibitor Cocktail (Sigma) and HaltTM Phosphatase Inhibitor Cocktail (Thermo) were dissolved. An 80% confluent 100-mm culture dish (Nunc$^{TM}$) was placed on ice and washed twice with ice-cold phosphate-buffered saline (PBS) followed by 25 min of incubation with 300 µl TDLB on ice. Lysed cells were collected into 1.5 ml tubes by an ice-cold cell lifter and centrifuged at 13,000 rpm for 10 min to remove cell

debris. Proteins were flash frozen and stored in -80˚C. Protein concentration was measured by Lowry method with the Bio-Rad DC protein assay kit as instructed by the manufacturer. Proteins were collected from cell lines of MEFs, *Capn4*$^{-/-}$ cells, MEFs transfected with control siRNA, and MEFs transfected with siRNA targeting *Bsg* gene.

20 μg of proteins were loaded onto a 4-20% gradient Tris–HEPES–SDS precast polyacrylamide gel system (Pierce) and resolved at 100 V for 1 hour. Proteins were then transferred onto an Immun-Blot PVDF Membrane (Bio-Rad) using a Trans-blot SD Semi-dry Transfer Cell (Bio-Rad) at 20 V for 30 min. Following transfer, the membrane was blocked for 1 hour with 5% milk in 1X phosphate-buffered saline (PBS) - 0.1% Tween (0.1% PBS/T) (for basigin antibody), 5% milk in 1X tris-buffered saline (TBS)-0.1% Tween (0.1% TBS/T) (for anti-actinin, anti-FLAG and anti-HA antibody), and then probed with the primary antibodies by incubation at 4˚C overnight with mild agitation. Primary polyclonal anti-basigin antibody (sc-9757, Santa Cruz) was diluted at 1:800 in 5% milk in 0.1% PBS/T, monoclonal anti-α-actinin antibody (A5044, Sigma) and monoclonal anti-FLAG antibody (F1804, Sigma) were diluted at 1:500 in 5% milk in 0.1% TBS/T, and monoclonal polyclonal anti-HA antibody (MMS-101P, Covance) was diluted at 1:1000 in 5% milk in 0.1% TBS/T. After washing 20 min for 3 times with 0.1% PBS/T, the membrane was incubated for 1 hour at room temperature with the secondary antibody. For the anti-basigin antibody, HRP-linked anti-goat IgG (sc-2020, Santa Cruz) was diluted at 1:2000 in 5% milk in 0.1% PBS/T; For anti-α-actinin, anti-FLAG, and anti-HA antibody, HRP-linked anti-mouse antibody (Fisher) was diluted at 1:10,000 in 5% milk in

0.1% TBS/T. After washing 20 min x3, the membrane was developed using ECL Plus Western Blotting Detection Reagents (Amersham).

**Cloning of *CAPN4* and *BSG*, and Immunoprecipitation**

Full length *CAPN4* was amplified by PCR from the pEGFP-CAPN4 plasmid and inserted into a pFLAG-CMV vector. *Bsg* gene lacking the sequence for the N-terminal 100 a.a. was amplified from the pJG4-5-BSG vector recovered from the yeast two hybrid assay and inserted into a pCDNA3 vector together with a HA sequence. The primers used for amplification of *Capn4* were: forward primer 5'-CCCAAGCTTATGTTCTTGGTGAATTCG-3' and reverse primer 5'-CCGGGATCCTCAGGAATACATAGTCAGCTGC-3'. The primers used for amplification of *Bsg* were: forward primer 5'-CGCGGATCCATGGAAGGGCCACCCAGGATCAA-3' and reverse primer 5'-CCGCTCGAGTCAGGTGGCGTTCCTCTGG-3'. Successful insertions were confirmed by sequencing (Applied Genomics Technology Center, Wayne State University).

293T cells were co-transfected with 10 μg of Flag-tagged *CAPN4* vector (full length) and 10 μg of HA-tagged *Bsg* vector. At 20 hour after transfection, cells were harvested and the immunoprecipitation assay was performed. Cells were lysed with ice-cold 1X lysis buffer (50 mM HEPES-NaOH pH 7.5, 100 mM NaCl, 0.5% NP-40, 2.5 mM EDTA, 10% glycerol, 1 mM DTT) with Protease Inhibitor Cocktail (Sigma) and HaltTM Phosphatase Inhibitor Cocktail (Thermo), and then collected and pelleted by

centrifugation. To 500 μg cell lysate, 10 μg anti-FLAG antibody or anti-HA antibody was added and then the lysates were incubated for 1 hour at 4˚C. 20 μl of Protein A/G PLUS Agarose (sc-2003, Santa Cruz) was added, and incubated at 4˚C on a rocker platform overnight. Immunoprecipitate was collected by centrifugation and the pellet was washed 4x with 1X lysis buffer. After final wash, the pellet was resuspended in 40μl of electrophoresis sample buffer. The sample was boiled for 3 minutes and analyzed by SDS-PAGE with correspondent antibodies.

**Preparation of Polyacrylamide Substrates**

A series of polyacrylamide substrates of different stiffnesses were prepared as described previously (Beningo et al., 2002). Briefly, a flexible 75μm x 22mm polyacrylamide substrate was made in a cell culture chamber dish in which 0.2μm fluorescent microbeads were embedded. The acrylamide (acryl, Bio-rad) concentration was fixed at 5% while N,N-methylene-bis-acrylamide (bis, Bio-rad) varied from 0.04% to 0.1% to attain different stiffnesses of the substrates. The substrates were then coated with 5 μg/cm$^2$ fibronectin (Sigma) at 4˚C overnight by crosslinking with Sulfo-sanpah (Thermo). Cells were seeded onto the substrates overnight prior to TFM or mechanosensing. The 5%/0.08% Acry/Bis substrates ($E$=1.41 kPa) were used in traction force microscopy (TFM), the 5%/0.1% Acryl/Bis substrates were used in mechanosensing assay to applied forces, and 5%/0.1% Acryl/Bis (hard) ($E$=2.11 kPa) and 5%/0.04% Acryl/Bis (soft) ($E$=0.41 kPa) substrates were used for the cell adhesion assay.

**Traction Force Microscopy**

Flexible polyacrylamide substrates of 5%/0.08% Acryl/Bis coated with 5 μg/cm$^2$ fibronectin were prepared as described above. Cells were seeded onto the flexible polyacrylamide substrates for 36 hrs. After the chamberdishes were kept in the incubator under regular cell culture conditions overnight, images for cells were collected as described previously (Beningo et al., 2002). Briefly, for a single cell, 3 images were taken under a 40X objective lens: a bright field image of the cell, an image for the fluorescent beads with the cell on the substrate, and another image for the fluorescent beads after the cell was removed by a pointed microneedle. DIM (Yu-li, Wang) was used to calculate bead displacement with or without the cell and the cell and nuclear boundaries. These data were used to generate and render traction stress values by using a custom made algorithm provided to our lab by Dr. Micah Dembo (Boston University) as described previously (Dembo and Wang, 1999; Marganski et al., 2003). Images of 14-22 cells for each cell line were collected.

**Mechanosensing Assay to Applied Mechanical Stimulation**

Flexible polyacrylamide substrates of 5/0.1% Acryl/Bis coated with fibronectin were prepared as described above. Cells were seeded onto the substrates and allowed to adhere overnight under regular cell culture conditions. As described previously (Lo et al., 2000), a cell was monitored for 10 min for its migration trajectory before a blunted microneedle was pressed onto the substrate in front of the direction the cell was migrating in order to generate a pushing force through the substrate to be interpreted by the cell. The pushing

force would release the tension on the substrate. Images were taken for cells with a 40X objective lens every 3 min continuously for 1 hour to record migrating trajectories of cells. If a cell responds to the pushing force that was applied by the microneedle by avoiding it, a "1" is recorded; if a cell continues to migrate on the same trajectory (ignoring the stimulus), a "0" is recorded. For each cell line, 6-8 cells were observed.

To explore the effect of homeostatic compliance on cellular morphology, polyacrylamide substrates of stiffness of 5%/0.1% Acryl/Bis (hard) and 5%/0.04% Acryl/Bis (soft) were made as described above. After solidification, the substrates were coated with 5μg/cm$^2$ fibronectin. Cells were coated onto the substrates and allowed to adhere overnight under regular cell culture conditions before the images were taken with 10X objective lens. The number of spread and round cells as observed visually by their area were counted from six random fields for each cell line on both stiffness of substrates. The cell numbers were plotted as bar graphs.

**Cell Adhesion Assay**

A centrifugation assay was used to measure cell-substrate adhesiveness. This assay was performed following the method described by Guo et al. (Guo et al., 2006) with a slight modification. Briefly, a hole was drilled in an air-tight culture dish (Pall Corporation), and a coverslip was attached to the culture dish. 5%/0.08% Acryl/Bis substrates were made on the coverslips as described above and then coated with 5 μg/cm$^2$ fibronectin. 2.5x10$^4$ cells were seeded onto fibronectin-coated substrates and allowed to adhere for 30 minutes at 37˚C. After incubation, the chambers were then inverted and

centrifuged for 5 minutes at 1800g. Ten random fields of cells were counted for each cell line immediately after centrifugation. Percentages of cells after centrifugation over before are expressed as bar graphs.

**Immunofluorescence**

After being flamed, no. 1.5 glass coverslips (Fisher) were attached to chamber dishes with vacuum grease. Then they were coated with 5 μg/cm$^2$ fibronectin (Sigma) at 4˚C overnight, and blocked with 1% BSA in PBS at 4˚C overnight. Cells were seeded onto the coverslips and allowed to attach overnight under incubation at 37˚C with 5% $CO_2$ in a humidified cell culture incubator. The cells were then fixed and permeabilized with the following steps: firstly, incubate for 10 min with 2.5% paraformaldehyde in 1X PBS at 37˚C; then incubate with 2.5% paraformaldehyde in 1X PBS with 0.1% Triton X-100 at 37˚C; followed by incubation of 5 min with 0.5 mg/ml $NaBH_4$ solution. After fixation and permeabilization, cells were blocked with 5% BSA in PBS for 1 hour at room temperature, and then incubated with anti-basigin antibody (sc-9757, Santa Cruz) at a 1:250 dilution for 3 hours at room temperature. Following 3 washes of 15 min, Alexa Fluor® 546 anti-goat secondary antibody was added at a 1:500 dilution in 5% BSA for 1 hour at room temperature. After the final washes (3 x 15 min each), mounting media (pH=7.8, 0.1% PPD, 1X PBS, 50% glycerol, 30% Q-$H_2O$) was added. Images were taken with appropriate filters for GFP signals.

**Cell Migration Assay**

After being flamed, no. 1.5 square glass coverslips (Fisher) were attached onto chamber dishes and the glass was coated with 5μg/cm$^2$ fibronectin (Sigma) diluted in 50 mM HEPES at 4˚C overnight. Cells were then seeded onto the coverslips and allowed to attach overnight under incubation at 37˚C with 5% $CO_2$ in a humidified cell culture incubator. Images were taken of a single cell for 2 hours at 2 min intervals with a 40x objective lens. All the collected images for one cell were imported into the custom built dynamic image analysis system software (DIM, Y-L. Wang) to calculate the linear speed and persistence of each cell. 15-18 cells were observed for each cell line.

**Microscopy**

Images of all experiments described above were acquired with an Olympus IX81 ZDC inverted microscope fitted with a custom-built stage incubator to maintain cells at 37˚C under 5% $CO_2$ for live cell imaging and a SPOT Boost EM-CCD-BT2000 back-thinned camera (Diagnostic Instruments Inc., Sterling Heights, MI). The camera was run by IPLab software (BD Biosciences).

## RESULTS

**Basigin is a Binding Partner of Calpain 4**

To study the mechanism utilized by calpain 4 to regulate the traction force production independently of the large catalytic subunits of calpains, we sought to identify direct binding partners of calpain 4 using the two-hybrid system. *CAPN4* gene was inserted into the plasmids of pCWX200 and pLexA and the whole gene was used as the

bait. The constructs were supplied by an outside company, ProteinLinks, to perform a yeast two-hybrid screen. Sequencing results identified basigin is one of the candidates as binding partners for calpain 4. The direct binding between calpain 4 and basigin was then confirmed by co-immunoprecipitation (Figure 3.1 *B*).

To observe the expression pattern of bagisin protein, immunofluorescence was performed with both MEFs and *Capn4*$^{-/-}$ cells with basigin antibody. Results indicated basigin spread more toward the cell body and diffusely to the cell edge (Figure 3.1 *C*). It seems that basigin failed to locate to the periphery of *Capn4*$^{-/-}$ cells. However, it is highly possible that this results from the thinness of the lamellipodia in *Capn4*$^{-/-}$ cells observed in our lab (Undyala et al., 2008). While comparing the expression level of basigin in both MEFs and *Capn4*$^{-/-}$ cells, we surprisingly found that basigin was expressed at a reduced level in *Capn4*$^{-/-}$ cells than in MEFs (Figure 3.1 *D*), supported by quantification of fluorescent signal strength in immunostaining (Figure 3.2 *B*). This result suggests that basigin is possibly functioning downstream of calpain 4 in the pathway that regulates traction force production through calpain 4.

**Silencing of Basigin through siRNA Reduced Basigin Expression Effectively**

To further study the function of basigin in cell migration, siRNA was used to silence the expression of basigin in mouse embryonic fibroblasts (MEFs). Nucleofection was used to transfect the oligonucleotides into cells and efficiency of inhibition was found to be 95% at 36 hrs as determined by western analysis (Figure 3.2 A). Furthermore, immunostaining confirmed silencing of basigin with a reduction in intensity of 82%

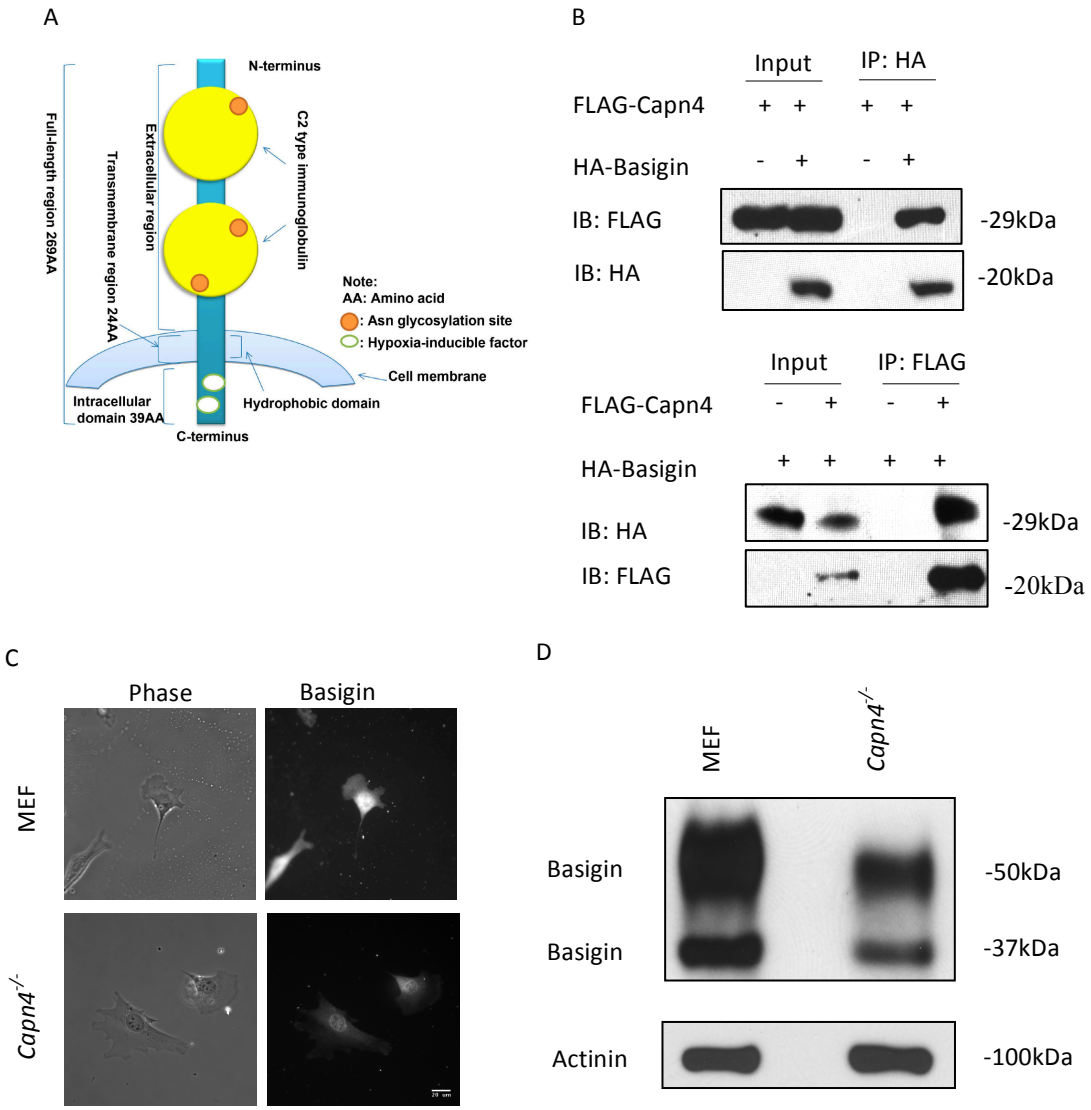



**FIGURE 3.1: Co-immunoprecipitation of calpain 4 and basigin proteins and the expression pattern of basigin protein.** *A*. The molecular structure of basigin (Xiong et al., 2014). *B*. *CAPN4* was inserted into a pFLAG-CMV vector, the *Bsg* gene, lacking 300bp encoding the N-terminus was inserted into a pCDNA3 vector containing a HA sequence. Lysates of 293T cells expressing these proteins were used for pull-down assays using either FLAG or HA antibody. *C*. Localization of basigin in MEFs and *Capn4*$^{-/-}$ cells. *D*. Basigin expression level is reduced in *Capn4*$^{-/-}$ cells compared to wildtype MEFs. Two bands of 50 and 37 kDa of basigin were found in western blots. Both bands showed reduced level of expression when calpain 4 was interrupted. α-Actinin was used as loading controls (Mag. bar = 20μm).

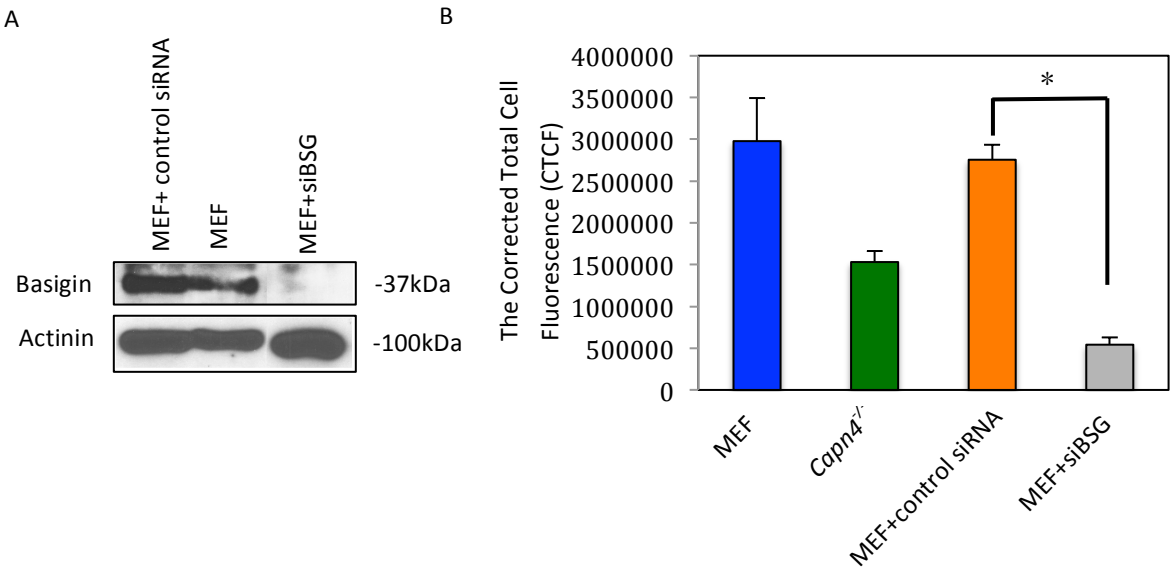



**FIGURE 3.2: Silencing of basigin through siRNA reduced basigin expression effectively.** *A.* Cell extracts were made 36 hrs after nucleofection with siRNA sequence targeting basigin. Western blots probed with anti-basigin antibody showed 95% reduction in basigin expression. *B.* A bar graph representing the corrected total cell fluoscence (CTCF) for each cell line calculated by ImageJ. Both *Capn4*$^{-/-}$ cells and MEFs in which basigin was inhibited have significantly reduced level of CTCF ($p$<0.005). Actinin was used as the loading control. Statistical analysis was performed by student's t-test. 11-12 cells were used for each cell line for calculation. * denotes $p$<0.05.

(p<0.005) when basigin was silenced in MEFs, as well as Capn4-/- cells showing a 49% reduction of signal intensity (p=0.01) (Figure 3.2 B).

**Inhibition of Basigin Resulted in Defects in Traction Force Production and Adhesion Strength in MEFs**

Previous studies in our lab determined that traction forces were reduced in *Capn4*$^{-/-}$ cells compared to wildtype MEF cells while inhibition of *CAPN1* or *CAPN2* or the overexpression of calpastatin had no effect on traction (Undyala et al., 2008). To learn whether basigin is involved with calpain 4 in the pathway for traction force, TFM was

performed on MEFs where basigin expression was knocked down, MEFs, and *Capn4*$^{-/-}$ control cells. The flexible polyacrylamide substrates used for TFM were covalently coated with fibronectin and the assay procedure used was as previously described. (Dembo & Wang, 1999) (Figure 3.3 *A*). As expected, *Capn4*$^{-/-}$ cells produced significantly reduced magnitude of traction forces (avg. 1.99 kPa) compared to wildtype MEFs (avg. 2.69kPa, $p$=0.03) and MEFs transfected with control siRNA (avg. 2.91kPa, $p$=0.04). Furthermore, silencing basigin expression in MEFs via siRNA also significantly reduced the magnitude of traction forces to 1.93 kPa ($p$=0.04) (Figure 3.3 *A*). These results suggest that silencing basigin leads to deficient traction force similar to the disruption of calpain 4 and thus is also implicated in the regulation of traction force.

To test the adhesion strength of focal adhesions to the substrates, we performed the centrifugation assay in the same set of cell lines as above using a previously described protocol (Guo et al., 2006; Undyala et al., 2008). Briefly, cells were seeded onto fibronectin coated flexible acrylamide substrates mounted onto chamber dishes and allowed to adhere for 30 min at 37˚C. The chamber dishes were inverted and centrifuged. The number of cells for each line attaching to the substrate was counted right before and after centrifugation. The results indicated that approximately 61% of *Capn4*$^{-/-}$ cells remained adhered to the substrates after centrifugation compared to 98% of MEFs that remained adhered (Figure 3.3 *B*). Similarly, silencing basigin through siRNA resulted in only approximately 63% of cells remaining adhered to the substrates (Figure 3.3 *B*). In comparison, 95% percent of MEFs treated with control siRNA remained adhered after

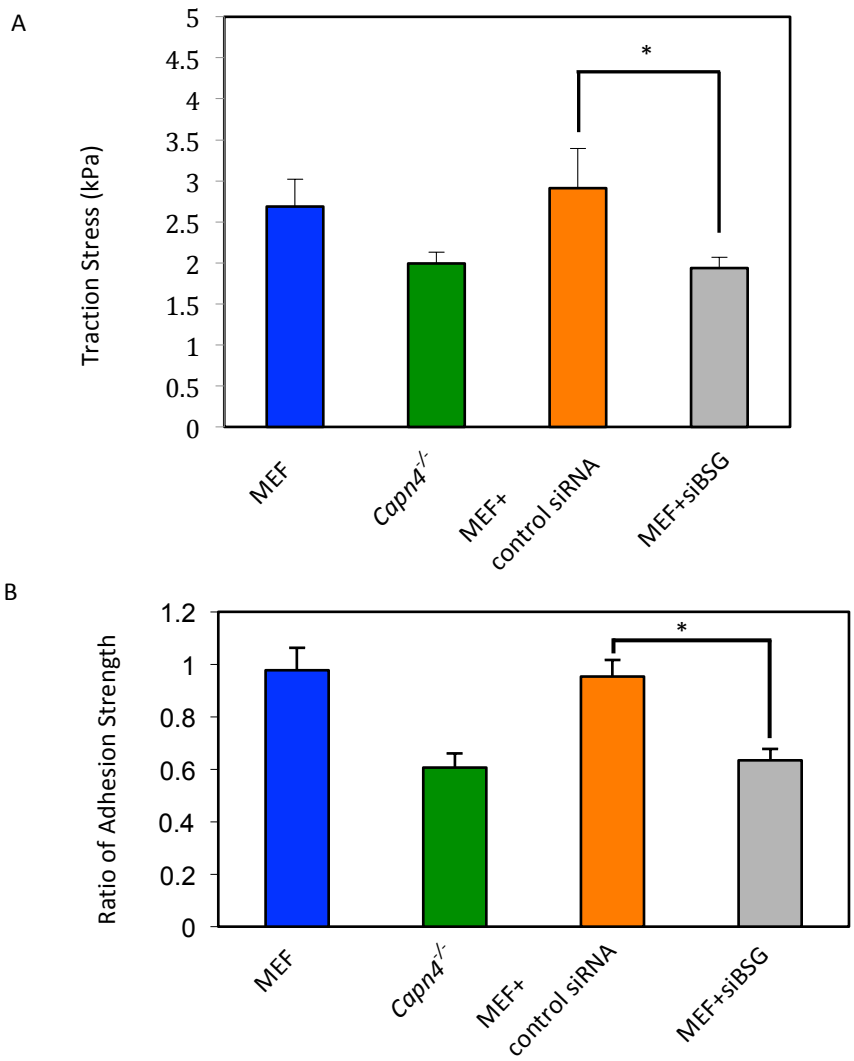



**FIGURE 3.3: Silencing basigin through siRNA resulted in reduced in traction force production and adhesion strength in MEFs**. Cells were seeded onto flexible polyacrylamide substrates covalently coated with fibronectin and allowed to attach overnight followed by traction force microscopy (TFM). *A.* A bar graph representing the average traction stress exerted by each cell line onto the substrate. Traction stress of basigin knock-down MEFs was compared with MEFs, *Capn4*$^{-/-}$ cells, and MEFs transfected with control siRNA. Averages from 14 MEFs, 22 *Capn4*$^{-/-}$ cells, 21 MEFs transfected with control siRNA, and 21 basigin knock-down MEFs were used for calculating the average traction stress from each cell type. *B.* A bar graph representing the adhesion strength by calculating the percentage of the number of cells that remained adhered onto the substrates after centrifugation. Compared to MEFs, *Capn4*$^{-/-}$ cells exhibited significantly reduced adhesion strength ($p$=0.02). When basigin was silenced throught siRNA in MEFs, a reduction of adhesion strength was also observed ($p$=0.02). Statistical analysis was performed by student's t-test. * denotes $p<0.05$.

centrifugation (Figure 3.3 *B*). These results suggest that basigin contributes to adhesion strength of focal adhesions in addition to regulating traction force production.

**Mechanosensing is Normal in Basigin Knockdown Cells**

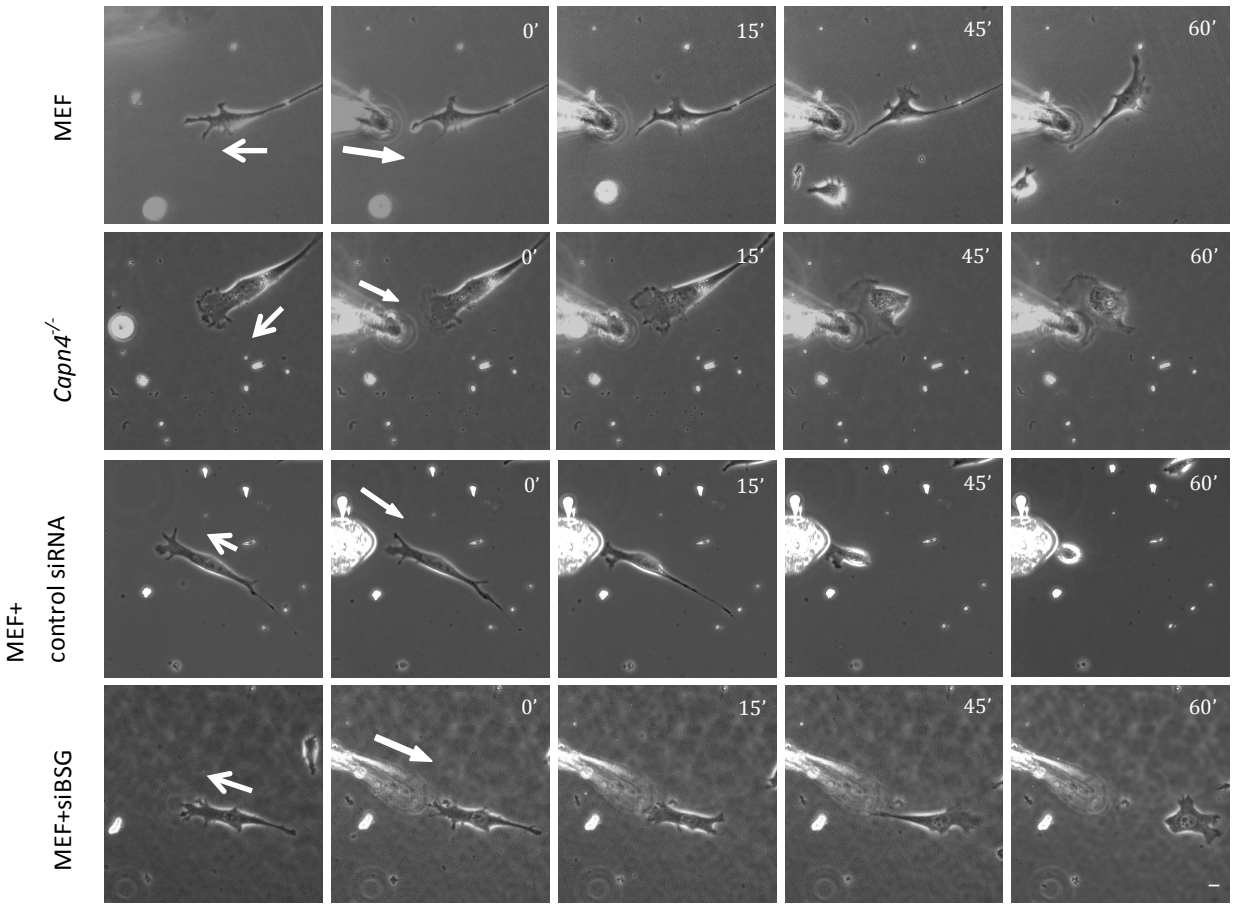
Cells are able to sense mechanical information from the environment including matrix elasticity, localized mechanical stimuli, and topography (Chang et al., 2013; Engler et al., 2006; Kenneth et al., 2011; Menon and Beningo, 2011; Mohammadi et al., 2014). These physical signals are transmitted from the outside of the cell inward, and lead to changes in the cytoskeletal networks, interaction with the extracellular matrix (ECM), cellular force production, differentiation, growth, and apoptosis (Chang et al., 2013; Engler et al., 2006; Guilak, 2009; Kenneth et al., 2011; Menon and Beningo, 2011). One previous study where cells were tested for their ability to sense localized stimulus shows that MEFs respond to it by changing the migratory trajectory or rounding up, and Calpain 1, 2, or 4 deficient cells continue to migrate along the same trajectory when a local stimulus is applied, meaning they are insensitive to localized stimuli (Undyala et al., 2008). In another assay, cells are evaluated by how well they spread on substrates of different stiffness. Previous results indicate that MEFs are able to sense the stiffness by spreading better on stiff substrates compared to on soft substrates (Pelham and Wang, 1997). Surprisingly, MEF cells deficient in any calpain 1, 2 or 4 are still able to sense the stiffness difference and spread differently on hard and soft substrates (Undyala et al., 2008). Traction forces were believed to not only function as the driving force for cell migration but also play equal roles in sensing the physical environment (Chang et al.,

2013). As our study indicated that silencing basigin in MEFs affected the generation of traction forces significantly, here we wanted to know whether mechanosensing was also affected in both assays.

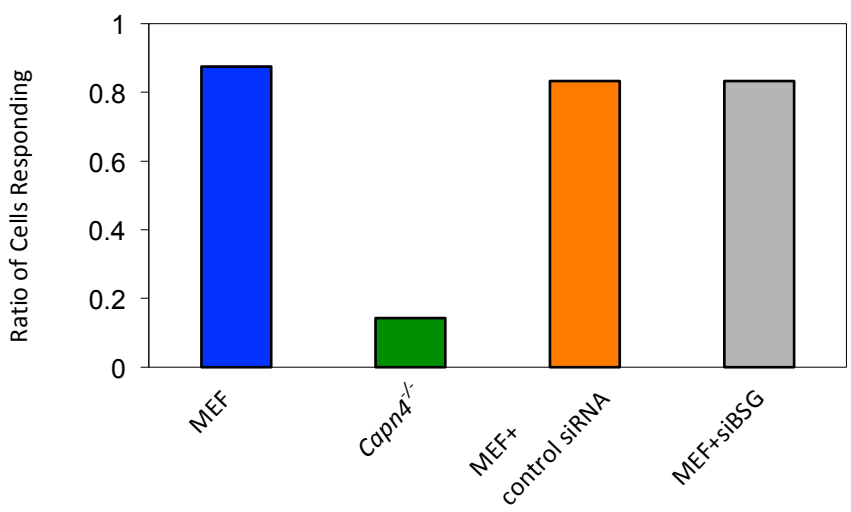
To test whether basigin plays a role in mechanosensing, cells were tested for their ability to respond to localized mechanical stimuli. In the assay, cells were seeded onto fibronectin-coated polyacrylamide substrates and a blunted microneedle was gently pushed onto the substrate, 100um or less, in front of the cell impeding the migratory path. As expected, 87.5% of MEF cells responded to the pushing force by changing trajectory to avoid it, while only 14.3% of *Capn4*$^{-/-}$ cells recognized the force and responded (Figure 3.4 *A, B*). As with MEF cells, 83.3% of MEF cells transfected with control siRNA reacted to the localized pushing force (Figure 3.4 *A, B*). When basigin was silenced in MEFs, 83.3% of cells still responded to the localized pushing force (Figure 3.4 *A, B*). These results indicate that basigin does not play a role in sensing the localized pushing stimulus.

To test whether basigin could be involved in sensing the stiffness of substrates, cells were seeded onto hard and soft flexible polyacrylamide substrates and allowed to adhere overnight. The morphology of cells on each stiffness of substrates was observed and recorded. As expected, when seeded on hard substrates, 87% of MEFs spread normally on hard substrates, as well as 91% of *Capn4*$^{-/-}$ cells. Meanwhile, 86% of MEFs treated with control non-target siRNA and 90% of MEFs treated with basigin targeting siRNA

A




B




| Cell Type | MEF | *Capn4*$^{-/-}$ | MEF+Control siRNA | MEF+BSG siRNA |
|---|---|---|---|---|
| Response | + | - | + | + |
| # of Cells | 7 of 8 | 1 of 7 | 5 of 6 | 5 of 6 |

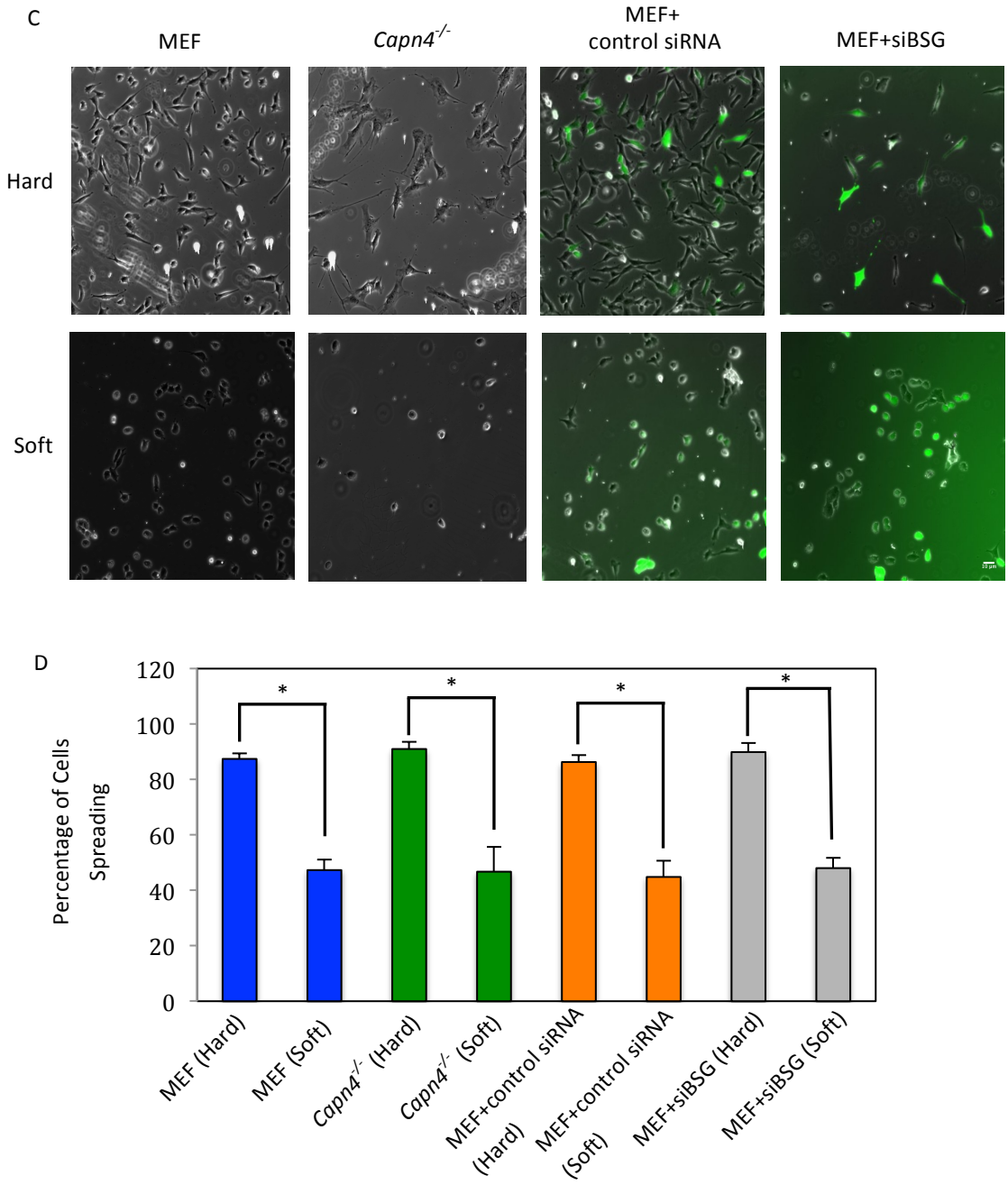



**FIGURE 3.4: Silencing basigin through siRNA does not affect the ability of MEFs to sense a localized stimulus or homeostatic tension of the underlying substrate.** *A*, Representative time-lapse images show the responses of cells to the applied localized stimulus including MEFs, *Capn4*$^{-/-}$ cells, MEFs transfected with control siRNA, and basigin knock-down MEFs. The thin arrows in the first column denote the cells starting trajectory; the bold arrows in the second column denote the direction the blunted needle is pushed. Cells were seeded onto flexible polyacrylamide substrates that were covalently coated with fibronectin and allowed to attach overnight. A blunted microneedle was

pressed toward the direction the cell was migrating to, and responses of cells were recorded for each cell line (Mag. bar = 10μm) and summarized in B. *B*. A bar graph indicates the percentage of cells of each cell line that respond to the localized pushing force applied by a blunted needle. The number of cells for each cell line and cells' responses were summarized in the table. If a cell migrates by avoiding the pushing force, it is marked with "+", denoting a positive response; if a cell continues to migrate toward the pushing force, it is marked with "-", denoting a negative response. *C.* Images were taken with 10X lens for each cell line after they were seeded on both hard (5%/0.1% Acryl/Bis) and soft (5%/0.04% Acryl/Bis) substrates and allowed to adhere overnight. Then numbers of cells were counted for each line based on the morphology (spread vs. round) as observed visually. The average cell counts for each line were graphed in *D*. Six random fields were counted for each cell line. Statistical analysis was performed by student's t-test. * indicates $p<0.05$.

also spread normally (Figure 3.4 *C, D*). In contrast, only 47% of MEFs, 42% of *Capn4*$^{-/-}$ cells, 45% of MEFs treated with control non-target siRNA, and 48% of MEFs treated with basigin targeting siRNA spread well when seeded on soft subtrates (Figure 3.4 *C, D*). The significant decrease of the number of cells spreading normally on substrates of different stiffness indicated that basigin was not implicated in sensing the stiffness of substrates. Taken together with the results from the localized stimulus assay, basigin does not appear to be involved in the mechanosensing process.

**Inhibiting Basigin Results in Reduced Cell Migration Speed without Affecting Migration Persistence**

Previous studies demonstrate that *Capn4*$^{-/-}$ cells have reduced migration speed compared to MEF cells, which is consistent with work in our lab (Dourdin et al., 2001; Undyala et al., 2008). To learn whether knockdown of basigin results in the same effect on cell migration, MEF cells where basigin was silenced by siRNA were seeded onto fibronectin coated glass coverslips and observed for 2 hours to track the locomotion of

the nuclei. Consistent with previous studies, *Capn4*$^{-/-}$ cells migrated at a significantly reduced linear speed (0.52 μm/min) compared to MEFs (0.77 μm/min, $p$=0.03). When basigin was silenced by siRNA, MEFs also migrated significantly lower than control MEFs (0.49 μm/min, $p$=0.02) at a similar speed as *Capn4*$^{-/-}$ cells ($p$=0.65) (Figure 3.5, *A*). Although migration speed was significantly affected by silencing basigin, migration persistence was similar in all lines (Figure 3.5 *B*). These results suggest that the pathway of calpain 4, which also involves basigin, not only regulates the generation of tractions, but also affects the linear migration speed.

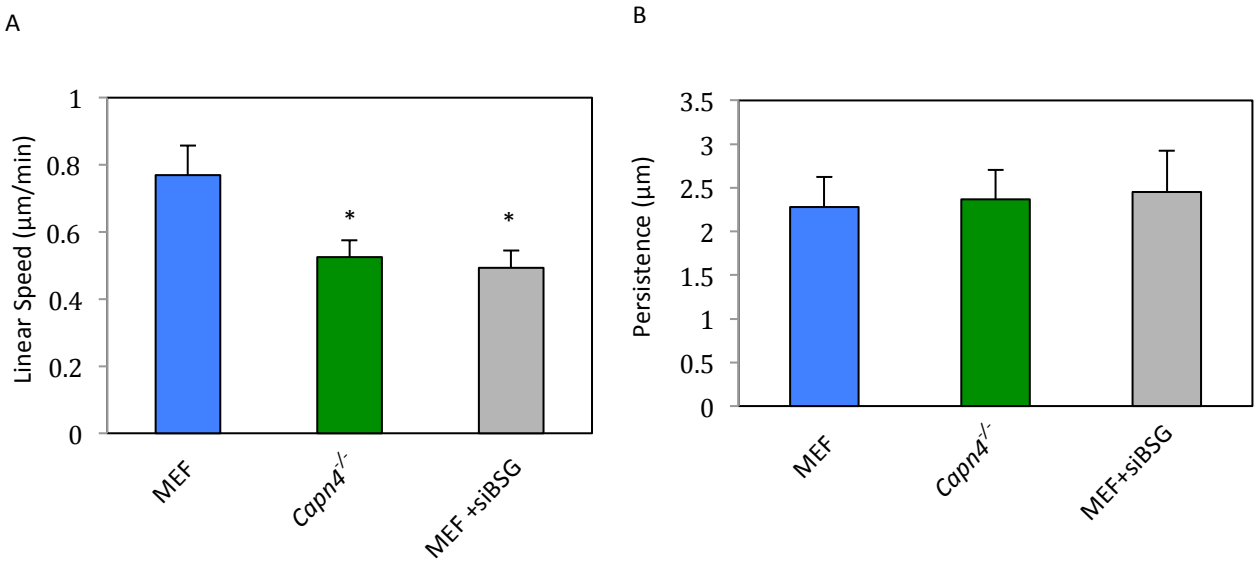



**FIGURE 3.5: Overexpression of DV promotes cell migration speed in *Capn4*$^{-/-}$ cells but not the persistence.** *A*. The bar graph represents the average migration speed of different cell lines: MEFs, *Capn4*$^{-/-}$ cells, MEFs with basigin knocked down. MEF cells migrated significantly faster than *Capn4*$^{-/-}$ cells. Inhibiting basigin in MEFs similarly inhibited the migration speed of cells significantly. *B*. The persistence of migration in each cell line was calculated. No significant difference in persistence was observed among 3 cell lines. 18 MEF cells, 15 *Capn4*$^{-/-}$ cells, and 15 basigin knock-down MEF cells were used for calculation in *A* and *B*. Statistical analysis was performed by student's t-test. * indicates $p$<0.05.

# DISCUSSION

Our lab has previously discovered that calpains are involved in both traction force production and sensing localized stimuli in MEF cells (Undyala et al., 2008). We found that both large and small subunits of calpain holoenzymes are required for cells to sense localized stimuli normally, while only the small subunit is required for traction force generation with no effect in production of traction forces when large subunits are silenced (Undyala et al., 2008). This suggests that the calpain small subunit functions independently of the proteolytic large subunits of calpain and the holoenzymes in the regulation of traction forces while all subunits are implicated in mechanosensing. Our recent study discovered that overexpressing domain V of calpain 4 in *Capn4*$^{-/-}$ cells rescued the deficient traction force while overexpressing domain VI in *Capn4*$^{-/-}$ cells restored the ability to sense the localized stimuli (see chapter 2). These results support our conclusion that calpain 4 regulates both traction force and mechanosensing within the same molecule.

To understand this pathway involving calpain 4 that regulates both traction force production and mechanosensing, we looked for direct binding partners for calpain 4 using the two-hybrid screen. Out of all candidates obtained through yeast two-hybrid assay, basigin raised our attention based on the fact that extensive study has already shown that basigin on the surface of tumor cells stimulates the production of MMPs in adjacent fibroblasts, and that it plays an important role in tumor cell motility and invasion (Sun and Hemler, 2001; Wang et al., 2015).

To understand the effect of basigin in cell migration, we performed a series of different assays. Although basigin was expected to co-localize with calpain 4 in cells, immunofluorescence staining failed to localize basigin protein to certain areas of cells (Figure 3.1 *C*). The almost eliminated signals for basigin locating to the periphery of *Capn4*$^{-/-}$ cells could be just a result of the thinness of lamellipodia at the cell periphery. The high expression level and multi functions of basigin in varied types of cells help explain the lack of localization of basigin staining to focal adhesions (Chen et al., 2011; Hori et al., 2000; Igakura et al., 1996; Igakura et al., 1998; Saxena et al., 2002).

Since the fragment of basigin used to confirm the direct binding with calpain 4 lacks the N-terminus 100 a.a, the binding of the two proteins occurs between C-terminus of basigin and calpain 4. Since basigin is a transmembrane protein, this interaction might facilitate the connecting of calpain holoenzymes to the cell membrane. In the future, efforts to understand how basigin interacts with calpain 4 and whether this interaction assists the localization of the calpain holoenzymes to the membrane will enhance our understanding of how traction force is regulated.

Previous research of basigin's function in cell migration focused on tumor cell motility and invasion. Basigin expression level is reportedly elevated in most types of tumor cells and is one of the most highly expressed proteins in disseminated cancer cells (Xiong et al., 2014). High levels of basigin expression on the surface of tumor cells induces increased level of MMP activity in both stromal cells and the tumor cells themselves (Gabison et al., 2005; Sun and Hemler; 2001; Zucker et al., 2001). Elevated

MMP activity then degrades the ECM and changes ECM turn-over dynamics, leading to increased tumor cell motility and invasion (Xiong et al., 2014). Consistent with these studies, we found that inhibiting basigin expression through siRNA in wildtype MEFs results in reduced traction force and adhesion strength (Figure 3.3 *A, B*) and decreased migration speed (Figure 3.5 *A*), suggesting that calpain 4 is a positive regulator for basigin in this pathway. Basigin is known to affect numerous targets in addition to MMPs (Gabison et al., 2005; Xiong et al., 2014). It is very likely that other proteins are involved in this signaling pathway for regulating traction force in addition to MMPs. It will be helpful to identify function further downstream of this signaling parthway.

Previous and recent research in our lab indicates deficient traction forces in *Capn4*$^{-/-}$ cells (Figure 3.3 *A*). Meanwhile, these *Capn4*$^{-/-}$ cells fail to respond to the localized stimuli comparing with MEFs but sense the stiffness of substrates normally as well as MEFs (Figure 3.4 *A, C*), suggesting that calpain 4 has provided a means to separate traction force generation and mechanosensing spatially and temporally. Moreover, MEFs in which basigin expression is silenced, respond to the localized mechanical stimuli and also sense the stiffness of substrates normally (Figure 3.4 *A*, *C*), suggesting that basigin functions only in the production of traction forces, but not in mechanosensing. Previous studies suggest that rigidity sensing mechanism is driven by traction forces in the frontal region of the migrating cell. This idea is based on the observation that localized softening of the substrate in the frontal region of the cell results in cellular retraction, reversal of cell polarity or cell immobilization (Wang, 2009). Our conclusion does not contradict this

observation since we only measured the overall level of traction force within a cell without measuring specific areas of a cell.

In summary, we identified basigin as a new binding partner for the calpain small subunit. We further tested basigin in several functional assays and concluded that basigin participates with calpain 4 in regulating the production of traction force and also affects substrate adhesion strength. However, basigin is not implicated in mechanosensing based on the normal response of basigin knockout MEF's in response to localized stimuli and homeostatic tension. Taken together, these results implicate basigin in the pathway in which calpain 4 is involved in regulating the generation of traction force independently of the large catalytic subunits of calpains.

## ACKNOWLEDGEMENTS

This work was supported by NIHR01GM084248 to K.A.B.

# CHAPTER 4 SUMMARY AND CONCLUSIONS

Mechanical aspects in the process of cell migration have attracted more and more attention in the last decade. Traction forces are mechanical forces that are generated by the acto-myosin cytoskeleton and transmitted onto the extracellular matrix via integrins and focal adhesions. These forces are detectable as deformation of elastic substrates or bending of microscopic elastomer pillars. Mechanosensing allows cells to collect mechanical input from the environment and translate them into changes of cell behavior and require the generation of contractile forces for this sensing. Both mechanosensing and generation of traction forces are integral parts of migration that play critical roles. The signaling pathway for the generation and regulation of traction force is not well understood. Calpains have long been implicated in the regulation of cell migration. Calpain 4 was previously known as a regulatory subunit for calpain catalytic activity. However, previous studies in our lab implicated calpain 4 in the function of regulating traction forces, and doing so independent of catalytic activity. In this dissertation I have addressed two different questions of calpain 4 in terms of its function in the regulation of traction force: 1) Can individual domains of calpain 4 regulate traction force or sensing of external stimuli independently? 2) Can binding partners of calpain 4 be identified that function in this signaling pathway for the regulation of traction force?

In Chapter 2, I have successfully demonstrated that domains of calpain 4 function independently in regulating traction force and sensing the external stimuli. To understand the function of each domain in migration, each domain was cloned into a GFP plasmid

and expressed in MEF cells followed by a series of functional tests. The results indicate that overexpression of domain V, but not domain VI in *Capn4*$^{-/-}$ cells can rescue 1) the traction force defect, 2) reduction of migration rate, and 3) abnormal focal adhesion organization in *Capn4*$^{-/-}$ cells. At the same time, overexpressing domain VI, but not domain V in *Capn4*$^{-/-}$ cells restores the ability to sense mechanical stimuli and the proteolytic activity. All of these results suggest that the 2 critical mechanical aspects, traction force and mechanosensing are regulated independently through different domains of calpain 4, and that the function of regulating traction force occurs in a signaling pathway that does not require the protease activity of the calpain holoenzyme.

In Chapter 3, basigin was identified as a binding partner for calpain 4, and appears to work with calpain 4 in the regulation of traction force. Basigin was identified by a yeast two-hybrid assay in which full-length calpain 4 served as the bait. This interaction was confirmed through co-immunoprecipitation. Further functional assays indicated that silencing of basigin in MEFs resulted in both a reduction in the magtitude of traction force and defective adhesion strength. On the other hand, silencing of basigin in MEFs did not interrupt the sensing of external stimuli and homeostatic tension of the substrate. Taken together these results implicate basigin in the calpain 4 mediated pathway for regulating traction force, which is separate from the mechanosensing signaling parthway.

In conclusion, I have shown that calpain 4, previously known as a regulatory component for calpain catalytic subunits, also has a critical independent function in the regulation of traction forces. This function is performed only through domain V of

calpain 4 while domain VI regulates mechanosensing together with the calpain catalytic subunits. The protein basigin is also involved in this traction force pathway and positively regulates the generation of traction forces. Further investigation of this regulatory pathway for the production of traction force can greatly increase our understanding of the mechanical aspects of cell migration and further benefit multiple normal and abnormal physiological processes.

## REFERENCES

1. Alberts, B., A. Johnson, J. Lewis, M. Raff, K. Roberts, and P. Walter. 2002. Molecular Biology of the Cell, 4e. Garland Science.

2. Alexandrova, A.Y., K. Arnold, S. Schaub, J.M. Vasiliev, J.J. Meister, A.D. B ershadsky, and A.B. Verkhovsky. 2008. Comparative dynamics of retrograde actin flow and focal adhesions: formation of nascent adhesions triggers transition from fast to slow flow. *PLoS ONE* 3(9):e3234.

3. Ananthakrishnan, R., and A. Ehrlicher. 2007. The Forces Behind Cell Movement. *Int J Biol Sci*. 3(5):303-317.

4. Arthur, J.S., J.S. Elce, C. Hegadorn, K. Williams, and P.A. Greer. 2000. Disruption of the murine calpain small subunit gene, Capn4: calpain is essential for embryonic development but not for cell growth and division. *Mol Cell Biol*. 20:4474-4481.

5. Azatov, M., S.M. Goicoechea, C.A. Otey, and A. Upadhyaya. 2016. The actin crosslinking protein palladin modulates force generation and mechanosensitivity of tumor associated fibroblasts. *Sci Rep*. 29;6:28805.

6. Bai, DS, Z. Dai, J. Zhou, Y.K. Liu, S.J. Qiu, C.J. Tan, Y.H. Shi, C. Huang, Z. Wang, Y.F. He, and J. Fan. 2009. Capn4 overexpression underlies tumor invasion and metastasis after liver transplantation for hepatocellular carcinoma. *Hepatology.*49(2):460-70.

7. Bassuk A.G., S. Yeh, S. Wu, D.F. Martin, S.H. Tsang, L. Gakhar, and V.B. Mahajan. 2015. Structural modeling of a novel CAPN5 mutation that causes uveitis and

neovascular retinal detachment. *PLoS One*. 10(4):e0122352.

8. Beckerle M.C., K. Burridge, G.N. DeMartino, and D.E. Croall. 1987. Colocalization of calcium-dependent protease II and one of its substrates at sites of cell adhesion. *Cell*. 51(4):569-77.

9. Beningo, K.A., M. Dembo, I. Kaverina, J.V. Small, and Y.L. Wang. 2001. Nascent focal adhesions are responsible for the generation of strong propulsive forces in migrating fibroblasts. *J Cell Biol*. 153:881-888.

10. Beningo, K.A. and Y.L. Wang. 2002. Flexible substrata for the detection of traction forces. *Trends in Cell Biol*. 112(2), 79-84.

11. Berginski M.E., E.A. Vitriol, K.M. Hahn, and S.M. Gomez. 2011. High-resolution quantification of focal adhesion spatiotemporal dynamics in living cells. *PLoS One*. 6(7): e22025.

12. Bershadsky, A.D., N.Q. Balaban, and B. Geiger. 2003. Adhesion-dependent cell mechanosensitivity. *Annu Rev Cell Dev Biol*. 2003;19:677-95.

13. Bhatt, A., I. Kaverina, C. Otey, and A. Huttenlocher. 2002. Regulation of focal complex composition and disassembly by the calcium-dependent protease calpain. *J. Cell Sci*.115, 3415-3425.

14. Bialkowska, K, S. Kulkarni, X. Du, D.E. Goll, T.C. Saido, and J.E. Fox. 2000. Evidence that beta3 integrin-induced Rac activation involves the calpain-dependent formation of integrin clusters that are distinct from the focal complexes and focal adhesions that form as Rac and RhoA become active. *J Cell Biol*. 151(3):685-96.

15. Biswas, C, Y. Zhang, R. DeCastro, H. Guo, T. Nakamura, H. Kataoka, and K. Nabeshima. 1995. The human tumor cell-derived collagenase stimulatory factor (renamed EMMPRIN) is a member of the immunoglobulin superfamily. *Cancer Res*. 55(2):434-9.

16. Brami-Cherrier, K, N. Gervasi, D. Arsenieva, K. Walkiewicz, M.C. Boutterin, A. Ortega, P.G. Leonard, B. Seantier, L. Gasmi, T. Bouceba, G. Kadaré, J.A. Girault, and S.T. Arold. 2014. FAK dimerization controls its kinase-dependent functions at focal adhesions. *EMBO J.* 33(4):356-70.

17. Brandenburg, K, F. Harris, S. Dennison, U. Seydel, and D. Phoenix. 2002. Domain V of m-calpain shows the potential to form an oblique-orientated alpha-helix, which may modulate the enzyme's activity via interactions with anionic lipid. *Eur J Biochem*. 269(22): 5414-22.

18. Browning, M.B., V. Guiza, B.H. Russell, J.J. Rivera, S. Cereceres, M. Hook, M. Hahn, and E. Cosgriff-Hernandez. 2014. Endothelial cell response to chemical, biological, and physical cues in bioactive hydrogels. *Tissue Eng Part A.* 20(23-24):3130-41.

19. Burridge, K., and M. Chrzanowska-Wodnicka. 1996. Focal adhesions, contractility and signaling. *Annu Rev Cell Dev Biol*.12:463-519.

20. Burridge, K, and C. Guilluy. 2016. Focal adhesions, stress fibers and mechanical tension. *Exp Cell Res.* 343(1):14-20.

21. Carafoli, E., and M. Molinari. 1998. Calpain: a protease in search of a function. *Biochem Biophys Res Commun*. 247(2): 193-203.

22. Carragher, N.O., B. Levkau, R. Ross, and E.W. Raines. 1999. Degraded collagen fragments promote rapid disassembly of smooth muscle focal adhesions that correlates with cleavage of pp125(FAK), paxillin, and talin. *J. Cell Biol*. 147, 619-630.

23. Chang, S.S., W.H. Guo, Y. Kim, and Y.L. Wang. 2013. Guidance of cell migration by substrate dimemsion. *Biophys J*. 104(2): 313-21.

24. Chen, H., K.L. Fok, S. Yu, J. Jiang, Z. Chen, Y. Gui, Z. Cai, and H.C. Chan. 2011. CD147 is required for matrix metalloproteinases-2 production and germ cell migration during spermatogenesis. *Mol. Hum. Reprod.*, 17, 405–414.

25. Cho, K, M.H. Cho, J.H. Seo, J. Peak, K.H. Kong, S.Y. Yoon, and D.H. Kim. 2015. Calpain-mediated cleavage of DARPP-32 in Alzheimer's disease. *Aging Cell*. 14(5):878-86.

26. Choi, C.K., M. Vicente-Manzanares, J. Zareno, L.A. Whitmore, A. Mogilner, and A.R. Horwitz. 2008. Actin and alpha-actinin orchestrate the assembly and maturation of nascent adhesions in a myosin II motor-independent manner. *Nat Cell Biol.* 10(9):1039-50.

27. Chrzanowska-Wodnicka, M., and K. Burridge. 1996. Rho-stimulated contractility drives the formation of stress fibers and focal adhesions. *J Cell Biol.*133(6): 1403-15.

28. Ciobanasu, C., B. Faivre, and C. Le Clainche. 2013. Intergrating actin dynamics, mechanotransduction and integrin activation: the multiple functions of actinbinding proteins in focal adhesions. *Eur J Cell Biol.* 92(10-11):339-48.

29. Clark, E.A., and J.S. Brugge. 1995. Integrins and signal transduction pathways: the road taken. *Science*. 268(5208):233-9.

30. Condeelis, J. 1993. Life at the leading edge: the formation of cell protrusions. *Annu Rev Cell Biol*. 9:411-444.

31. Crawford, C., N.R. Brown, and A.C. Willis. 1990. Investigation of the structural basis of the interaction of calpain II with phospholipid and with carbohydrate. *Biochem J*. 265(2): 575-9.

32. Croce, K., R. Flaumenhaft, M. Rivers, B. Furie, B.C. Furie, I.M. Herman, and D.A. Potter. 1999. Inhibition of calpain blocks platelet secretion, aggregation, and spreading. *J. Biol. Chem*. 274, 36321-36327.

33. Crowley, E., and A.F Horwitz. 1995. Tyrosine phosphorylation and cytoskeletal tension regulate the release of fibroblast adhesions. *J. Cell Biol*., 131, 525–537.

34. Cuevas, B.D., A.N. Abell, J.A. Witowsky, T. Yujiri, N.L. Johnson, K. Kesavan, M. Ware, P.L. Jones, S.A. Weed, R.L. DeBiasi, Y. Oka, K.L. Tyler, and G.L. Johnson. 2003. MEKK1 regulates calpain-dependent proteolysis of focal adhesion proteins for rear-end detachment of migrating fibroblasts. *EMBO J*. 22:3346-3355.

35. Dai, Z., S.L. Zhou, Z.J. Zhou, D.S. Bai, X.Y. Xu, X.T. Fu, Q. Chen, Y.M. Zhao, K. Zhu, L. Yu, G.H. Yang, Z. Wang, W.Z. Wu, J. Zhou, and J. Fan. 2014. Capn4 contributes to tumor growth and metastasis of hepatocellular carcinoma by activation of FAK-Src signaling pathways. *J Pathol.* 234(3):316-28.

36. Daman, A, F. Harris, S. Biswas, J. Wallace, and D.A. Phoenix. 2001. A theoretical

investigation into the lipid interaction of m-calpain. *Mol Cell Biochem*. 223(1-2): 159-63.

37. Dedieu, S., G. Mazeres, S. Poussard, J.J. Brustis, and P. Cottin. 2003. Myoblast migration is prevented by a calpain-dependent accumulation of MARCKS. *Biol. Cell.* 95(9):615-623.

38. Dembo, M. and Y.L. Wang. 1999. Stresses at the cell-to-substrate interface during locomotion of fibroblasts. *Biophys J.* 76(4):2307-2316.

39. Diepenbroek, M., N. Casadei, H. Esmer, T.C. Saido, J. Takano, P.J. Kahle, R.A. Nixon, M.V. Rao, R. Melki, L. Pieri, S. Helling, K. Marcus, R. Krueger, E. Masliah, O. Riess, and S. Nuber. 2014. Overexpression of the calpain-specific inhibitor calpastatin reduces human alpha-Synuclein processing, aggregation and synaptic impairment in [A30P]αSyn transgenic mice. *Hum Mol Genet.* 23(15): 3975-89.

40. Discher, D.E., P. Janmey, and Y.L. Wang. 2005. Tissue cells feel and respond to the stiffness of their substrate. *Science* 310:1139–43.

41. Dourdin, N., A.K. Bhatt, P. Dutt, P. Greer, J. S. Arthur, J.S. Elce, and A. Huttenlocher. 2001. Reduced cell migration and disruption of the actin cytoskeleton in calpain deficient-embryonic fibroblasts. *J. Biol. Chem*. 276(51):48382-48388.

42. Dumbauld, D.W., H. Shin, N.D. Gallant, K.E. Michael, H. Radhakrishna, and A.J. García. 2010. Contractility modulates cell adhesion strengthening through focal adhesion kinase and assembly of vinculin-containing focal adhesions. *J Cell Physiol.* 223(3):746-56.

43. Engler, A.J., S. Sen, H.L. Sweeney, and D.E. Discher. 2006. Matrix elasticity directs stem cell lineage specification. *Cell.* 126(4): 677-89.

44. Fackler, O.T., and R. Grosse. 2008. Cell motility through plasma membrane blebbing. *J. Cell Biol*. 181:879–884.

45. Fang, K.P., J.L. Zhang, Y.H. Ren, and Y.B. Qian. 2014. Talin-1 correlates with reduced invasion and migration in human hepatocellular carcinoma cells. *Asian Pac J Cancer Prev*. 15(6):2655-61.

46. Fischer, I., G. Romano-Clarke, and F. Grynspan. 1991. Calpain-mediated proteolysis of microtubule associated proteins MAP1B and MAP2 in developing brain. *Neurochem Res.*16(8):891-8.

47. Flevaris, P., A. Stojanovic, H. Gong, A. Chishti, E. Welch, and X. Du. 2007. A molecular switch that controls cell spreading and retraction. *J. Cell Biol*. 179(3):553-565.

48. Fogh, B.S., H.A. Multhaupt, and J.R. Couchman. 2014. Protein kinase C, focal adhesions and the regulation of cell migration. *J Histochem Cytochem* 62(3):172-84.

49. Fouchard, J., C. Bimbard, N. Bufi, P. Durand-Smet, A. Proag, A. Richert, O. Cardoso, and A. Asnacios. 2014. Three-dimensional cell body shape dictates the onset of traction force generation and growth of focal adhesions. *Proc Natl Acad Sci USA*. 111(36):13075-80.

50. Franco, S.J., and A. Huttenlocher. 2005. Regulating cell migration: calpains make the cut. *J Cell Sci*. 118(Pt 17): 3829-38.

51. Franco, S., B. Perrin, and A. Huttenlocher. 2004a. Isoform specific function of calpain 2 in regulating membrane protrusion. *Exp. Cell. Res*. 299, 179-187.

52. Franco, S.J., M.A. Rodgers, B.J. Perrin, J. Han, D.A. Bennin, D.R. Critchley, and A. Huttenlocher. 2004b. Calpain-mediated proteolysis of talin regulates adhesion dynamics. *Nat. Cell Biol*. 6(10):977-983.

53. Frangioni, J.V., A. Oda, M. Smith, E.W. Salzman, and B.G. Neel. 1993. Calpain-catalyzed cleavage and subcellular relocation of protein phosphotyrosine phosphatase 1B (PTP-1B) in human platelets. *EMBO J.* 12(12):4843-56.

54. Friedl, P. 2004. Prespecification and plasticity: shifting mechanisms of cell migration. *Curr Opin Cell Biol*. 16:14-23.

55. Friedl, P., and S. Alexande. 2011. Cancer invasion and the microenvironment: plasticity and reciprocity. *Cell*. 147(5): 992-1009.

56. Friedl, P., and K. Wolf. 2010. Plasticity of cell migration: a multiscale tuning model. *J Cell Biol*. 188(1): 11–19.

57. Friedl, P., K. Wolf, and J. Lammerding. 2011. Nuclear mechanics during cell migration. *Curr Opin Cell Biol*. 23(1):55-64.

58. Gabison, E.E., T. Hoang-Xuan, A. Mauviel, and S. Menashi. 2005. EMMPRIN/CD147, an MMP modulator in cancer, development and tissue repair. *Biochimie.* 87:361–368.

59. Gadea, G., V. Sanz-Moreno, A. Self, A. Godi, and C.J. Marshall. 2008. DOCK10-mediated Cdc42 activation is necessary for amoeboid invasion of melanoma cells. *Curr Biol* 18:1456–1465.

60. Gardel, M.L., I.C. Schneider, Y. Aratyn-Schaus, and C.M. Waterman. 2010. Mechanical integration of actin and adhesion dynamics in cell migration. *Annu Rev Cell Dev Biol.* 26:315-33.

61. Gasiorowski, J.Z., C.J. Murphy, and P.F. Nealey. 2013. Biophysical cues and cell behavior: the big impact of little things. *Annu Rev Biomed Eng*. 15:155-76.

62. Gates, R. E. and L.E. King Jr. 1983. Proteolysis of the epidermal growth factor receptor by endogenous calcium-activated neutral protease from rat liver. *Biochem. Biophys. Res. Commun*. 113, 255-261.

63. Georges, P.C., and P.A. Janmey. 2005. Cell type-specific response to growth on soft materials. *J Appl Physiol (1985)*. 98(4):1547-53.

64. Gil-Parrado, S., O. Popp, T.A. Knoch, S. Zahler, F. Bestvater, M. Felgenträger, A. Holloschi, A. Fernández-Montalván, E.A. Auerswald, H. Fritz, P. Fuentes-Prior, W. Machleidt, and E. Spiess. 2003. Subcellular localization and in vivo subunit interactions of ubiquitous mu-calpain. *J Biol Chem*. 278(18):16336-46.

65. Glading, A., D.A. Lauffenburger, and A. Wells. 2002. Cutting to the chase: calpain proteases in cell motility. *Trends Cell Biol*. 12:46-54.

66. Goetz, J.G., B. Joshi, P. Lajoie, S.S. Strugnell, T. Scudamore, L.D. Kojic, and I.R. Nabi. 2008. Concerted regulation of focal adhesion dynamics by galectin-3 and tyrosine-phosphorylated caveolin-1. J Cell Biol. 180:1261-1275.

67. Gokhin, D.S., M.T. Tierney, Z. Sui, A. Sacco, and V.M. Fowler. 2014. Calpain-mediated proteolysis of tropomodulin isoforms leads to thin filament elongation in dystrophic skeletal muscle. *Mol Biol Cell*. 25(6):852-65.

68. Goldmann, W.H. 2014. Mechanosensation: a basic cellular process. *Prog Mol Biol Transl Sci*. 126:75-102.

69. Goll, D. E., V.F. Thompson, H. Li, W. Wei, and J. Cong, 2003. The calpain system. *Physiol. Rev*. 83, 731-801.

70. Goodarzi, M.O., K.D. Taylor, X. Guo, M.J. Quiñones, J. Cui, Y. Li, M.F. Saad, H. Yang, W.A. Hsueh, H.N. Hodis, and J.I. Rotter. 2005. Association of the diabetes gene calpain-10 with subclinical atherosclerosis: the Mexican-American Coronary Artery Disease Study. *Diabetes.* 54(4):1228-32.

71. Guilak, F., D.M. Cohen, B.T. Estes, J.M. Gimble, W. Liedtke, and C.S. Chen. 2009. Control of stem cell fate by physical interactions with the extracellular matrix. *Cell Stem Cell*. 5(1):17-26.

72. Guo, W.H., M.T. Frey, N.A. Burnham, and Y.L. Wang. 2006. Substrate rigidity regulates the formation and maintenance of tissues. *Biophys. J.* 90(6):2213-2220.

73. Gupta, M., B. Doss, C.T. Lim, R. Voituriez, and B. Ladoux. 2016. Single cell rigidity sensing: A complex relationship between focal adhesion dynamics and large-scale actin

cytoskeleton remodeling. *Cell Adh Migr*. 2;10(5):554-567.

74. Guyon, J.R., E. Kudryashova, A. Potts, I. Dalkilic, M.A. Brosius, T.G. Thompson, J.S. Beckmann, L.M. Kunkel, and M.J. Spencer. 2003. Calpain 3 cleaves filamin C and regulates its ability to interact with gamma- and delta-sarcoglycans. *Muscle Nerve.* 28(4):472-83.

75. Hopkinson, S.B., K.J. Hamill, Y. Wu, J.L. Eisenberg, S. Hiroyasu, and J.C. Jones. 2014. Focal Contact and Hemidesmosomal Proteins in Keratinocyte Migration and Wound Repair. *Adv Wound Care (New Rochelle)*. 3(3):247-263.

76. Hori, K., N. Kitayama, S. Kachi, M. Kondo, K. Kadomatsu, J. Usukura, T. Muramatsu, S. Mori, and Y. Miyake. 2000. Retinal dysfunction in basigin deficiency. *Invest. Ophthalmol*. *Vis. Sci*. 41, 3128-3133.

77. Horikawa, Y., N. Oda, N.J. Cox, X. Li, M. Orho-Melander, M. Hara, Y. Hinokio, T.H. Lindner, H. Mashima, P.E. Schwarz, L. del Bosque-Plata, Y. Horikawa, Y. Oda, I.Yoshiuchi, S. Colilla, K.S. Polonsky, S. Wei, P. Concannon, N. Iwasaki, J. Schulze, L.J.Baier, C. Bogardus, L. Groop, E. Boerwinkle, C.L. Hanis, and G.I. Bell. 2000. Genetic variation in the gene encoding calpain-10 is associated with type 2 diabetes mellitus, *Nat. Genet*. (26) 163–175.

78. Hotchin, N.A., and A. Hall. 1995. The assembly of integrin adhesion complexes requires both extracellular matrix and intracellular rho/rac GTPases. *J. Cell Biol.* 131:1857–65.

79. Hotulainen, P., and P. Lappalainen. 2006. Stress fibers are generated by two distinct actin assembly mechanisms in motile cells. *J Cell Biol*. 173(3): 383–394.

80. Hua, Y., and S. Nair. 2015. Proteases in cardiometabolic diseases: Pathophysiology, molecular mechanisms and clinical applications. *Biochim Biophys Acta.* 1852(2):195-208.

81. Huttenlocher, A., S.P. Palecek, Q. Lu, W. Zhang, R.L. Mellgren, D.A. Lauffenburger, M.H. Ginsberg, and A.F. Horwitz. 1997. Regulation of cell migration by the calcium-dependent protease calpain. *J. Biol. Chem*. 272, 32719-32722.

82. Hynes, R.O. 2002. Integrins: bidirectional, allosteric signaling machines. *Cell*. 110(6): 673-87.

83. Igakura, T., K. Kadomatsu, T. Kaname, H. Muramatsu, Q.W. Fan, T. Miyauchi, Y. Toyama, N. Kuno, S. Yuasa, M. Takahashi, T. Senda, O. Taguchi, K. Yamamura, K. Arimura, and T. Muramatsu. 1998. A null mutation in basigin, an immunoglobulin superfamily member, indicates its important roles in peri-implantation development and spermatogenesis. *Dev. Biol*. 194, 152-165.

84. Igakura, T., K. Kadomatsu, O. Taguchi, H. Muramatsu, T. Kaname, T. Miyauchi, K. Yamamura, K. Arimura, and T. Muramatsu. 1996. Roles of besigin, a member of the immunoglobulin superfamily, in behavior as to an irritating odor, lymphocyte response and bloodbrain barrier. *Biochem. Biophys. Res. Commun.* 224, 33-36.

85. Imajoh, S., H. Kawasaki, and K. Suzuki. 1986. The amino-terminal hydrophobic region of the small subunit of calcium-activated neutral protease (CANP) is essential for its activation by phosphatidylinositol. *J Biochem.* 99(4): 1281-4.

86. Jin, N., X. Yin, J. Gu, X. Zhang, J. Shi, W. Qian, Y. Ji, M. Cao, X. Gu, F. Ding, K. Iqbal, C.X. Gong, F. Liu. 2015. Truncation and Activation of Dual Specificity Tyrosine Phosphorylation-regulated Kinase 1A by Calpain I: A MOLECULAR MECHANISM LINKED TO TAU PATHOLOGY IN ALZHEIMER DISEASE. *J Biol Chem.* 290(24):15219-37.

87. Johnson, G.V., and R.P. Guttmann. 1997. Calpains: intact and active? Bioessays. 19(11): 1011-8.

88. Kambayashi, J., Y. Kajiwara, M. Sakon, T. Ohshiro, and T. Mori. 1986. Possible participation of calpain in myosin light chain phosphorylation of human platelets. *Biochem. Int.* 13, 571-578.

89. Kanekura, T., X. Chen, and T. Kanzaki. 2002. Basigin (CD147) is expressed on melanoma cells and induces tumor cell invasion by stimulating production of matrix metalloproteinases by fibroblasts, *Int. J. Cancer.* 99 (4):520–528.

90. Keller, R. 2005. Cell migration during gastrulation. *Curr Opin Cell Biol*. 17(5):533-41.

91. Kim, T. 2015. Determinants of contractile forces generated in disorganized actomyosin bundles. *Biomech Model Mechanobiol*. 14(2):345-55.

92. Krakhmal, N.V., M.V. Zavyalova, E.V. Denisov, S.V. Vtorushin, and V.M. Perelmuter. 2015. Cancer Invasion: Patterns and Mechanisms. *Acta Naturae*. 7(2):17-28.

93. Kshitiz, J. Park, P. Kim, W. Helen, A.J. Engler, A. Levchenko, and D.H. Kim. 2012. Control of stem cell fate and function by engineering physicalmicroenvironments. *Integr Biol (Camb)*. 4(9):1008-18.

94. Kudo-Sakamoto, Y., H. Akazawa, K. Ito, J. Takano, M. Yano, C. Yabumoto, A.T. Naito, T. Oka, J.K. Lee, Y. Sakata, J. Suzuki, T.C. Saido, and I. Komuro. 2014. Calpain-dependent cleavage of N-cadherin is involved in the progression of post-myocardial infarction remodeling. *J Biol Chem.* 9(28):19408-19.

95. Kulkarni, S., D.E. Goll, and J.E. Fox. 2002. Calpain cleaves RhoA generating a dominant-negative form that inhibits integrin-induced actin filament assembly and cell spreading. *J. Biol. Chem.* 277, 24435-24441.

96. Lämmermann, T., B.L. Bader, S.J. Monkley, T. Worbs, R. Wedlich-Söldner, K. Hirsch, M. Keller, R. Förster, D.R. Critchley, R. Fässler, and M.Sixt. 2008. Rapid leukocyte migration by integrin-independent flowing and squeezing. *Nature*. 453:51–55.

97. Lauffenburger, D.A., and A.F. Horwitz. 1996. Cell migration: a physically integrated molecular process. *Cell*. 84(3):359-69.

98. Laukaitis, C.M., D.J. Webb, K. Donais, and A.F. Horwitz. 2001. Differential dynamics of alpha 5 integrin, paxillin, and alpha-actinin during formation and disassembly of adhesions in migrating cells. *J Cell Biol*. 153(7):1427-40.

99. Lazzara, C.A., R.R. Riley, A. Rane, J.K. Andersen, and Y.H. Kim. 2015. The combination of lithium and l-Dopa/Carbidopa reduces MPTP-induced abnormal involuntary movements (AIMs) via calpain-1 inhibition in a mouse model: Relevance for Parkinson′s diseasetherapy. *Brain Res*. 5;1622:127-36.

100. Li, F.Z., P.C. Cai, L.J. Song, L.L. Zhou, Q. Zhang, S.S. Rao, Y. Xia, F. Xiang, J.B. Xin, P.A. Greer, H.Z. Shi, Y. Su, W.L. Ma, and H. Ye. 2015. Crosstalk between calpain activation and TGF-β1 augments collagen-I synthesis in pulmonary fibrosis. *Biochim Biophys Acta*. 1852(9):1796-804.

101. Li, Y., J. Wu, F. Song, J. Tang, S.J. Wang, X.L. Yu, Z.N. Chen, and J.L. Jiang. 2012. Extracellular membrane-proximal domain of HAb18G/CD147 binds to metal ion-dependent adhesion site (MIDAS) motif of integrin β1 to modulate malignant properties of hepatoma cells. *J Biol Chem*. 287(7):4759-72.

102. Liedtke, W., and C. Kim. 2005. Functionality of the TRPV subfamily of TRP ion channels: add mechano-TRP and osmo-TRP to the lexicon. *Cell Mol Life Sci*. 62(24): 2985-3001.

103. Litersky, J.M., and G.V. Johnson. 1992. Phosphorylation by cAMP-dependent protein kinase inhibits the degradation of tau by calpain. *J Biol Chem*. 267(3):1563-8.

104. Liu, F., L. Cui, Y. Zhang, L. Chen, Y. Wang, Y. Fan, T. Lei, F. Gu, R. Lang, G.A. Pringle, X. Zhang, Z. Chen, and L. Fu. 2010. Expression of HAb18G is associated with tumor progression and prognosis of breast carcinoma. Breast Cancer Res Treat 124(3):677–688.

105. Lo, C.M., H.B. Wang, M. Dembo, and Y.L. Wang. 2000 Cell movement is guided by the rigidity of the substrate. *Biophys J.* 79:144-152.

106. Loie, E., L.E. Charrier, K. Sollier, J.Y. Masson, and P. Laprise. 2015. CRB3A Controls the Morphology and Cohesion of Cancer Cells through Ehm2/p114RhoGEF-Dependent Signaling. *Mol Cell Biol*. Oct;35(19):3423-35.

107. Lokuta, M.A., P.A. Nuzzi, and A. Huttenlocher. 2003. Calpain regulates neutrophil chemotaxis. *Proc. Natl. Acad. Sci. USA* 100(7):4006-4011.

108. Maki, M., Y. Maemoto, Y. Osako, and H. Shibata. 2012. Evolutionary and physical linkage between calpains and penta-EF-hand $Ca^{2+}$ proteins. *FEBS J.* 279(8):1414-21.

109. Mamoune, A., J.H. Luo, D.A. Lauffenburger, and A. Wells. 2003. Calpain-2 as a target for limiting prostate cancer invasion. *Cancer Res.* 63(15): 4632-40.

110. Mannowetz, N., P. Wandernoth, and G. Wennemuth. 2012. Basigin interacts with both MCT1 and MCT2 in murine spermatozoa. *J Cell Physiol*. 227(5):2154-62.

111. Marganski, W.A., M. Dembo, and Y.L. Wang. 2003. Measurements of cell-generated deformations on flexible substrata using correlation-based optical flow. *Meth. Enzymol*. 361, 197-121.

112. Menon, S. 2012. Biophysical and biochemical factors in the cellular microenvironment; effects on cell migration and invasion. Wayne State University Dissertations. Paper 576.

113. Menon, S., and K.A. Beningo. 2011. Cancer cell invasion is enhanced by applied mechanical stimulation. *PLoS One*. 6(2):e17277.

114. Menon, S., C.M. Kang, and K.A. Beningo. 2011. Galectin-3 secretion and tyrosine phosphorylation is dependent on the calpain small subunit, Calpain 4. *Biochem Biophys Res Commun.* 410(1):91-6.

115. Mogilner, A., and G. Oster. 2003. Cell biology. Shrinking gels pull cells. *Science*. 302(5649):1340-1.

116. Mohammadi, H., P.A. Janmey, and C.A. McCulloch. 2014. Lateral boundary mechanosensing by adherent cells in a collagen gel system. *Biomaterials*. 35(4):1138-49.

117. Moore, S.W., and M.P. Sheetz. 2011. Biophysics of substrate interaction: influence on neural motility, differentiation, and repair. *Dev Neurobiol.* 71(11):1090-101.

118. Mui, K.L., C.S. Chen, and R.K. Assoian. 2016. The mechanical regulation of integrin-cadherin crosstalk organizes cells, signaling and forces. *J Cell Sci*. 15;129(6):1093-100.

119. Müller, A., B. Homey, H. Soto, N. Ge, D. Catron, M.E. Buchanan, T. McClanahan, E. Murphy, W. Yuan, S.N. Wagner, J.L. Barrera, A. Mohar, E. Verástegui, and A. Zlotnik. 2001. Involvement of chemokine receptors in breast cancer metastasis. *Nature.* 410(6824):50-6.

120. Muramatsu, T., and T. Miyauchi. 2003. Basigin (CD147): a multifunctional transmembrane protein involved in reproduction, neural function, inflammation and tumor invasion. *Histol Histopathol*. 18(3):981-7.

121. Myers, K.A., K.T. Applegate, G. Danuser, R.S. Fischer, and C.M. Waterman. 2011. Distinct ECM mechanosensing pathways regulate microtubule dynamics to control endothelial cell branching morphogenesis. *J Cell Biol.* 192(2): 321–334.

122. Na, S., O. Collin, F. Chowdhury, B. Tay, M.X. Ouyang, Y.X. Wang, and N. Wang. 2008. Rapid signal transduction in living cells is a unique feature of mechanotransduction, *Proc. Natl. Acad. Sci. U. S. A*. 105(18):6626-31.

123. Nakahara, S., and A. Raz. 2006. On the role of galectins in signal transduction. *Methods Enzymol*. 417:273-289.

124. Nassar, D., E. Letavernier, L. Baud, S. Aractingi, and K. Khosrotehrani. 2012. Calpain activity is essential in skin wound healing and contributes to scar formation. *PLoS One*. 7(5):e37084.

125. Oda, A., B.J. Druker, H. Ariyoshi, M. Smith, and E.W.Salzman. 1993. pp60src is an endogenous substrate for calpain in human blood platelets. *J. Biol. Chem*. 268, 12603-12608.

126. Ono, Y., H. Sorimachi. 2011. Calpains: an elaborate proteolytic system. *Biochim Biophys Acta*. 1824(1):224-36.

127. Palecek, S.P., J.C. Loftus, M.H. Ginsberg, D.A. Lauffenburger, and A.F. Horwitz. 1997. Integrin–ligand binding properties govern cell migration speed through cell-substratum adhesiveness. *Nature.*385:537–540.

128. Panková, K., D. Rösel, M. Novotný, and J. Brábek. 2010. The molecular mechanisms of transition between mesenchymal and amoeboid invasiveness in tumor cells. *Cell Mol Life Sci*. 67(1):63-71.

129. Papusheva, E. and C.P. Heisenberg. 2010. Spatial organization of adhesion: force-dependent regulation and function in tissue morphogenesis. *EMBO J*. 29(16): 2753–2768.

130. Parent, C.A., and P.N. Devreotes. 1999. A cell's sense of direction. *Science*. 284(5415):765-70.

131. Partha, S.K., R. Ravulapalli, J.S. Allingham, R.L. Campbell, and P.L. Davies. 2014. Crystal structure of calpain-3 penta-EF-hand (PEF) domain - a homodimerized PEF family member with calcium bound at the fifth EF-hand. *FEBS J.* 281(14):3138-49.

132. Pasapera, A.M., I.C. Schneider, E. Rericha, D.D. Schlaepfer, and C.M. Waterman. 2010. Myosin II activity regulates vinculin recruitment to focal adhesions through FAK-mediated paxillin phosphorylation. J Cell Biol. 188(6): 877–890.

133. Pasapera, A.M., S.V. Plotnikov, R.S. Fischer, L.B. Case, T.T. Egelhoff, C.M. Waterman. 2015. Rac1-dependent phosphorylation and focal adhesion recruitment of myosin IIA regulates migration and mechanosensing. *Curr Biol*. 25(2):175-86.

134. Paulhe, F., A. Bogyo, H. Chap, B. Perret, and C. Racaud-Sultan. 2001. Vascular smooth muscle cell spreading onto fibrinogen is regulated by calpains and phospholipase C. *Biochem. Biophys. Res. Commun*. 288, 875-881.

135. Pelham, R.J., Jr., and Y. Wang. 1997. Cell locomotion and focal adhesions are regulated by substrate flexibility. *Proc Natl Acad Sci U S A*. 94:13661-13665.

136. Perrin, B.J., K.J. Amann, and A. Huttenlocher. 2006. Proteolysis of cortactin by calpain regulates membrane protrusion during cell migration. *Mol Biol Cell*. 17(1):239-50.

137. Pfaff, M., X. Du, and M.H. Ginsberg. 1999. Calpain cleavage of integrin beta cytoplasmic domains. *FEBS Lett*. 460 (1), 17–22.

138. Plotnikov, S.V., A.M. Pasapera, B. Sabass, and C.M. Waterman. 2012. Force Fluctuations within Focal Adhesions Mediate ECM-Rigidity Sensing to Guide Directed Cell Migration. *Cell*. 151(7): 1513-27.

139. Potter, D. A., J.S. Tirnauer, R. Janssen, D.E. Croall, C.N. Hughes, K.A. Fiacco, J.W. Mier, M. Maki, and I.M. Herman. 1998. Calpain regulates actin remodeling during cell spreading. *J. Cell Biol.* 141, 647-662.

140. Potts, A.J., D.E. Croall, and M.E. Hemler. 1994. Proteolytic cleavage of the integrin beta 4 subunit. *Exp Cell Res*. 212(1):2-9.

141. Pushkarsky, T., V. Yurchenko, A. Laborico, and M. Bukrinsky. 2007. CD147 stimulates HIV-1 infection in a signal-independent fashion. *Biochem Biophys Res Commun.* 363(3):495-9.

142. Pushkarsky, T., G. Zybarth, L. Dubrovsky, V. Yurchenko, H. Tang, H. Guo, B. Toole, B. Sherry, and M. Bukrinsky. 2001. CD147 facilitates HIV-1 infection by interacting with virus-associated cyclophilin A, *Proc. Natl. Acad. Sci USA*. 98(11): 6360–

6365.

143. Raimbourg, Q., J. Perez, S. Vandermeersch, A. Prignon, G. Hanouna, J.P. Haymann, L. Baud, and E. Letavernier. 2013. The calpain/calpastatin system has opposing roles in growth and metastatic dissemination of melanoma. *PLoS One.* 8(4):e60469.

144. Ridley, A.J., M.A. Schwartz, K. Burridge, R.A. Firtel, M.H. Ginsberg, G. Borisy, J.T. Parsons, and A.R. Horwitz. 2003. Cell migration: integrating signals from front to back. *Science*. 302 (5651): 1704-9.

145. Rios-Doria, J., R. Kuefer, S.P. Ethier, and M.L. Day. 2004. Cleavage of beta-catenin by calpain in prostate and mammary tumor cells. *Cancer Res*.64, 7237-7240.

146. Rosenberger, G., A. Gal, and K. Kutsche. 2005. AlphaPIX associates with calpain 4, the small subunit of calpain, and has a dual role in integrin-mediated cell spreading. *J Biol Chem*. 280:6879-6889.

147. Sahai, E., and C.J. Marshall. 2003. Differing modes of tumour cell invasion have distinct requirements for Rho/ROCK signaling and extracellular proteolysis. *Nat Cell Biol.* 5:711–719.

148. Saido, T.C., K. Mizuno, and K. Suzuki. 1991. Proteolysis of protein kinase C by calpain: effect of acidic phospholipids. *Biomed Biochim Acta*.50(4-6):485-9.

149. Samantaray, S., V.H. Knaryan, D.C. Shields, A.A. Cox, A. Haque, and N.L. Banik. 2015. Inhibition of Calpain Activation Protects MPTP-Induced Nigral and Spinal Cord Neurodegeneration, Reduces Inflammation, and Improves Gait Dynamics in Mice. *Mol*

*Neurobiol*. 52(2):1054-66.

150. Sato, K., and S. Kawashima. 2001. Calpain function in the modulation of signal transduction molecules. *Biol Chem*. 382(5): 743-51.

151. Saxena, D.K., T. Oh-Oka, K. Kadomatsu, T. Muramatsu, and K. Toshimori. 2002. Behaviour of a sperm surface transmembrane glycoprotein basigin during epididymal maturation and its role in fertilization in mice. *Reproduction* 123, 435-444.

152. Schmidt, C.E., A.F. Horwitz, D.A. Lauffenburger, and M.P. Sheetz. 1993. Integrin–cytoskeletal interactions in migrating fibroblasts are dynamic, asymmetric, and regulated. *J. Cell Biol*. 123, 977–991.

153. Schoenwaelder, S.M., and K. Burridge. 1999. Bidirectional signaling between the cytoskeleton and integrins. *Curr. Opin. Cell Biol*. 11: 274–286.

154. Selliah, N., W.H. Brooks, and T.L. Roszman. 1996. Proteolytic cleavage of alpha-actinin by calpain in T cells stimulated with anti-CD3 monoclonal antibody. *J Immunol*. 156(9):3215-21.

155. Serrano, K., and D.V. Devine. 2004. Vinculin is proteolyzed by calpain during platelet aggregation: 95 kDa cleavage fragment associates with the platelet cytoskeleton. *Cell Motil. Cytoskeleton* 58, 242-252.

156. Shi, M.L., M.H. Cao, J. Song, Q.H. Liu, H.L. Li, F. Meng, Z.Q. Pan, J. Bai, and J.N. Zheng. 2015. PinX1 inhibits the invasion and metastasis of human breast cancer via suppressing NF-κB/MMP-9 signaling pathway. *Mol Cancer*. 14:66.

157. Sorimachi, H., H. Mamitsuka, and Y. Ono. 2012. Understanding the substrate specificity of conventional calpains. *Biol Chem*. 393(9):853-71.

158. Stenzinger, A., D. Wittschieber, M.von Winterfeld, B. Goeppert, C. Kamphues, W. Weichert, M. Dietel, A. Rabien, and F. Klauschen. 2012. High extracellular matrix metalloproteinase inducer/CD147 expression is strongly and independently associated with poor prognosis in colorectal cancer. *Hum Pathol*. 43(9):1471–1481.

159. Stricker, J., Y. Aratyn-Schaus, P.W. Oakes, and M.L. Gardel. 2011. Spatiotemporal constraints on the force-dependent growth of focal adhesions. *Biophys J.* 100(12):2883-93.

160. Sullivan, S.J., G. Daukas, and S.H. Zigmond. 1984. Asymmetric distribution of the chemotactic peptide receptor on polymorphonuclear leukocytes. *J. Cell Biol*. 99, 1461–1467.

161. Sun, J., and M.E. Hemler. 2001. Regulation of MMP-1 and MMP-2 production through CD147/extracellular matrix metalloproteinaseinducer interactions. *Cancer Res*. 61(5):2276-81.

162. Suzuki, K., S. Hata, Y. Kawabata, and H. Sorimachi. 2004. Structure, activation, and biology of calpain. *Diabetes*. 53 Suppl 1:S12-8.

163. Suzuki, R., T. Oka, Y. Tamada, T.R. Shearer, and M. Azuma. 2014. Degeneration and dysfunction of retinal neurons in acute ocular hypertensive rats: involvement of calpains. *J Ocul Pharmacol Ther*. 30(5):419-28.

164. Tompa, P., P. Buzder-Lantos, A. Tantos, A. Farkas, A. Szilagyi, Z. Banoczi, F. Hudecz, and P. Friedrich. 2004. On the sequential determinants of calpain cleavage. *J. Biol. Chem*. 279, 20775-20785.

165. Trepat, X., L. Deng, S.S. An, D. Navajas, D.J. Tschumperlin, W.T. Gerthoffer, J.P. Butler, and J.J. Fredberg. 2007. Universal physical responses to stretch in the living cell. *Nature* 447:592–95.

166. Undyala, V., M. Dembo, K. Cembrola, B.J. Perrin, A. Huttenlocher, J.S. Elce, P.A. Greer, Y.L. Wang, and K.A. Beningo. 2008. The calpain small subunit regulates cell-substrate mechanical interactions during fibroblast migration. *J Cell Sci*. 121(Pt 21): 3581–3588.

167. Vogel, V. 2006. Mechanotransduction involving multimodular proteins: converting force into biochemical signals. *Annu Rev Biophys Biomol Struct.* 35:459-88.

168. Walters, N.J., and E. Gentleman. 2015. Evolving insights in cell-matrix interactions: elucidating how non-soluble properties of the extracellular niche direct stem cell fate. *Acta Biomater*. 11:3-16.

169. Wanaguru, M, W. Liu, B.H. Hahn, J.C. Rayner, and G.J. Wright. 2013 RH5-Basigin interaction plays a major role in the host tropism of Plasmodium falciparum. *Proc Natl Acad Sci U S A*. 110(51):20735-40.

170. Wang, Y.L. 2009. Traction forces and rigidity sensing of adherent cells. *Conf Proc IEEE Eng Med Biol Soc*. 2009:3339-40.

171. Wang, Y., L. Yuan, X.M. Yang, D. Wei, B. Wang, X.X. Sun, F. Feng, G. Nan, Y. Wang, Z.N. Chen, and H. Bian. 2015. A chimeric antibody targeting CD147 inhibits hepatocellular carcinoma cell motility via FAK-PI3K-Akt-Girdin signaling pathway. *Clin Exp Metastasis*. 32(1):39-53.

172. Watanabe, A., M. Yoneda, F. Ikeda, Y. Terao-Muto, H. Sato, and C. Kai. 2010. CD147/EMMPRIN acts as a functional entry receptor for measles virus on epithelialcells. *J. Virol*. 84(9): 4183–4193.

173. Webb, D.J., K. Donais, L.A. Whitmore, S.M. Thomas, C.E. Turner, J.T. Parsons, and A.F. Horwitz. 2004. FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. *Nat Cell Biol*. 6(2):154-61.

174. Wert, K.J., A.G. Bassuk, W.H. Wu, L. Gakhar, D. Coglan, M. Mahajan, S. Wu, J. Yang, C.S. Lin, S.H. Tsang, and V.B. Mahajan. 2015. CAPN5 mutation in hereditary uveitis: the R243L mutation increases calpaincatalytic activity and triggers intraocular inflammation in a mouse model. *Hum Mol Genet.* 24(16):4584-98.

175. Whelan, D., N.M. Caplice, and A.J. Clover. 2014. Fibrin as a delivery system in wound healing tissue engineering applications. *J Control Release*. 196:1-8.

176. Wolf, K., M. Te Lindert, M. Krause, S. Alexander, J. Te Riet, A.L. Willis, R.M. Hoffman, C.G. Figdor, S.J. Weiss, and P. Friedl. 2013. Physical limits of cell migration: Control by ECM space and nuclear deformation and tuning by proteolysis and traction force. *J Cell Biol*. 201(7): 1069–1084.

177. Wu, C.Y., S.B. Asokan, M.E. Berginski, E.M. Haynes, N.E. Sharpless, J.D. Griffith,

S.M. Gomez, and J.E. Bear. 2012. Arp2/3 complex is critical for lamellipodia and organization of cell-matrix adhesion but dispensable for fibroblast chemotaxis. *Cell.* 148(5): 973–987.

178. Xiong, L., C.K. 3rd Edwards, and L. Zhou. 2014. The Biological Function and Clinical Utilization of CD147 in Human Diseases: A Review of the Current Scientific Literature. *Int J Mol Sci.* 15(10):17411-41.

179. Xu, L., and X. Deng. 2006. Suppression of cancer cell migration and invasion by protein phosphatase 2A through dephosphorylation of mu- and m-calpains. *J Biol Chem*. 281(46):35567-75.

180. Yamada, M., Y. Yoshida, D. Mori, T. Takitoh, M. Kengaku, H. Umeshima, K. Takao, T. Miyakawa, M. Sato, H. Sorimachi, A. Wynshaw-Boris, and S. Hirotsune. 2009. Inhibition of calpain increases LIS1 expression and partially rescues in vivo phenotypes in a mouse model of lissencephaly. *Nat Med.* 15(10):1202-7.

181. Yan, B., D.A. Calderwood, B. Yaspan, and M.H. Ginsberg. 2001. Calpain cleavage promotes talin binding to the beta 3 integrin cytoplasmic domain. J Biol Chem. 276(30):28164-70.

182. Yang, M., Y. Yuan, H. Zhang, M. Yan, S. Wang, F. Feng, P. Ji, Y. Li, B. Li, G. Gao, J. Zhao, and L. Wang. 2013. Prognostic significance of CD147 in patients with glioblastoma. *J. Neurooncol*., 115, 19–26.

183. Yao, X., A. Thibodeau, and J.G. Forte. 1993. Ezrin-calpain I interactions in gastric parietal cells. *Am J Physiol.* 265(1 Pt 1):C36-46.

184. Yoshizawa, T., H. Sorimachi, S. Tomioka, S. Ishiura, and K. Suzuki. 1995. A catalytic subunit of calpain possesses full proteolytic activity, *FEBS Lett*. 358(1):101-3.

185. Zaidel-Bar, R., C. Ballestrem, Z. Kam, and B. Geiger. 2003. Early molecular events in the assembly of matrix adhesions at the leading edge of migrating cells. *J Cell Sci.* 116(Pt 22):4605-13.

186. Zaidel-Bar, R., S. Itzkovitz, A. Ma'ayan, R. Iyengar, and B. Geiger. 2007. Functional atlas of the integrin adhesome. *Nat Cell Biol*. 9:858-867.

187. Zheng, P.C., X. Chen, H.W. Zhu, W. Zheng, L.H. Mao, C. Lin, J.N. Liu, and M. Zheng. 2014. Capn4 is a marker of poor clinical outcomes and promotes nasopharyngeal carcinoma metastasis via nuclear factor-κB-induced matrix metalloproteinase 2 expression. *Cancer Sci*. 105(6):630-8.

188. Zucker, S., M. Hymowitz, E.E. Rollo, R. Mann, C.E. Conner, J. Cao, H.D. Foda, D.C. Tompkins, and B.P. Toole. 2001. Tumorigenic potential of extracellular matrix metalloproteinase inducer. *Am. J. Pathol*. 158:1921–1928.

# ABSTRACT

## IDENTIFYING THE FUNCTION OF THE CALPAIN SMALL-SUBUNIT IN THE MECHANICS OF CELL MIGRATION

by

**BINGQING HAO**

**May 2017**

**Advisor:** Dr. Karen A. Beningo

**Major:** Biological Sciences

**Degree:** Doctor of Philosophy

Cell migration plays a vital role in many physiological events including: morphogenesis, wound healing, and immune response. Dysfunctional cell migration results in multiple disease states including chronic inflammation, vascular disease, and tumor metastasis, to name a few. Progress in understanding the mechanism of cell migration had been slow until the turn of the century when rapid technological advances in microscopy and omics burst to the forefront. These advances led to the realization that physical factors (dimensions, fluid shear stress, hydrostatic pressure, compression stress, environmental stiffness, and topography) have profound effects on cell migration. This study of cell mechanics has expanded extensively in the past 20 years as with the application of multidisciplinary approaches in nanotechnology, biophysics, and modern cell biology.

Given the importance of focal adhesion dynamics in migration and mechanics, we focused on the function of calpain proteases on cell migration. Previously we discovered

that when compared to wildtype MEFs, *Capn4*$^{-/-}$ cells displayed reduced traction force and this was not observed when the large catalytic subunits were silenced respectively or when the endogenous inhibitor calpastatin was overexpressed. In comparison, mechanosensing of localized tension was defunct in cells lacking the large subunits, or calpain 4, or when the holoenzyme activity was inhibited by calpastatin. These results together formed our conclusion that the regulatory small subunit calpain 4 must modulate the production of traction forces independent of the catalytic activity of the calpain holoenzymes, but function together to regulate the mechanosensing of localized tension.

In gaining understanding of the mechanics of traction force and mechanosensing of cell migration, we asked how calpain 4 protein regulates traction force. By overexpressing each domain in *Capn4*$^{-/-}$ cells, we have found that only the overexpression of domain V in *Capn4*$^{-/-}$ cells rescues the traction force defect, the reduced migration rate, and the abnormal focal adhesion organization. However, only the overexpression of domain VI in *Capn4*$^{-/-}$ cells restores both the ability to sense mechanical stimuli and the proteolytic activity. These results suggest that domains of calpain 4 function independently in regulating the traction force and sensing the external stimuli. We also asked what other players also function in regulating traction force through calpain 4. We performed a yeast two-hybrid assay and identified basigin to be one of the binding proteins. Further results indicated that inhibition of basigin in MEFs resulted in reduced level of traction force and defective adhesion strength without interfering with the sensing of external stimuli and homeostatic tension of the substrate.

Together these results further elucidate the mechanism of cell migration and interplay of traction force and mechanosensing, and establish calpain 4 to be a critical player in the regulation of traction force. Further investigation into this signaling pathway will greatly expand our scope of the mechanical aspects of cell migration and further benefit cell migration related diseases studies.

# AUTOBIOGRAPHICAL STATEMENT

## BINGQING HAO

### Educational Qualification:

2007-2017: Doctor of Philosophy, Wayne State University, Detroit, MI, USA

2003-2006: Master of Science (Biology), Sichuan University, Chengdu, China

1999-2003: Bachelor of Science (biotechnology), Sichuan University, Chengdu, China

### Honors and Awards:

Dissertation Fellowship, the Graduate School, Wayne State University, summer 2014

Graduate Research Assistant Recognition Award, Wayne State University, 2011

Outstanding Graduate Student Award, Sichuan University, 2006

### Publications:

Bingqing Hao, and Karen Beningo. Traction force and mechanosensing can be functionally distinguished through the use of specific domains of the calpan small subunit. Submmited.

Bingqing Hao, Li Wang, Linchun Mu, Weikai Bao et al. 2006. A study of conservation genetics in Cupressus chengiana S.Y.Hu, an endangered endemic of China, using ISSR markers. Biochemical Genetics Vol. 44: 31-45.

Linchun Mu, Li Wang, Li Yao, Bingqing Hao, and Qin Luo. 2005. The application of petG-trnP sequence to the systematic study of Chinese Cupressus species. Journal of Sichuan University (Natural Science Edition), Vol. 42, No.5, 2005: 1033-1037.

### Poster Presentations:

Bingqing Hao, and Karen A. Beningo, "Specific Domains of the Calpain Small Subunit Regulate Either Traction Force or the Response to External Mechanical Stimuli", 2013, ASCB annual conference

Bingqing Hao, and Karen A. Beningo, "A Direct Interaction between the Calpain Regulatory Subunit and an Inducer of Matrix-Metalloproteinases; A Potential Role in Cellular Traction Forces", 2011, ASCB annual conference