MEKK1-dependent phosphorylation of calponin-3 tunes cell contractility

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ABSTRACT

MEKK1 (also known as MAP3K1), which plays a major role in MAPK signaling, has been implicated in mechanical processes in cells, such as migration. Here, we identify the actin-binding protein calponin-3 as a new MEKK1 substrate in the signaling that regulates actomyosin-based cellular contractility. MEKK1 colocalizes with calponin-3 at the actin cytoskeleton and phosphorylates it, leading to an increase in the cell-generated traction stress. MEKK1-mediated calponin-3 phosphorylation is attenuated by the inhibition of myosin II activity, the disruption of actin cytoskeletal integrity and adhesion to soft extracellular substrates, whereas it is enhanced upon cell stretching. Our results reveal the importance of the MEKK1–calponin-3 signaling pathway to cell contractility.

KEY WORDS: Actomyosin, Mechanotransduction, Phosphorylation, Substrate rigidity

INTRODUCTION

MEKK1 (also known as MAP3K1), is a mitogen-activated protein kinase (MAPK) kinase kinase that activates the c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) MAPK signaling pathways (Yujiri et al., 1998; Hagemann and Blank, 2001). Full-length MEKK1 contributes to pro-survival signaling, whereas its cleavage by caspase 3 generates a C-terminal fragment that promotes apoptosis (Pham et al., 2013). In vivo analysis using MEKK1-deficient mice, which exhibit a failure in eyelid closure caused by impaired epithelial migration (Yujiri et al., 2000; Zhang et al., 2003), highlights regulation of cell migration as a physiological role of MEKK1. This is further supported by in vitro studies showing that MEKK1 is crucially involved in directed migration of various types of cells (Yujiri et al., 2000; Zhang et al., 2003, 2005; Cuevas et al., 2003, 2006; Deng et al., 2006).

In migrating cells, actomyosin-generated contractile forces pull the cell body forward and promote retraction of the cell rear (Ridley et al., 2003; Parsons et al., 2010). Thus, actomyosin contraction is essential for directed cell migration (Parsons et al., 2010). MEKK1 has been reported to contribute to the formation of actin stress fibers (Zhang et al., 2003, 2005), a major contractile apparatus in non-muscle cells, as well as the retraction of cell rear tails (Cuevas et al., 2003). This implies that MEKK1 might promote cell migration by regulating actomyosin contractility. Although MEKK1 localizes to the actin cytoskeleton (Christerson et al., 1999; Cuevas et al., 2003) and functions downstream of RhoA in stress fiber formation (Zhang et al., 2005), little is known about the MEKK1-mediated signaling that regulates cell contractility.

Calponins, which comprise a family of actin-binding proteins, are reportedly involved in the regulation of a variety of cell motile behaviors such as migration, contraction and morphogenesis (Rozenblum and Gimona, 2008; Wu and Jin, 2008). There are three genetic isoforms of calponin: h1 or basic calponin (calponin-1; CNN1), h2 or neutral calponin (calponin-2; CNN2), and h3 or acidic calponin (calponin-3; CNN3). CNN1 is exclusively expressed in smooth muscle cells, whereas CNN2 and CNN3 are expressed more ubiquitously. The amino acid sequences are highly conserved among these isoforms; only the C-terminal tails differ to a large extent (Fig. S1). CNN1 bound to actin filaments inhibits actin-activated myosin ATPase activity without affecting phosphorylation of the myosin regulatory light chain (MLC), and attenuates contraction of smooth muscle (Abe et al., 1990; Winder and Walsh, 1990; Itoh et al., 1994; Horowitz et al., 1996; Obara et al., 1996, 2000; Shibukawa et al., 2010, 2013). However, it remains unclear whether...
phosphorylation of CNN3 at the other sites is implicated in actin- or actomyosin-related cell functions.

Here, we show that MEKK1 regulates cellular contractility. Furthermore, we find that MEKK1 phosphorylates CNN3 at Thr288. MEKK1-dependent phosphorylation of CNN3 increases the traction stress that cells generate. Importantly, the CNN3 phosphorylation, which depends on myosin II activity and actin cytoskeletal integrity, is enhanced upon adhesion to rigid extracellular substrates or application of external stretching force. This suggests that the MEKK1–CNN3 signaling is mechanically regulated. Collectively, we propose that MEKK1 and CNN3 comprise a new pathway involved in a positive-feedback regulatory mechanism of cellular contractility.

RESULTS
MEKK1 mediates traction stress generation of cells
To examine the role of MEKK1 in regulating cellular contractility, we used traction force microscopy to measure the traction stress exerted by cells on extracellular substrates (Pelham and Wang, 1999; Dembo and Wang, 1999). RNA interference (RNAi)-mediated depletion of MEKK1 expression (short hairpin RNA (shRNA) for MEKK1) in mouse myoblastic C2C12 cells significantly reduced the magnitude of cell-generated traction stress (Fig. 1B,C). Notably, phosphorylation of MLC (myosin regulatory light chain 2, MYL2), a crucial step in the activation of non-muscle myosin II (Tan et al., 1992), was not affected by depleting MEKK1 expression (Fig. 1A), suggesting that MEKK1 regulation of cellular contractility does not involve alteration of MLC phosphorylation.

Phosphorylation of CNN3 at Thr288, a new phosphorylation site, is blocked by inhibition of myosin II
We then sought molecular details behind the regulation of cellular contractility by MEKK1. Contractility of non-muscle cells is reportedly modulated by the mechanical properties of the surrounding environments (Geiger et al., 2009; Zaidel-Bar et al., 2015), including the substrate rigidity, as we have observed (Fig. 1C), and as we and others have reported previously (Lo et al., 2000; Paszek et al., 2005; Saez et al., 2005; Yip et al., 2013). We postulated that MEKK1 might be involved in the positive-feedback mechanism that mediates substrate-rigidity-dependent regulation of cell contractility (Giannone and Sheetz, 2006; Buxboim et al., 2010; Trichet et al., 2012), and examined whether the activity of MEKK1 was modulated by actomyosin contraction, the major source of cellular contractility (Beningo et al., 2006). MEKK1 activity was assessed by evaluating the level of MEKK1 phosphorylation at Thr1381 in its activation loop, the autophosphorylation that reportedly represents MEKK1 activation (Matsuzawa et al., 2008; Enzler et al., 2009; Saha et al., 2014). In an immunoblot analysis of C2C12 cell lysate using an antibody that was raised against Thr1381-phosphorylated MEKK1 (Fig. S2), we did not detect a distinct band at the molecular mass of full-length MEKK1 (196 kDa) (asterisk in Fig. S3A). Instead, the anti-Thr1381-phospho-MEKK1 antibody blot demonstrated a band at the apparent molecular mass of 39 kDa, which disappeared in the lysate from myosin-II-inhibited cells (arrow in Fig. S3A). To identify the protein represented by the 39-kDa band, immunoprecipitates with the anti-Thr1381-phosphorylated MEKK1 antibody were resolved by SDS-PAGE. A mass spectrometric analysis of the excised gel piece containing the 39-kDa band (indicated by a square parenthesis in Fig. S3B) revealed that the sample included peptides that share the sequences with CNN3 (Fig. S3C). Given that CNN3 contains the sequence (S285QGTG289), which is similar to the region containing the phosphorylation site (Thr1381) in MEKK1 (S1378KGTG1382), we hypothesized that the 39-kDa band detected by the anti-Thr1381-phosphorylated MEKK1 antibody were resolved by SDS-PAGE. A mass spectrometric analysis of the excised gel piece containing the 39-kDa band (indicated by a square parenthesis in Fig. S3B) revealed that the sample included peptides that share the sequences with CNN3 (Fig. S3C). Given that CNN3 contains the sequence (S285QGTG289), which is similar to the region containing the phosphorylation site (Thr1381) in MEKK1 (S1378KGTG1382), we hypothesized that the 39-kDa band detected by the anti-Thr1381-phosphorylated MEKK1 antibody blot might represent CNN3 phosphorylated at Thr288.

To test this hypothesis, we conducted anti-Thr1381-phosphorylated MEKK1 immunoblot analysis of anti-CNN3 immunoprecipitates and observed a distinct 39-kDa band.
(Fig. S3D), suggesting that the anti-Thr1381-phosphorylated MEKK1 antibody could cross-react to phosphorylated CNN3. To further examine whether Thr288 of CNN3 was phosphorylated in cells, we raised a polyclonal antibody against Thr288-phosphorylated CNN3. When we analyzed C2C12 cell lysate by immunoblotting using this antibody, we detected a distinct 39-kDa band (Fig. 2A). Furthermore, the intensity of the 39-kDa band was markedly decreased in the lysate from CNN3-depleted cells (Fig. 2B). In addition, exogenously expressed FLAG-tagged CNN3 wild-type (FLAG–CNN3 WT), but not its threonine-replaced mutant (FLAG–CNN3 T288A), could be detected by anti-Thr288-phosphorylated CNN3 immunoblotting (Fig. 2C). These results indicate that Thr288 of CNN3 is phosphorylated in cells.

**CNN3 is phosphorylated by MEKK1**

Thr1381 is the autophosphorylation site of MEKK1 (Deak et al., 1996), and tested whether actomyosin contraction modulated MEKK1 activity and CNN3 phosphorylation. Although inhibition of MEKK1 forms a complex with CNN3 in cells.

We next examined whether modulation of MEKK1 expression altered CNN3 phosphorylation. When expression of MEKK1 was depleted using shRNA, CNN3 phosphorylation was greatly attenuated (Fig. 3E). By contrast, overexpression of wild-type MEKK1, but not its kinase-dead mutant (MEKK1 D1369A) (Xu et al., 1996), increased CNN3 phosphorylation (Fig. 3F). Furthermore, when immunopurified HA–MEKK1 was incubated with a GST-fused recombinant protein of the C-terminal region of human CNN3 (amino acids 243–329; GST–CNN3243-329; Fig. S4) in vitro, GST–CNN3243-329 was phosphorylated in an ATP-dependent manner (Fig. 3G). These results strongly suggest that Thr288 of CNN3 is phosphorylated by MEKK1.

**Phosphorylation of CNN3 is involved in traction force generation by cells**

We next examined whether Thr288 phosphorylation of CNN3 was involved in cellular contractility regulated by MEKK1. To test whether CNN3 phosphorylation modulated cellular contractility, we depleted endogenous CNN3 expression from C2C12 cells using shRNA, and introduced either a wild-type (WT) or phospho-defective (T288A) mutant FLAG-tagged shRNA-resistant form of CNN3 (Fig. 4A), and measured the cell-generated traction stresses on substrates with different rigidities. We found that CNN3-T288A-expressing cells exerted smaller traction stress compared with CNN3-WT-expressing cells on both the 6 kPa and the 24 kPa substrates (Fig. 4B,C). We speculate that a lack of statistical significance in the difference on the 14 kPa substrate might be due to the large variability in traction stresses that cells generate on ~15 kPa substrates (Yip et al., 2013). Taken together, these results from our traction force microscopy analysis indicate that CNN3 phosphorylation at Thr288 positively regulates cellular contractility.

The phosphorylation level of MLC was not affected by the phosphorylation status of CNN3 Thr288 (Fig. 4A) in the same manner as it was not by the expression of MEKK1 (Fig. 1A). Therefore, CNN3 phosphorylation-mediated regulation of cellular contractility appears to be distinct from modulation of MLC phosphorylation.

**CNN3 phosphorylation depends on cytoskeletal integrity and tension**

We then asked whether the MEKK1–CNN3 pathway participates in the positive-feedback mechanism that regulates cellular contractility (Giannone and Sheetz, 2006; Buxboim et al., 2010; Trichet et al., 2012), and tested whether actomyosin contraction modulated MEKK1 activity and CNN3 phosphorylation. Although inhibition
of myosin II did not apparently affect the activity of MEKK1 (Fig. 5A), it did significantly decrease CNN3 phosphorylation (Fig. 5B). Disruption of the actin cytoskeleton (Fig. 5C) and adhesion to softer substrates (Fig. 5D) also attenuated the CNN3 phosphorylation. Furthermore, higher cell density, which gives rise to attenuated actin stress fiber formation (Bereiter-Hahn and Kajstura, 1988), resulted in lower CNN3 phosphorylation levels (Fig. 5E). These results suggest that CNN3 phosphorylation at Thr288 is attenuated under the conditions where development of actomyosin-based cytoskeletal tension is hampered. By contrast, sustained equibiaxial stretching (3%, 5 min) of substrates to which cells adhered (Ursekar et al., 2014) caused an increase in CNN3 phosphorylation (Fig. 5F). Collectively, we suggest that Thr288 phosphorylation of CNN3 depends upon cytoskeletal tension. Notably, even though the distributions of CNN3 and MEKK1 along the stress fibers became less punctate upon myosin II inhibition, their colocalization appeared to be preserved (Fig. 5G). This suggests that the formation of the complex between CNN3 and MEKK1 is neither based on actomyosin contractility nor dependent on CNN3 phosphorylation.
DISCUSSION

Actomyosin contraction primarily depends on MLC phosphorylation, which is regulated by Rho to Rho kinase and/or myosin light chain kinase signaling (Fukata et al., 2001). In addition to this well-documented mechanism, we have revealed in this study that MEKK1 and CNN3 comprise a new pathway for regulation of cellular contractility; MEKK1 mediates CNN3 phosphorylation at Thr288, which results in an increase in cellular contractility.

At present, it is unclear how Thr288 phosphorylation of CNN3 instigates an increase in cellular contractility. However, studies on smooth muscle CNN1 might provide an insight into the underlying mechanism. CNN1 bound to actin filaments decreases the ATPase activity of MLC-phosphorylated myosin (Abe et al., 1990; Winder and Walsh, 1990). Given that both the actin-binding and the myosin-ATPase-inhibitory regions are highly conserved between CNN1 and CNN3 (Fig. S1) (Winder and Walsh, 1990; Itoh et al., 1994; Obara et al., 1996; Tang et al., 1996), Thr288 phosphorylation of CNN3 might also attenuate its inhibitory activity on the myosin ATPase activity, thereby increasing the actomyosin contractility.

Apart from these expected similarities among calponins, it is noteworthy that Thr288 resides in the C-terminal tail region of CNN3, where the sequence is not conserved in other calponin isoforms (Fig. S1). Therefore, the regulatory mechanism of cellular contractility through Thr288 phosphorylation by MEKK1 appears to be specific to CNN3. At present, however, it is unclear how cytoskeletal contractility promotes MEKK1-dependent phosphorylation of Thr288 in CNN3. Because MEKK1 phosphorylation was not decreased by myosin II inhibition (Fig. 5A), it is unlikely that actomyosin contraction enhances CNN3 phosphorylation by increasing the kinase activity of MEKK1. Given that the interaction of CNN3 and MEKK1 appears to be independent of actomyosin contraction (Fig. 5G), the susceptibility of CNN3 to phosphorylation by MEKK1 might be enhanced by mechanical extension as we previously reported in the case of phosphorylation of p130Cas (also known as BCAR1) by Src (Sawada et al., 2006). Alternatively, increased cell contractility might lead to inactivation of phosphatase(s) responsible for dephosphorylation of CNN3. Although further studies are needed to uncover the mechanism behind the cytoskeletal-tension-dependent regulation of CNN3 phosphorylation, localization of the MEKK1–CNN3 complex on stress fibers designates stress fibers per se as a distinct signaling platform for mechanotransduction (Hirata et al., 2015).

Although CNN3 phosphorylation by MEKK1 increases cellular contractility, the resulting development of cytoskeletal tension in turn promotes CNN3 phosphorylation, which leads to formation of a positive-feedback loop concerning the mechanical regulation of cell functions. In addition to CNN3, CNN2 is also expressed in non-muscle cells (Wu and Jin, 2008). Interestingly, expression of CNN2 is reportedly upregulated under conditions in which cytoskeletal tension is higher (Hossain et al., 2005, 2006; Jiang et al., 2014). Given that CNN2 stabilizes actin stress fibers (Hossain et al., 2005), the cytoskeletal-tension-dependent increase in CNN2 expression might also participate in a positive feedback type of cellular contractility regulation. In contrast, CNN3 expression was not affected by the status of cytoskeletal tension (Fig. 5). These observations suggest that there is a difference between CNN2 and CNN3 in the mechanisms by which they contribute to the cytoskeletal-tension-associated regulation of cellular contractility, and indicate the importance of threonine phosphorylation in the tail region, which is unique for CNN3.

Cellular contractility is implicated in various fundamental cell functions including migration, morphogenesis, survival, proliferation and differentiation (Clark et al., 2007). The in vivo contribution of MEKK1 and CNN3 to wound closure (Deng et al., 2006; Daimon et al., 2013), in which actomyosin contraction plays an important role (Shaw and Martin, 2009), might involve the MEKK1-dependent CNN3 phosphorylation that we have demonstrated in this study.
MATERIALS AND METHODS

Cell culture, transfection and retroviral infection

C2C12, NIH3T3 and 293T cells were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (Life Technologies) at 37°C in 5% CO₂. For transient transfection of cells with plasmids, the Lipofectamine 2000 transfection reagent (Life Technologies) was used according to the manufacturer’s instruction. Retroviral infection was conducted as described previously (Kawauchi et al., 2008). Infected cells were selected with 4 µg/ml puromycin and/or 1000 µg/ml hygromycin.

Plasmids

pcDNA3-HA-mouse MEKK1 was a gift from Isao Naguro (University of Tokyo, Tokyo, Japan). Human CNN3 cDNA was a gift from Yoshinao Wada and Yukinao Shibukawa (Osaka Medical Center and Research Institute for Maternal and Child Health, Osaka, Japan). pcDNA3-α-actinin-mCherry was a gift from Hiroaki Machiyama (National University of Singapore, Singapore). The F-Tractin-EGFP construct (Johnson and Schell, 2009) was a gift from Michael J. Schell (Uniformed Services University, Bethesda, MD). The FLAG and human CNN3 sequences were subcloned into the pBabe.hygro vector. Site-directed mutants of MEKK1 (D1369A) and CNN3 (T288A and shRNA-resistant mutants) were generated by the

Fig. 5. CNN3 phosphorylation at Thr288 depends on cytoskeletal tension. (A) NIH3T3 cells transfected with HA–MEKK1 were treated with DMSO (control) or 100 µM blebbistatin (Blebb) for 30 min. The cells were lysed and immunoblotted for Thr1381-phosphorylated MEKK1 (pMEKK1), MEKK1, HA and β-actin. (B,C) Top panels, NIH3T3 cells treated with DMSO (control), 100 µM blebbistatin (Blebb) or 1 µM cytochalasin D (CytoD) for 30 min were lysed and immunoblotted for Thr288-phosphorylated CNN3 (pCNN3) and CNN3. Bottom panels, quantification of the densitometric ratio of pCNN3 against CNN3. Each bar represents the mean±s.d. (n=3). *P<0.01 (two-tailed, paired t-test). (D) NIH3T3 cells grown on polyacrylamide gel substrates with rigidity of 72 and 9 kPa were lysed and immunoblotted for Thr288-phosphorylated CNN3 (pCNN3) and CNN3. Similar results were obtained in two independent experiments. (E) Left, differential interference contrast images of NIH3T3 cells grown at different cell densities (1×10⁴, 3×10⁴ and 8×10⁴ cells/cm²). Scale bar: 200 µm. Right, NIH3T3 cells grown at different cell densities (1×10⁴, 3×10⁴ and 8×10⁴ cells/cm²) were lysed and immunoblotted for Thr288-phosphorylated CNN3 (pCNN3), CNN3 and β-actin. Similar results were obtained in two independent experiments. (F) C2C12 cells grown on collaged-coated PDMS chambers were treated with 100 µM blebbistatin (Blebb) for 30 min, and then the chambers were equibiaxially stretched (3% for 5 min) in the presence of blebbistatin. The cells were lysed and immunoblotted for Thr288-phosphorylated CNN3 (pCNN3) and CNN3. Similar results were obtained in two independent experiments. The position of molecular mass markers is indicated for each blot. (G) C2C12 cells co-transfected with HA–MEKK1 and FLAG–CNN3 were treated with DMSO (control) or 100 µM blebbistatin (Blebb) for 15 min and then immunostained for HA and FLAG. Scale bar: 10 µm.
QuickChange mutagenesis method (Agilent Technologies, Santa Clara, CA). For shRNA-mediated depletion of protein expression, the target sequence was inserted into the pSUPER.neo.puro vector (Oligogenein, Seattle, WA). The target sequences used were: 5′-GGAAACCGGTGCAGAGGATG-3′ for mouse MEKK1 (Map3k1), and 5′-GTATGCGAGAAAACAAACAA-3′ for mouse CNN3 (Cnn3). For bacterial expression of GST–CNN3243-329, the amino acid 243–329 region of CNN3 was amplified by PCR and cloned into the pGEX-5X-2 vector.

**Antibodies and inhibitors**

Rabbit polyclonal antibody (pAb) against Thr288-phosphorylated CNN3 was raised against the phospho-peptide NGSQG(pT)GTVNS (Abmart, Shanghai, China). The rabbit pAb against CNN3 (cat. no. sc-28546) and control rabbit IgG (cat. no. sc-2027) were purchased from Santa Cruz Biotechnology (Dallas, TX). The rabbit pAb against Thr1400-phosphorylated human MEKK1 (cat. no. PAB0513) was from Abnova (Taipei, Taiwan). The rabbit pAb against MEKK1 (cat. no. A302-396A) was from Bethyl Laboratories (Montgomery, TX). The mouse monoclonal antibodies (mAbs) against β-actin (cat. no. A5441) and FLAG (cat. no. F1804) were from Sigma-Aldrich (St. Louis, MO). The rabbit pAbs against Ser19-phosphorylated myosin light chain 2 (cat. no. 3671) and total myosin light chain 2 (cat. no. 3672) were from Cell Signaling Technology (Danvers, MA). The rat mAb against HA (cat. no. 11867423001) was from Roche (Basel, Switzerland). The rabbit pAb against GST (cat. no. PM013) was from Medical & Biological Laboratories (Nagoya, Japan). Horseradish peroxidase (HRP)-conjugated anti-mouse and -rabbit-IgG antibodies were from GE Healthcare (Little Chalfont, UK). Alexa-Fluor-488-conjugated goat anti-mouse-IgG, Alexa-Fluor-488-conjugated goat anti-rat-IgG and Alexa-Fluor-546-conjugated goat anti-mouse-IgG antibodies were from Life Technologies. Blebbistatin and cytochalasin D were from Toronto Research Chemicals (North York, Canada) and Sigma-Aldrich, respectively.

**Immunoblotting**

Cells were lysed with 2× lithium dodecyl sulfate sample buffer (Life Technologies) containing 2.5% β-mercaptoethanol. The lysate samples were resolved by SDS-PAGE (4–12% Bis-Tris gel; Life Technologies), transferred onto nitrocellulose membranes (Merck Millipore, Billerica, MA), and probed with antibodies. Immunoreactive bands were detected with SuperSignal West Pico or Femto Chemiluminescent Substrate (Thermo Fisher Scientific, Rockford, IL). Antibodies were diluted to 1:10,000 in Tris-buffered saline containing 0.1% Tween 20 and 1% skimmed milk.

**Immunoprecipitation**

Primary antibodies and control rabbit IgG (50 µg/ml) were covalently coupled to protein-G-conjugated magnetic beads (Life Technologies) with 20 mM dimethyl pimelimidate (DMP) and 0.1% formaldehyde. The beads were washed with PBS, and the purified recombinant protein was eluted with 20 mM reduced glutathione (GSH). Mass spectrometric analysis

**Mass spectrometric analysis**

SDS-PAGE-resolved immunoprecipitates were visualized by negative staining (Wako Pure Chemical Industries, Osaka, Japan), and the gel piece containing the 39-kDa protein band was excised. The gel piece was washed with 50% acetonitrile, reduced with 10 mM dithiothreitol for 45 min at 37°C, alkylated with 50 mM iodoacetamide for 30 min at room temperature, and digested with 5 ng/µl trypsin (Promega, Madison, WI) overnight at 37°C. Digested peptides were extracted by 50% acetonitrile and 0.1% formic acid, vacuum dried, and analyzed by nano-liquid chromatography tandem mass spectrometry (nanoLC-MS/MS) using a TripleTOF 5600 mass spectrometry system (AB SCIEX, Foster City, CA) on-line coupled with an in-house packed ReproSil-Pur C18-AQ column driven by an Ultimate 3000 RSLC nano system (Thermo Fisher Scientific) as previously described (Iwasaki et al., 2012).

Peptide identification was performed by Mascot version 2.3.01 (Matrix Science, Boston, MA) against the SwissProt database (version 2011.06) containing 16,376 mouse protein sequences. Peptides were considered to be identified using the criteria as previously described (Iwasaki et al., 2012).

**Preparation of polyacrylamide gel substrates**

Polyacrylamide gel substrates, to which fibronectin (Sigma-Aldrich) was conjugated using sulfo-SANPAH (Thermo Fisher Scientific), were prepared as described previously (Dembo and Wang, 1999). The Young’s modulus of the gels was measured by an AFM indentation assay (Vedula et al., 2014). For traction force microscopy, we used polyacrylamide gels co-polymerized with N-acryloyloxy-6-aminocaproic acid (ACA; Tokyo Chemical Industry, Tokyo, Japan), in which fluorescent beads (0.2 µm; Polysciences, Warrington, PA) were embedded. The ACA-copolymerized gels with Young’s moduli of 6, 14 and 23 kPa were prepared on glass-bottom dishes, as described previously (Yip et al., 2013). Collagen type I (Koken, Tokyo, Japan) was covalently conjugated to the gel surface through ACA (Yip et al., 2013).

**Traction force microscopy**

Cells were grown overnight on collagen-conjugated gel substrates in HEPES-buffered Dulbecco’s modified Eagle’s medium (Life Technologies) supplemented with 10% fetal bovine serum. Cells and fluorescent beads embedded in the gels were observed with a spinning-disc confocal microscopy system (UlirView VoX; PerkinElmer, Waltham, MA) equipped with an inverted microscope (IX81; Olympus, Tokyo, Japan), a 60× water immersion objective (NA 1.2, UPlanSapo; Olympus) and an electron multiplying charge-coupled device camera (C9100-13; Hamamatsu Photonics, Hamamatsu, Japan) at 37°C. Differential interference contrast images of cells as well as fluorescent images of embedded beads were acquired before and after detaching the cells from the gel substrate by treatment with trypsin and EDTA. From the bead images before and after cell detachment, the entire displacement field in the gel substrate was calculated using MATLAB software (MathWorks, Natick, MA). The traction stress field was then obtained by solving the inverse Boussinesq problem as described previously (Yip et al., 2013).

**In vitro kinase assay**

The GST–CNN3243-329 protein was expressed in Escherichia coli BL21 cells. The cells were lysed with 1% Triton X-100, 1.9 mg/ml lysozyme, 9 mM dithiothreitol, 20 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl and protease inhibitors (pH 8.0). The soluble fraction of the bacterial lysate was applied to GST SpinTrap columns (GE Healthcare). After repeated washing with PBS, the purified recombinant protein was eluted with 20 mM reduced L-glutathione (Sigma-Aldrich) and 50 mM Tris-HCl (pH 8.0).

HA–MEKK1 expressed in NIH3T3 cells was immunoprecipitated with anti-HA-antibody-coupled magnetic beads. As a control, the lysate of NIH3T3 cells transfected with the empty pcDNA3 vector was incubated with the anti-HA-antibody-coupled magnetic beads. After washing with washing buffer (20 mM Tris-HCl pH 8.0, 1 mM EDTA, 150 mM NaCl), the HA–MEKK1–magnetic-bead complexes were incubated with purified GST–CNN3243-329 in 25 mM HEPES, 50 mM NaCl, 5 mM MgCl2, 0.5 mM dithiothreitol, protease inhibitors and phosphatase inhibitors (pH 7.4) either in the presence or absence of 10 µM ATP for 30 min at 30°C. The products were analyzed by immunoblotting.

**Stretching cells**

Cells were equibiaxially stretched using the device reported elsewhere (Ursekar et al., 2014). In brief, cells were grown overnight on
polydimethylsiloxane (PDMS) stretch chambers coated with 10 µg/ml collagen type I. After treatment of the cells with 100 µM blebbistatin for 30 min at 37°C, the chambers were then equilibrated by stretching for 3% in the presence of blebbistatin. The cells were analyzed by immunoblotting.

Immunofluorescence

Cells were fixed and permeabilized for 30 min with 4% formaldehyde and 0.2% Triton X-100 in cytoskeleton-stabilizing buffer (137 mM NaCl, 5 mM KCl, 1.1 mM Na2HPO4, 0.4 mM KH2PO4, 4 mM NaHCO3, 2 mM MgCl2, 5.5 mM glucose, 2 mM EGTA, and 5 mM PIPES, pH 6.1) (Hirata et al., 2008). This was followed by blocking with 1% bovine serum albumin (BSA) in cytoskeleton-stabilizing buffer for 30 min. The cells were then incubated with primary antibodies for 40 min, washed and further incubated with secondary antibodies for 40 min. Both primary and secondary antibodies were diluted to 1:100 in the cytoskeleton-stabilizing buffer containing 1% BSA. The stained cells were observed with an epi-fluorescence inverted microscope (IX81, OLYMPUS, Tokyo, Japan) equipped with an oil microscope (IX81, OLYMPUS, Tokyo, Japan) equipped with an oil objective (Olympus, Tokyo, Japan). Metamorph software (Molecular Devices, Sunnyvale, CA) was used for image acquisition.

Statistical analysis

Statistical analyses were performed using Student’s two-tailed t-test (either paired or unpaired).

Author contributions

The authors declare no competing or financial interests.

Competing interests

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Supplementary information

Supplementary information available online at http://jcs.biologists.org/lookup/doi/10.1242/jcs.189415.supplemental

References


**Fig. S1. Calponin isoforms.** (A) Domain structures of calponins. S100; S100 binding site, CH; calponin homology domain, ABS1; actin binding site 1, R1; calponin-like repeat 1, R2; calponin-like repeat 2, R3; calponin-like repeat 3. (B) Amino acid sequences of C-terminal tail regions of calponins. The SQGTG sequence and Thr288 of CNN3 are indicated by an underline and in red, respectively.
**Fig. S2. Detection of MEKK1 phosphorylation at Thr1381 by immunoblotting.** NIH3T3 cells transfected with either the empty vector (vector), HA-tagged wild-type (WT) MEKK1 or HA-tagged threonine-replaced (T1381A) MEKK1 were lysed and immunoblotted with anti-Thr1381-phosphorylated MEKK1 (α-pMEKK1) and anti-MEKK1 (α-MEKK1) antibodies.
**Fig. S3. CNN3 is detected with the anti-Thr1381-phosphorylated MEKK1 antibody.** (A) C2C12 cells treated with DMSO (control) or 100 µM blebbistatin (Blebb) for 30 min were lysed and immunoblotted with anti-Thr1381-phosphorylated MEKK1 (α-pMEKK1) and anti-β-actin antibodies. The arrow indicates the 39-kDa band that has been detected with the anti-Thr1381-phosphorylated MEKK1 antibody. (B) Immunoprecipitates from the C2C12 cell lysate with the anti-Thr1381-phosphorylated MEKK1 antibody (α-pMEKK1) or control rabbit IgG (IgG) were resolved by SDS-PAGE. Protein bands were visualized by silver staining. A gel piece containing the 39-kDa band (indicated by a square parenthesis) was excised and subjected to a mass spectrometric analysis. (C) Peptide sequences in mouse CNN3 that have been detected with the mass spectrometric analysis are shown in red. (D) Immunoprecipitates from the C2C12 cell lysate with the anti-CNN3 antibody (α-CNN3) or control rabbit IgG (IgG) were immunoblotted with anti-Thr1381-phosphorylated MEKK1 (α-pMEKK1) and anti-CNN3 (α-CNN3) antibodies.
Fig. S4. The recombinant GST-CNN3243-329 protein subjected to SDS-PAGE and visualized by Coomassie staining.