Regulation of Traction Force through the Direct Binding of Basigin and Calpain 4

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ABSTRACT

Traction force and mechanosensing (the ability to sense mechanical attributes of the environment) are two important factors used by a cell to modify behavior during migration. Previously it was determined that the calpain small subunit, calpain 4, regulates the production of traction force independent of its proteolytic holoenzyme. A proteolytic enzyme is formed by calpain4 binding to either of its catalytic partners, calpain 1 and 2. To further understand how calpain 4 regulates traction force, we used two-hybrid analysis to identify more components of the traction pathway. We discovered that basigin, an integral membrane protein and a documented matrix-metalloprotease (MMP) inducer binds to calpain 4 in two-hybrid and pull-down assays. Traction force was deficient when basigin was silenced in MEF cells, and defective in substrate adhesion strength. Consistent with Capn4$^{-/-}$ MEF cells, the cells deficient in basigin responded to localized stimuli. Together these results implicate basigin in the pathway in which calpain 4 regulates traction force 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 significant to technological applications such as tissue engineering (Lauffenburger and Horwitz, 1996, Friedl and Alexander, 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 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 inactivate μ-calpain and m-calpain and result 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 of 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 partners 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 have 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 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 (directly or indirectly) with a number of 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 its known interacting proteins are implicated

in cell migration. Basigin's functions in tumorigenesis and 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, MEFs in which basigin is silenced are able to respond to localized stimuli and also sense the stiffness of substrates normally, suggesting a function of basigin only in the production of traction forces but not in mechanosensing. 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 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. Mouse embryonic fiborolasts (MEFs), *Capn4*$^{-/-}$ cells and 293T cells were used in this study. MEFs were purchased from ATCC. 293T cells were a gift from Dr. Xiangdong Zhang, University of Buffalo. 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% CO2 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 ended by adding complete media. 293T cells were maintained and split similarly with 1% Pen/Strep (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 pCWX200 and pLexA 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 provided to ProteinLinks Inc. (Pasadena, CA) for yeast two-hybrid assay. The candidates obtained via yeast two-hybrid assay were identified by DNA sequencing (Applied Genomics Technology Center, Wayne State University).

Small Interfering RNA (siRNA) Nucleofection

Wild-type MEFs were used for selectively silencing *Capn4* via siRNA. The knock-down was generated through transient transfection with either control siRNA oligonucleotides or siRNA oligonucleotides targeting *Bsg* gene using the siGENOME SMARTpool system (Dhamacon). The siRNA oligonucleotides targeting *Bsg* gene were: GAUUGGUUCUGGUUUAAGA, CAUCAGCAACCUUGACGUA,GCAAGUCCGAUGCAUCCUA,GGACAAGAAUGUACGC CAG. Nucleofection was performed using the Amaxa MEF2 Nucleofector Kit (Lonza) following

the manufacturer's suggested protocol. Briefly, MEF 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 addition of up to 5 ug of control siRNA or siRNA targeting *Bsg* gene. The total volume of the MEF 2 Nucleofector solution and supplement 1 mixture and the siRNA 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 cuvette instantly after the running the program to avoid the damage to cells. Nucleofected cells were then seeded according to the requirements of the following procedures. Inhibition of basigin expression reaches maximum at 36 hrs after nucleofection.

Protein Extraction and Western Blotting

Proteins were extracted from each cell line with triple detergent lysis buffer (TDLB): pH8 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 (Thermofisher) 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 into 1.5 ml tubes by an ice-cold cell lifter and centrifuged at 13,000 rpm for 10 min to get rid of cell debris. To be stocked, proteins were flash frozen with liquid N_2 and stored in -80°C. Protein concentration was measured by Lowry high 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 was 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 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 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-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 for 3 times, the membrane was detected 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 resulting from 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 4 times with 1X lysis buffer. After final wash, the pellet was resuspended in 40 ul 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 75 μm x 22 mm polyacrylamide substrate was made in a cell culture chamber dish 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. 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% Acryl/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. 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, 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 onto 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 applied by the needle 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, 6-10 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 5 $\mu g/cm^2$ 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 into 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 $\mu g/cm^2$ 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 1800 g. Ten random fields of cells were counted for each cell line right away after centrifugation. Percentages of cells after centrifugation over before are expressed in column graphs.

Immunofluorescence

After being flamed, No. 1.5 glass coverslips (Fisher) were attached to chamber dishes. Then they were coated with 5 $\mu g/cm^2$ fibronectin (Sigma) at $4^{\circ}C$ overnight, and blocked with 1% BSA in PBS at $4^{\circ}C$ overnight. Cells were seeded onto the coverslips and allowed to attach overnight under incubation at 37 ˚C with 5% CO2 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 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 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 GFP signals.

Cell Migration Assay

After being flamed, No. 1.5 square glass coverslips (Fisher) were attached onto chamber dishes and 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% CO2 in a humidified cell culture incubator. Images were taken to describe the migration trajectory 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 further elucidate the mechanism utilized by calpain4 to regulate the production of traction force, the *Capn4* gene was inserted into the plasmids of pCWX200 and pLexA and the whole gene was used as the bait to identify binding partners for calpain 4 protein by yeast two-hybrid. Sequencing identified basigin as one candidate binding partner for calpain 4. This direct binding between calpain 4 and basigin was confirmed by co-immunoprecipitation (Fig. 1 *A*). The direct binding between calpain 4 and basigin suggests the possibility that basigin is involved in the pathway of the generation of traction forces regulated by calpain 4.

To observe the expression pattern of the basigin protein and look for co-localization with calpain4, immunofluorescence was performed in both MEFs and *Capn4-/-* cells with basigin antibody. Basigin localized predominantly pericellular and diffuse at the cell edge (Fig. 1 *B*). Basigin failed to locate to the periphery of *Capn4-/-* cells. However, it is very likely that this results from the thinness of the lamellipodia in *Capn4-/-* cells described previously (Undyala et al., 2008). When comparing the expression level of basigin in both MEFs and *Capn4-/-* cells, we observed that basigin was expressed at a reduced level in *Capn4-/-* cells compared to MEFs (Fig. 1 *C*), as supported by quantification of fluorescent signal strength in immunostaining (Fig. 2 *B*). Combined we find evidence of direct interaction of basigin with calpain4 via two-hybrid and co-immunoprecipitation and some correlation between expression patterns and signal strength in immunofluorescence.

Knockdown of Basigin Resulted in Defects in Traction Force and Adhesion Strength in MEFs

To study further address the function of basigin in cell migration we used siRNA to knockdown expression of basigin in mouse embryonic fibroblasts (MEFs). Nucleofection resulted

in a high efficiency of inhibition which reached 95% at 36 hours after nucleofection as demonstrated by western blots (Fig. 2 *A*). Thus the following assays were conducted at 36 hours after nucleofection accordingly. The quantification of the strength of immunostaining signal with basigin antibody indicated a 82% reduction in intensity ($p<0.005$) when basigin was silenced through siRNA in MEFs, as well as in *Capn4*$^{-/-}$ cells showing a 49% reduction in signal intensity ($p=0.01$) (Fig. 2 *B*).

Previous studies in cell migration revealed a deficiency in traction forces in *Capn4*$^{-/-}$ cells compared to wild-type MEF cells, while inhibition of *Capn1* or *Capn2* or the overexpression of calpastatin did not affect traction forces (Undyala et al., 2008). We tested if basigin is involved in the production of traction force using basigin knock down MEFs, MEFs, and *Capn4*$^{-/-}$ control cells. Cells were seeded on flexible polyacrylamide substrate covalently coated with fibronectin and subjected to analysis by traction force microscopy (TFM) (Dembo & Wang, 1999). Vector maps are used to indicate the strength of traction forces in each cell line illustrated by the length and density of vectors (Fig. 3 *A*). As expected, *Capn4*$^{-/-}$ cells produced significantly reduced magnitude of traction forces (avg. 1.99 kPa) compared to wild-type MEFs (avg. 2.69kPa, $p=0.03$) and MEFs transfected with control siRNA (avg. 2.91kPa, $p=0.04$). Furthermore, silencing basigin in MEFs also significantly reduced the magnitude of traction forces to 1.93 kPa ($p=0.04$) (Fig 3. *B*). These results suggest that silencing basigin leads to deficient traction force production and is consistent with the traction data obtained from the disruption of calpain 4.

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 (Guo et al., 2006; Undyala et al., 2008). Cells were seeded onto fibronectin coated flexible acrylamide substrate mounted onto chamber dishes and allowed to adhere for 30 minutes at 37°C. Then 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. We discovered that approximately 61% of *Capn4*$^{-/-}$ cells remained

adhered to the substrates after centrifugation compared to 98% of MEFs that remained adhered (Fig. 3 *C*). Similarly, silencing basigin resulted in only approximately 63% of cells remaining adhered on the substrates (Fig. 3 *C*). In comparison, 95% percent of MEFs treated with control siRNA remained adhered after centrifugation (Fig. 3 *C*). These results suggest that basigin contributes to adhesion strength of focal adhesions in addition to regulating traction force production and these observations align with the behavior of the *Capn4-/-*.

Neither Local Stimulation or Homeostatic Tension are affected by Knockdown of Basigin

Cells are able to sense mechanical information from the environment including matrix elasticity, localized mechanical forces, 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 outside-in 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). Previous study where cells were tested for their ability to sense localized stimulus found that MEFs respond by changing their migratory trajectory or rounding up, and Calpain 1, 2 or 4 deficient cells continue to migrate along the same trajectory meaning they are insensitive to localized stimulus (Undyala et al., 2008). In another assay to compare how well cells spread on substrates of different stiffness, 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 mechanosensing assays.

To test whether basigin plays a role in mechanosensing, knockdown cells were tested for their ability to respond to localized mechanical stimuli. In the assay, cells were seeded onto flexible polyacrylamide substrates coated with fibronectin and a blunted needle was gently pushed onto the substrate against the direction the cell was migrating toward. As expected, 87.5% of MEF cells responded to the pushing force by avoiding it while only 14.3% of *Capn4*$^{-/-}$ cells responded (Fig. 4 *A, B*). Similarly as MEF cells, 83.3% of MEF cells transfected with control siRNA reacted to the localized pushing force as MEF cells (Fig. 4 *A, B*). When basigin was silenced in MEFs, 83.3% of cells still responded to the localized pushing force (Fig. 4 *A, B*). Our results indicate that basigin does not play a role in sensing the localized mechanical stimulus.

To test whether basigin is implicated in sensing the stiffness of subtrates, 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 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 also spread normally (Fig. 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 substrates (Fig. 4 *C, D*). The significant decrease of the number of cells spreading normally on subtrates of different stiffness indicated that basigin was not implicated in sensing the stiffness of substrates. Together with the results with from a localized stimuli, it is suggested that basigin is not involved in the mechanosensing process in terms of both localized stimuli and substrate stiffness.

Knockdown of Basigin Results in Reduced Cell Migration Speed without Affecting Migration Persistence

The speed and persistence of cell migration are affected by multiple factors, including dimension, matrix stiffness, cell–cell and cell–matrix adhesion, traction forces, cytoskeletal polarity, and the

capacity to degrade ECM by proteolytic enzymes (Plotnikov et al., 2012; Wolf et al., 2013). Previous observation indicates that *Capn4*$^{-/-}$ cells have reduced migration speed compared to MEF cells (Dourdin et al., 2001; Undyala et al., 2008). To learn whether inhibition of basigin also results in the same effect on cell migration, basigin knockdown cells 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 they also migrated significantly lower than control MEFs (0.49 μm/min, p=0.02) at a similar speed as *Capn4*$^{-/-}$ cells (p=0.65) (Fig. 5, *A*). Although migration speed was significantly affected by silencing basigin, migration persistence was similar in all lines (Fig. 5, *B*). These results suggest that this pathway of calpain 4, which also involves basigin, not only regulates the generation of tractions, but also affects the linear migration speed.

DISCUSSION

Our lab has previously discovered that Calpains participate in both production of traction force 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 independent of the proteolytic large subunits of calpain and the holoenzymes in the regulation of traction forces while all subunits are implicated in mechanosensing. In this study we have looked for insight into how this mechanism might work by looking for direct binding partners of Calpain4.

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In this study we have discovered a previously unknown physical interaction between Basigin and Calpain4 as determined by two-hybrid and co-immunoprecipitation. We also looked for co-localization by immunofluorescence. On one hand we were surprised to find a lack of co-localization between basigin and calpain4 via conventional immunofluorescnce (Fig. 1 *B*). On the other, Calpain4 localization is diffuse on its own. What was worth noting was the diminished signals for basigin locating to the periphery of *Capn4-/-* cells. As suggested this could result from the thinness of lamellipodia at the cell periphery. In addition, the high expression level and multi functions of basigin in varied types of cells could explain the lack of localization of basigin to focal adhesions in our study (Chen et al., 2011; Hori et al., 2000; Igakura et al., 1996; Igakura et al., 1998; Saxena et al., 2002).

Studies of basigin's function in cell migration focuses on its function in tumor cell motility and invasion. It has been reported that basigin expression level is commonly 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 level of basigin expression on the surface of tumor cells induces increased level of MMP activity in both of stromal cells and tumor cells themselves (Gabison et al., 2005; Sun and Hemler; 2001; Zucker et al., 2001). Elevated MMP activity then degrades ECM and changes ECM turn-over dynamics, potentially leading to tumor cell motility and invasion (Xiong et al., 2014). Consistent with these studies, our current study found that inhibiting basigin expression through siRNA in wild-type MEFs results in reduced traction force and adhesion strength (Fig. 3 *B*, *C*) and decreased migration speed (Fig. 5 *A*), suggesting that calpain 4 functions as a positive regulator for basigin in this pathway. However, basigin affects 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 pathway of traction force regulation. Further studies are needed to identify what components function further downstream of this signaling pathway.

Previous research indicates deficient traction force in *Capn4-/-* cells (Fig. 3 *B*). Further, these *Capn4-/-* cells respond to localized stimuli and sense the stiffness of substrates normally (Fig. 4 *B*,

D), suggesting that calpain 4 has provided a means to separate traction force and mechanosensing spatially and temporally. Moreover, MEFs in which basigin is silenced are able to respond to the localized stimuli and also sense the stiffness of substrates normally (Fig. 4 *B*, *D*), suggesting a function of basigin only in the production of traction forces but not in mechanosensing. Previous studies suggest substrate rigidity sensing is driven by traction forces in the cells leading edge since localized softening of the frontal region results in retraction of the cell, 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 have identified a direct binding partner for Calpain4 in Basigin. Further we have determined that Basigin and Calpain4 contributes to the regulation of traction force and the strength of substrate adhesion. However, Basigin and Calpain4 are not implicated in mechanosensing and do not respond to localized stimuli and homeostatic tension. Together, these results implicate basigin in an unknown pathway in which calpain 4 is involved in the regulation of traction force independent of the Calpain 1 and 2 catalytic large subunits.

Acknowledgements: The authors would like to acknowledge Dr. Alexander Gasparski for writing and technical assistance. Portions of this work were developed from the dissertations of Dr. Bingqing Hao. Funding for this project was initially provided to KAB through NIH R01-GM084248 and start-up funds from Wayne State University.

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Figure Legend

FIGURE 1. Co-immunoprecipitation to confirm the direct binding between calpain4 and basigin proteins and the expression pattern of basigin protein. *A*. Full gene of *Capn4* was inserted into a pFLAG-CMV vector and the *Bsg* gene lacking the sequence for the N-terminal 100 a.a. was inserted into a pCDNA3 vector together with a HA sequence. Both plasmids were transfected into 293T cells. Pull-down assays were performed with either FLAG or HA antibody to confirm the direct binding between calpain 4 and basigin. *B*. Localization of basigin in MEFs and *Capn4-/-* cells. Cells were seeded onto fibronectin coated coverslips, allowed to attach overnight and treated by immunostaining. *C*. Basigin expression level is reduced in *Capn4-/-* cells compared to control MEF cells. Two bands of 50 and 37 kDa were stained by targeting basigin in western blots. Both bands showed reduced level of expression when calpain4 was interrupted. Actinin was used as the loading control (Mag. bar = 20μm).

FIGURE 2. Silencing of basigin through siRNA reduced basigin expression effectively. *A.* Cell extracts were made 36 hours 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 fluorescence (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.

FIGURE 3. Silencing basigin through siRNA resulted in defects in traction force production and adhesion strength in MEFs. *A.* Cells were seeded onto flexible polyacrylamide substrates covalently coated with fibronectin and allowed to attach overnight followed by traction force microscopy (TFM). Images were taken for the fluorescent microbeads that were embedded in substrates with or without the attached cell on the substrate generating traction forces onto the

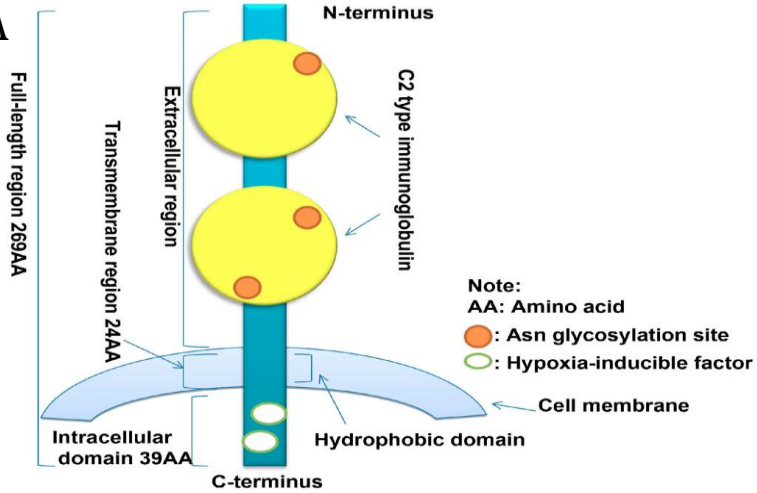
substrate. The information of magnitude of bead displacements and the cell and nuclear boundaries were collected and used to calculate traction stress values by using a custom made algorithm. A vector map was generated to indicate the magnitude and direction of traction stress exerted by a single cell. In the vector map (*A*. right), red and pink colors represent the areas of stronger traction stress, and blue and gray colors represent the areas of weaker traction stress (Mag. bar = 20μm). *B.* 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. When basigin was silenced by siRNA, traction stress level was significantly reduced as well as in *Capn4*$^{-/-}$ cells, compared to MEFs and MEFs transfected with control siRNA. 14 MEFs, 22 *Capn4*$^{-/-}$ cells, 21 MEFs transfected with control siRNA, and 21 basigin knock-down MEFs were used for traction force calculation. *C.* 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 through siRNA in MEFs, a reduction of adhesion strength was also observed (p=0.02). Statistical analysis was performed by student's *t* test.

FIGURE 4. Silencing basigin through siRNA does not affect the ability of MEFs to sense the localized pushing force and 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 original cell migration direction; the bold arrows in the second column denote the direction the blunted needle is pushed toward. Cells were seeded onto flexible polyacrylamide substrates that were covalently coated with fibronectin and allowed to attach overnight. A blunted needle 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

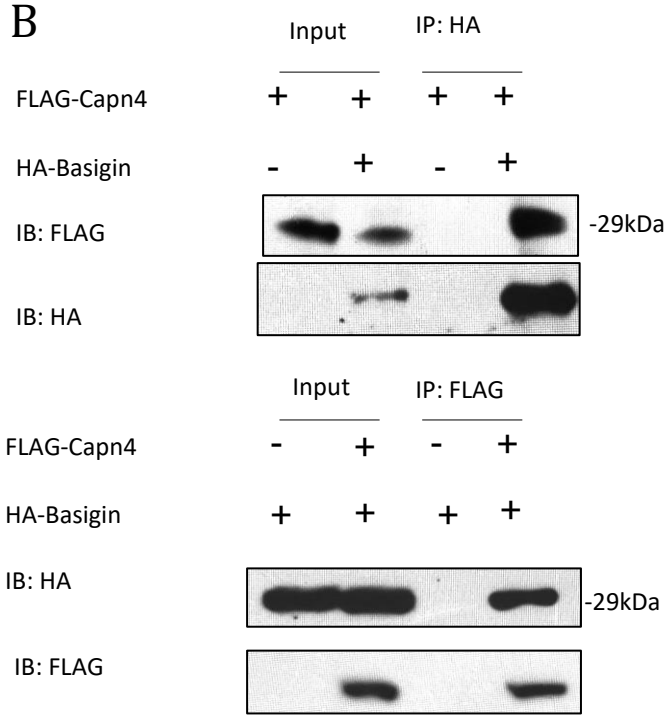
bar graph indicates the percentage of cells of each cell line that respond to the localized pushing force applied by a blunted needle. The number of cells for each cell line and cells' responses were summarized in the table. If a cell migrates by avoiding the pushing force, it is marked with "+", denoting a positive reaction; if a cell continues to migrate toward the pushing force, it is marked with "-", denoting a negative reaction. *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 $p<0.05$.

FIGURE 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 same 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.

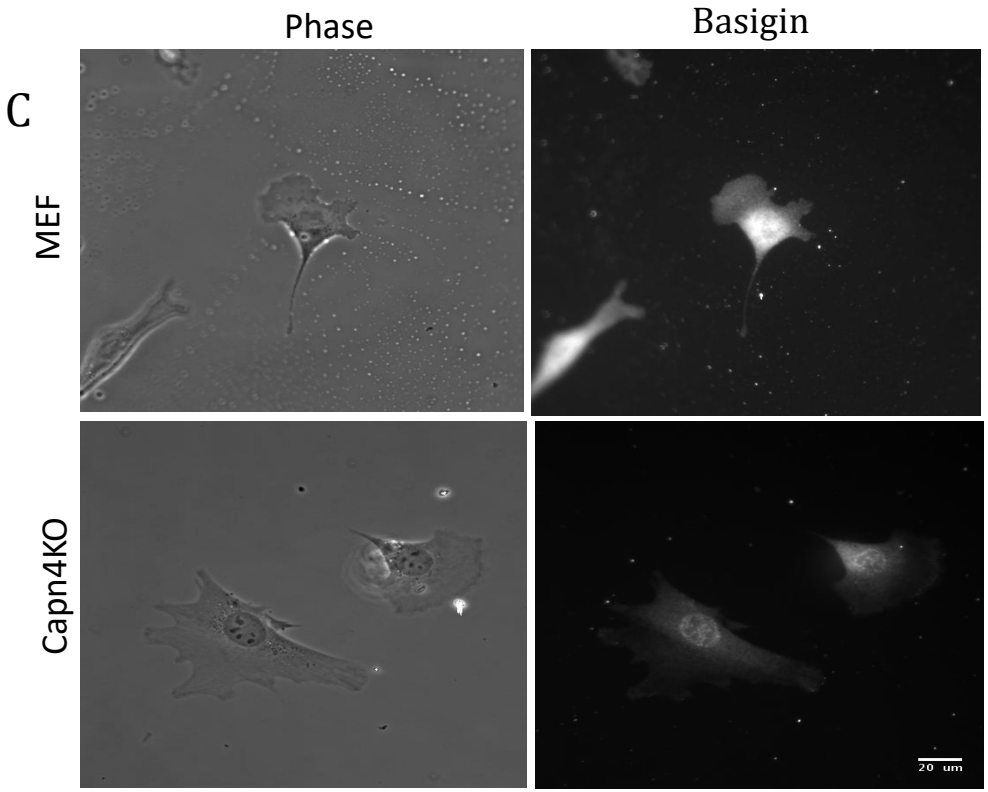
A



B



C



D

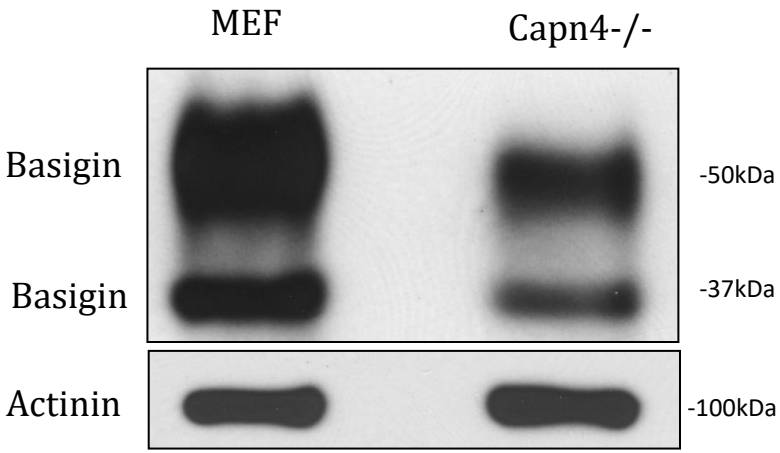
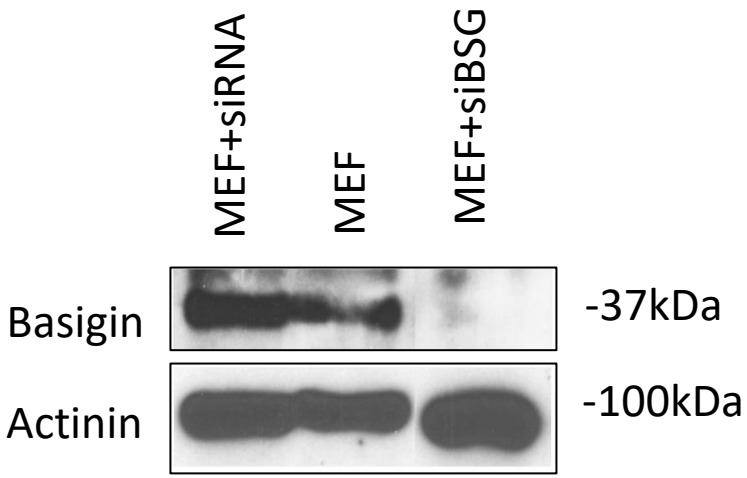


Figure 1.

Figure 2.

A


MEF+siRNA
MEF
MEF+siBSG
Basigin
Actinin
-37kDa
-100kDa

B

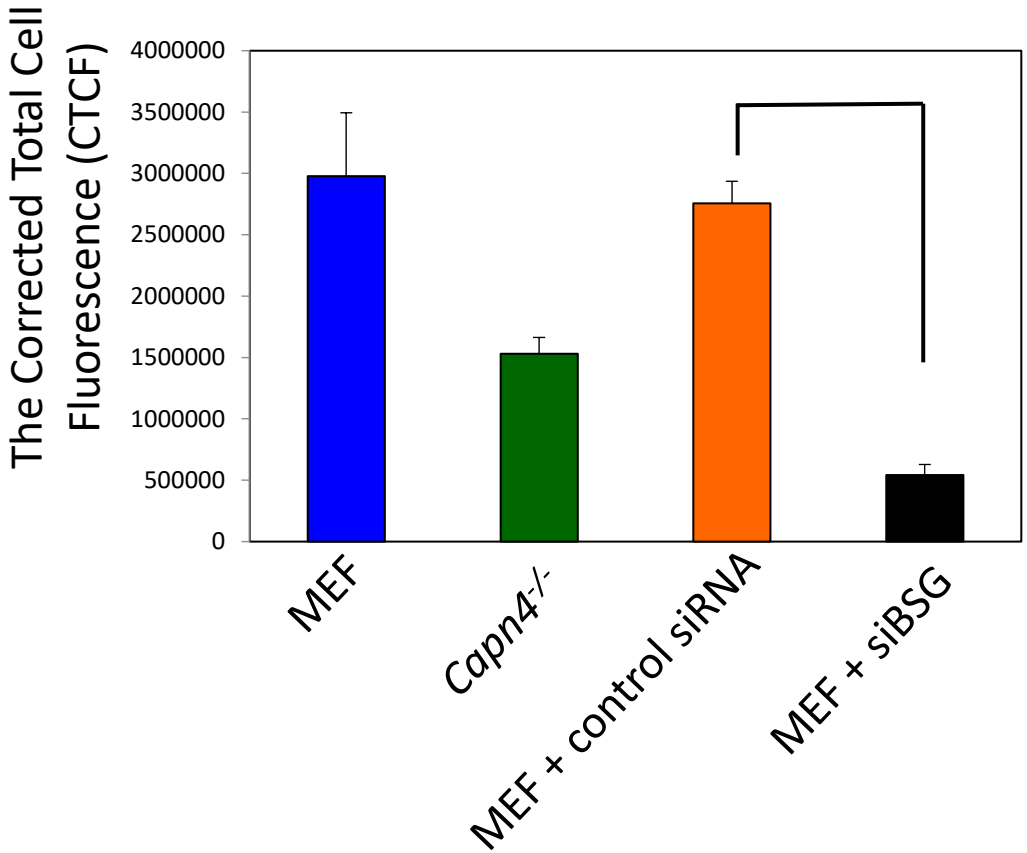
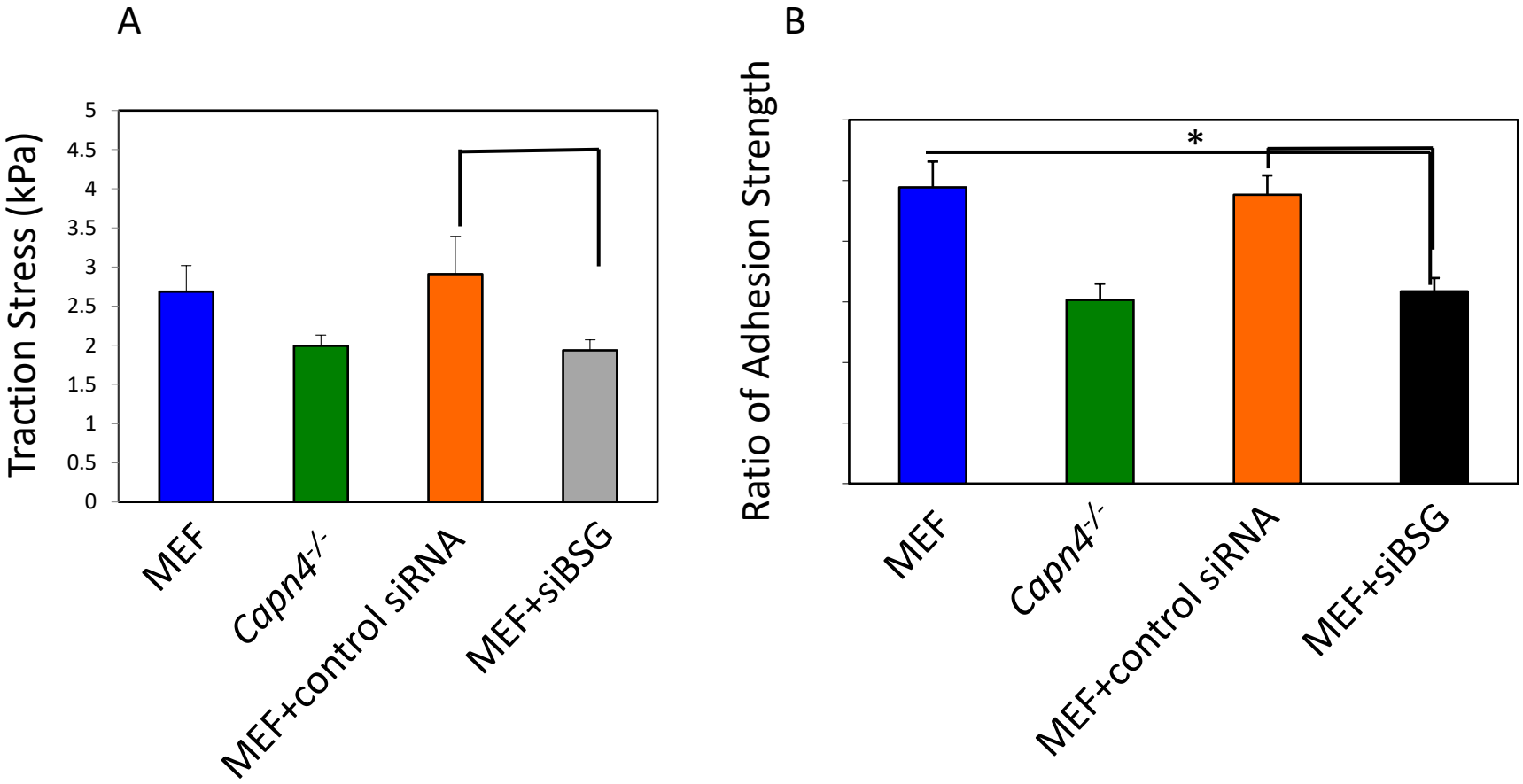
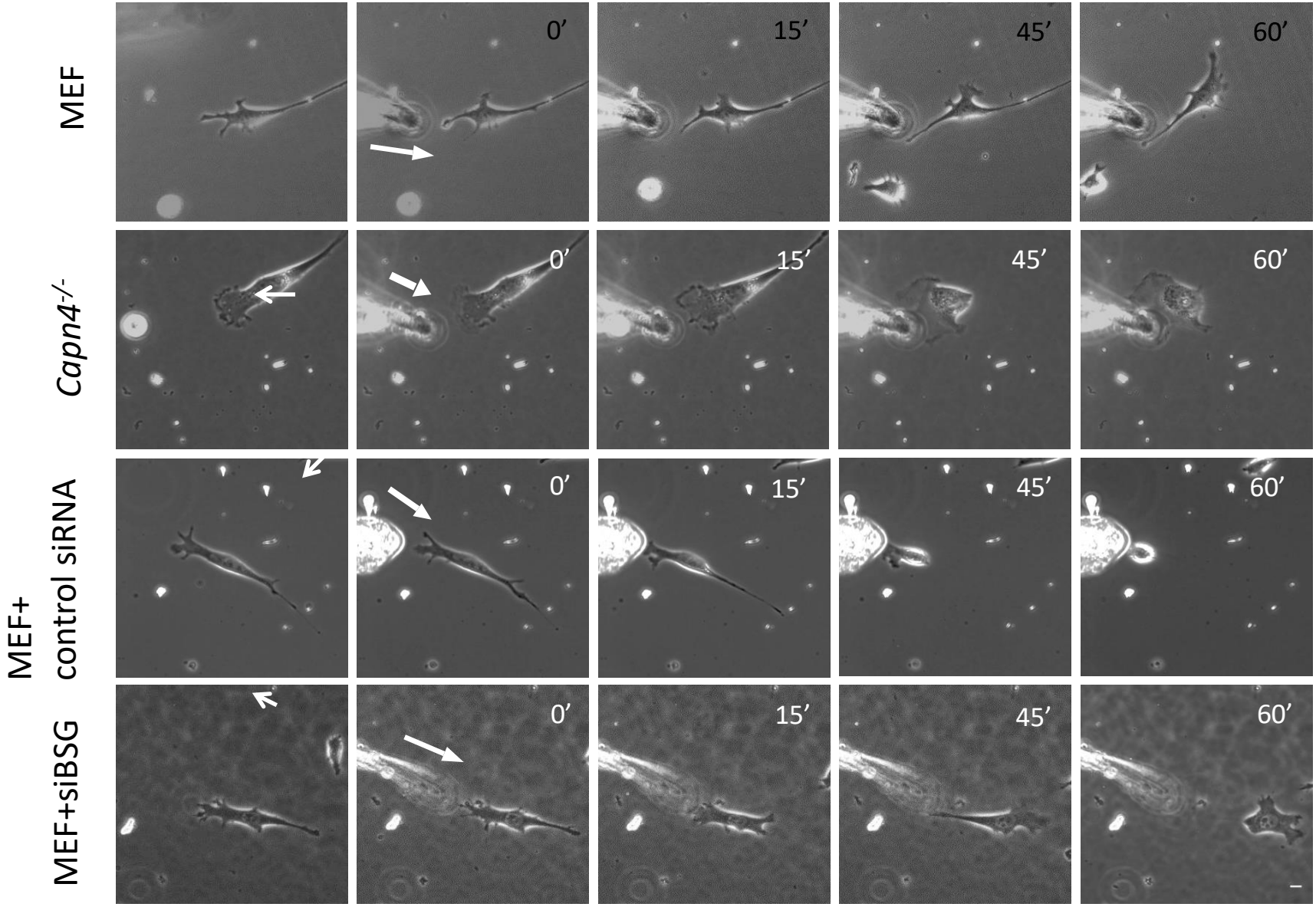

The Corrected Total Cell Fluorescence (CTCF)
4000000
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
MEF
Capn4-/-
MEF + control siRNA
MEF + siBSG

Figure 3.


A
Traction Stress (kPa)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
MEF
Capn4-/-
MEF+control siRNA
MEF+siBSG
B
Ratio of Adhesion Strength
*
MEF
Capn4-/-
MEF+control siRNA
MEF+siBSG

A

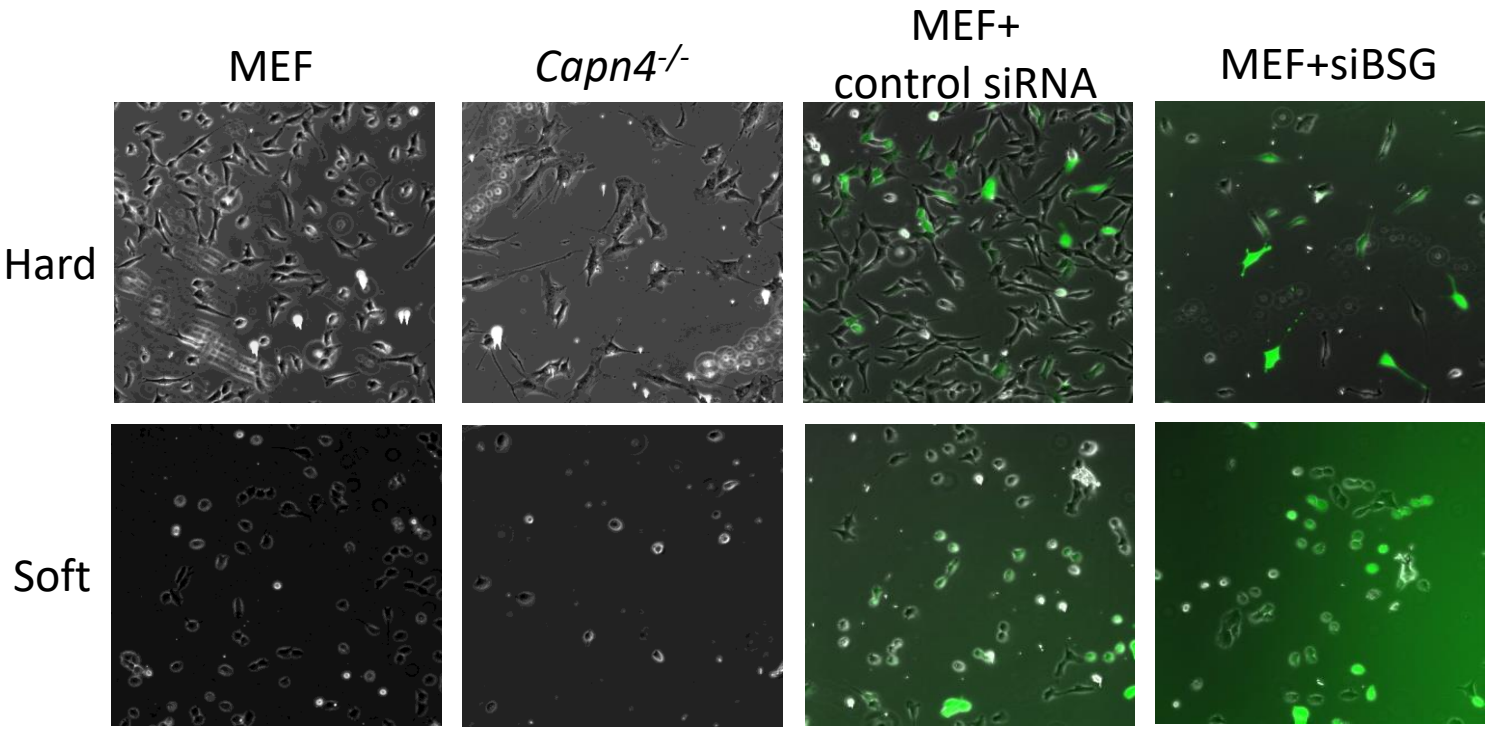


B

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 4.

C


MEF
Capn4-/-
MEF+
control siRNA
MEF+siBSG
Hard
Soft

D

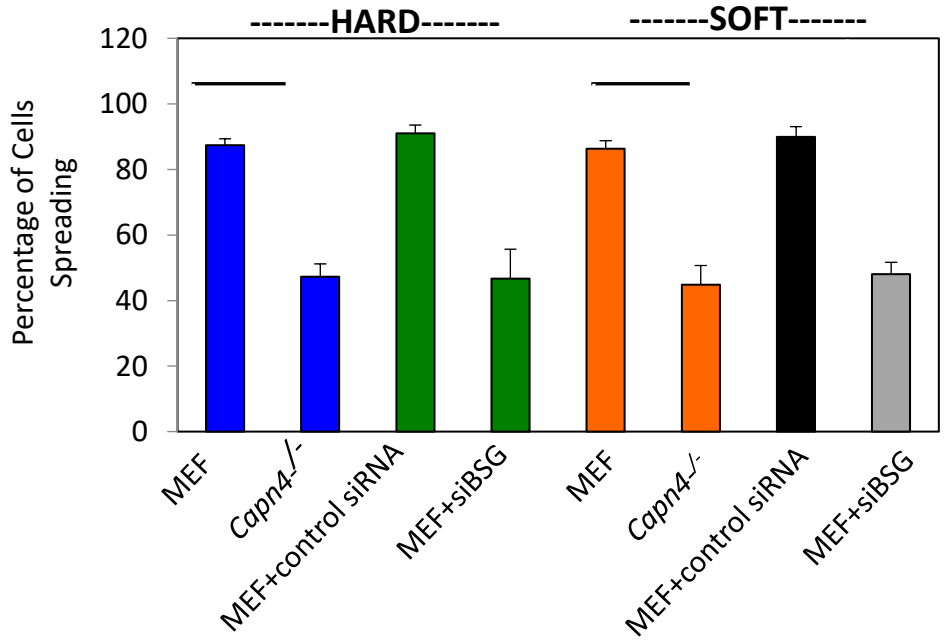
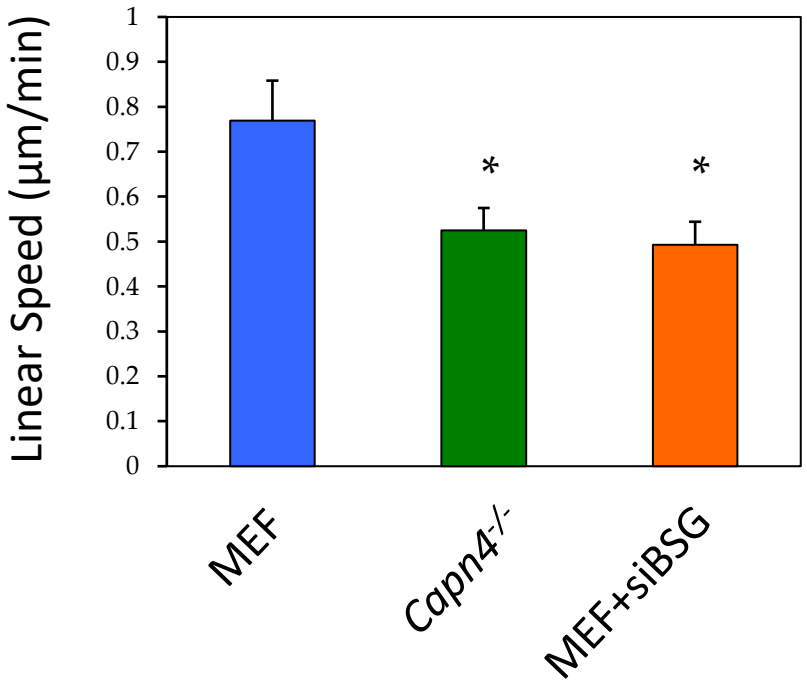

-------HARD-------
-------SOFT-------
Percentage of Cells Spreading
120
100
80
60
40
20
0
MEF
Capn4-/-
MEF+control siRNA
MEF+siBSG
MEF
Capn4-/-
MEF+control siRNA
MEF+siBSG

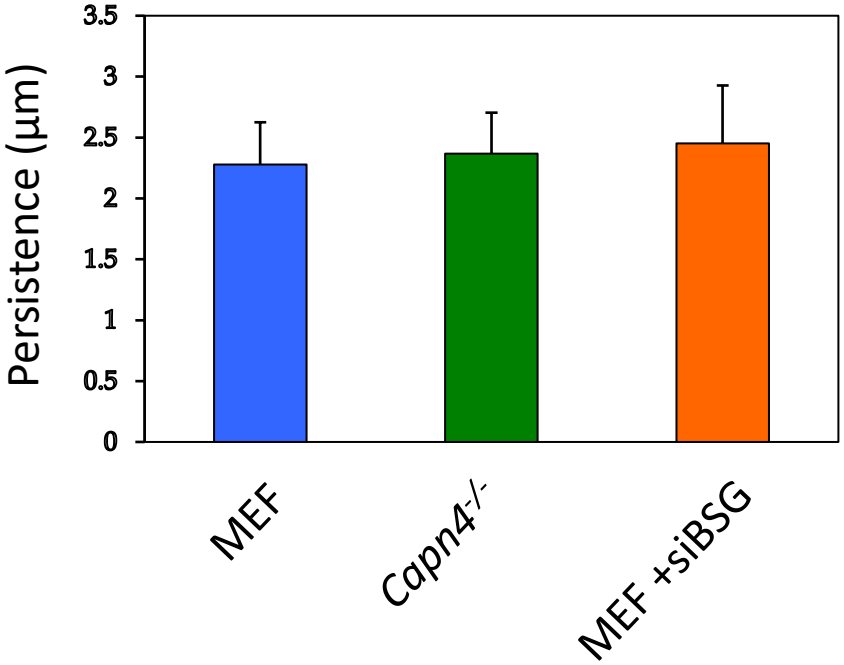
Figure 4

Figure 5.

A


Linear Speed (µm/min)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
*
MEF
Capn4-/-
MEF+siBSG

B


Persistence (µm)
3.5
3
2.5
2
1.5
1
0.5
0
MEF
Capn4-/-
MEF +siBSG