**Substrate stiffness induces nuclear localization of myosin regulatory light chain to suppress apoptosis**

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**Abstract**

Stiffness of the extracellular matrix regulates various biological responses, but the response mechanisms are poorly understood. Here, we found that the nuclear di-phosphorylated myosin regulatory light chain (2P-MRLC) is a critical mechano-mediator that suppresses apoptosis in response to substrate stiffness. Stiff substrates promoted the nuclear localization of 2P-MRLC. Zipper-interacting protein kinase [ZIPK; also known as death-associated protein kinase 3 (DAPK3)], a kinase for MRLC, was localized in the nucleus in response to stiff substrates and promoted the nuclear localization of 2P-MRLC. Moreover, actin fiber formation induced by substrate stiffness promoted the nuclear localization of 2P-MRLC via ZIPK. 2P-MRLC in response to substrate stiffness suppressed the expression of MAF bZIP transcription factor B (MafB) and repressed apoptosis. These findings reveal a newly identified role of MRLC in mechanotransduction.

**Key words: myosin regulatory light chain, substrate stiffness, mechanotransduction, apoptosis, MafB, ZIPK, actin filament, extracellular matrix, nuclear localization**

**Abbreviations**

**1P-MRLC**, mono-phosphorylated myosin regulatory light chain

**2P-MRLC**, di-phosphorylated myosin regulatory light chain

**AFM**,atomic force microscopy

**ChIP**, chromatin immunoprecipitation

**DAPK1**, death-associated protein kinase 1

**DMSO**, dimethyl sulfoxide

**ECM**, extracellular matrix

**GAPDH**,glyceraldehyde 3-phosphate dehydrogenase

**HUVEC**, human umbilical vein endothelial cell

**ICAM-1**, intercellular adhesion molecule 1

**KD**, knockdown

**LZ**, leucine zipper

**MafB**, MAF bZIP transcription factor

**MSC**, mesenchymal stem cell

**NC**, negative control

**Nf-kB**, nuclear factor-kB

**MLCK**, myosin light-chain kinase

**MRLC,** myosin regulatory light chain

**MRTF-A**, myocardin related transcription factor-A

**PCR**, polymerase chain reaction

**qPCR**, quantitative polymerase chain reaction

**ROCK**, Rho-associated protein kinase

**siRNA**, small interfering RNA

**TWIST1**, twist family bHLH transcription factor 1

**YAP**, Yes-associated protein

**ZIPK/DAPK3**, zipper-interacting protein kinase/death-associated protein kinase 3

**Introduction**

The cells *in vivo* interact with the surrounding extracellular matrix (ECM) and respond to its physical properties, which are determined by the structure and composition of the ECM[1,2]. The structure and composition of the ECM are strictly regulated to optimize the cell function for each tissue[3]. Appropriate tissue stiffness regulates stem cell differentiation and morphogenesis[4-6], whereas an aberrant increase in ECM stiffness causes abnormal cell behavior during aging and the pathological progression of cancer, fibrosis, and cardiovascular disease[7]. In addition, substrate stiffness promotes progressive cancer phenotype*s in vitro*, including proliferation[8] and epithelial–mesenchymal transition[9]. Thus, understanding the cellular response to substrate stiffness is important for understanding homeostasis and disease mechanisms. Tumor stiffness has traditionally been used as a diagnostic marker and more recently as a prognostic factor[10]. However, the cellular responses induced by substrate stiffness remain largely unknown.

Cell responses to mechanical properties include a process that converts mechanical stimuli into biochemical signals, known as mechanotransduction[11]. Cellular response to stiffness is mediated by the nuclear translocation of stiffness-responsive transcription factors and the regulation of gene expression. -catenin[12], myocardin related transcription factor-A (MRTF-A)[13], nuclear factor-B (Nf-kB)[14], SNAIL1[15], twist family bHLH transcription factor 1 (TWIST1)[9], and Yes-associated protein (YAP)[16] are transcription factors that respond to substrate stiffness and are localized in the nucleus. Matrix stiffness contributes to cancer progression by regulating transcription factors[17]. However, cellular responses to substrate stiffness have not been completely explained by the currently known transcription factors. Therefore, there is a need to identify novel transcription factors that respond to substrate stiffness.

Myosin regulatory light chain (MRLC, also known as MLC2, RLC, or LC20) localizes to the stress fiber as a subunit of myosin II. Mono-phosphorylated MRLC (1P-MRLC) at Ser19 or di-phosphorylated MRLC (2P-MRLC) at Thr18/Ser19 induces thick stress fiber formation and enhances contractile force by enforcing the ATPase activity of myosin II[18,19]. However, recent studies have shown that 2P-MRLC exists not only in stress fibers, but also in the nucleus[20]. MRLC functions as a transcription factor[20-22]. It binds to the intercellular adhesion molecule 1 (ICAM-1) core promoter[20]. In addition, phosphorylation/dephosphorylation of MRLC regulates ICAM-1 promoter activity[20]. Ser19-phosphorylated MRLC binds to the XO promoter and activates *XO* gene transcription[22]. However, the mechanisms regulating the nuclear localization of 2P-MRLC remain unclear. Previous reports have indicated that phosphorylation is a key event in the function of nuclear MRLC. Nuclear levels of MRLC di-phosphorylation are enhanced during myocardial ischemia/reperfusion[22,23]. However, the mechanism regulating the localization and function of nuclear MRLC is not yet fully understood. Substrate stiffness promotes the diphosphorylation of MRLC[14]. Thus, substrate stiffness may also regulate the nuclear localization of MRLC.

In this study, we found that substrate stiffness mediates the nuclear localization of MRLC and triggers apoptosis by preventing the MAF bZIP transcription factor B (MafB) expression. These findings identify nuclear 2P-MRLC as a critical mechano-mediator that suppresses apoptosis in response to substrate stiffness.

**Materials and Methods**

**Cell culture**

A431 (RRID: CVCL\_0037) human epidermoid carcinoma cell line was obtained from the American Type Culture Collection**.** A549 (RRID: CVCL\_0030) human lung adenocarcinoma cell line and HeLa (RRID: CVCL\_0023) human cervical adenocarcinoma cell line were obtained from Riken Cell Bank. Human umbilical vein endothelial cells (HUVECs) were obtained from Kurabo. Mouse mesenchymal stem cells (MSCs), kindly gifted by Dr. Suzanne Ponik (University of Wisconsin-Madison), were obtained from BALBc mice (BALB/cByJ, female, 7 weeks old, from Jackson laboratory)[24]. A431, A549, and HeLa cells were cultured in Dulbecco's modified Eagle’s medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (172012, Sigma-Aldrich) and 1% antibiotic/antimycotic solution (Sigma-Aldrich). MSCs were cultured in Dulbecco's modified Eagle’s medium (Sigma-Aldrich) supplemented with 20% fetal bovine serum (172012, Sigma-Aldrich) and 1% antibiotic/antimycotic solution (Sigma-Aldrich). HUVECs were cultured in endothelial cell growth medium 2 (C-22111, Promocell Co., Heidelberg, Germany) at 37 °C and 5% CO2. Cell line authentication has not been validated. All cells and cell lines used for experiments were routinely screened for mycoplasma contamination by Venor GeM Classic Mycoplasma Detection Kit for conventional PCR (Minerva Biolabs GmbH, Berlin, Germany) or MycoAlert™ Mycoplasma Detection Kit (LONZA, Basel, Switzerland).

**Cell culture substrates**

Plastic or glass dishes coated with 300 g/mL collagen-I (Nitta Gelatin, Osaka, Japan) were used as stiff substrates. Polyacrylamide gels were prepared using the following reagents (0.4 kPa: 0.05% N,N′-methylenebisacrylamide (BIS) and 5.0% acrylamide; 271 kPa: 0.6% BIS and 12% acrylamide. The procedure for preparing the polyacrylamide gel conformed to a previous study[25]. The surface stiffness of the polyacrylamide gels was measured by atomic force microscopy (Table S1).

**Atomic force microscopy (AFM)**

The surface stiffness of the gels was measured by atomic force microscopy AFM (Nanowizard4, Bruker, Billerica, MA, USA) using a microscope (TE300, NIKON SOLUTIONS CO., LTD., Tokyo, Japan). Pyramidal silicon nitride cantilevers (MLCT; Bruker) with a spring constant of 0.01 N/m, calibrated by thermal tuning using a simple harmonic oscillator model, were used. Samples were indented with a calibrated force of 0.2 nN (for 0.4 kPa gel) or 0.5 nN (for 1.2, 2.3, 25, 61, 83, 134, or 271 kPa gels) in a scan area of 1 µm2 (4 pixels×4 lines for 0.4, 1.2, 2.3, 25, 61, 83, 134 kPa gels or 8 pixels×8 lines for 271 kPa gel). The elastic properties (Young’s modulus) were estimated after the application of the Hertzian model with an assumed Poisson's ratio of 0.5. using JPK Data Processing software (Bruker). The elastic properties and representative force-indentation curves, including the fit of the Hertzian model, are presented in Table S1 and Fig. S1, respectively.

**Antibody**

Antibodies against Phospho-Myosin Light Chain 2 (Thr18/Ser19) (3674S, Cell Signaling Technology, Inc., Danvers, MA, USA) Phospho-Myosin Light Chain 2 (Ser19) (3671S, Cell Signaling Technology), Myosin Light Chain 2 Antibody (3672S, Cell Signaling Technology), Caspase3 (9662S, Cell Signaling Technology), Collagen type I (c2456, Sigma-Aldrich), GAPDH (AM4300, Invitrogen, Carlsbad, CA, USA), Lamin A/C (4777S, Cell Signaling Technology), MAFB (30919, Cell Signaling Technology), α-Tubulin (T9026, Sigma-Aldrich), ZIPK (Z0134, Sigma-Aldrich) were used.

**Inhibitor treatment**

HeLa cells were treated with inhibitors for 24 h. 25 μM of Blebbistatin (+/-) (BML-EI315, Enzo Life Sciences, Inc., Farmingdale, NY, USA), 0.1 μM of Jasplakinolide (J4580, Sigma-Aldrich), 10 μM of Latrunculin A (L5163, Sigma-Aldrich), 10 μM of ML-7 (I2764 Sigma-Aldrich), 10 μM of Y-27632 (Y0503, Sigma-Aldrich), 200 μM of ZIPK inhibitor (324788, Sigma-Aldrich) or dimethyl sulfoxide (046-21981, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), were added. Experiments were performed 24 h after inhibitor administration.

**Immunofluorescence**

The cells on the substrates were washed once with phosphate buffered saline (PBS) and then fixed with 4% paraformaldehyde (Nacalai tesque, INC., Kyoto, Japan) in PBS for 10 min at room temperature. After three times washes with PBS, permeabilization with 1.0 % Triton-X100 in PBS was performed for 10 min at room temperature. After three times wash with PBS, blocking was performed using 1% bovine serum albumin (FUJIFILM Wako Pure Chemical Corporation) in PBS for 1 h at room temperature. The cells were incubated with a primary antibodies (Total-MRLC 1:200, 1P-MRLC 1:200 or 2P-MRLC 1:150 in PBS) overnight at 4°C. After three washes with PBS, the cells were incubated with a secondary antibody solution with or without Alexa Fluor-546 phalloidin (1:500, Invitrogen) and hoechst33342 in PBS for 1 h at room temperature. Alexa Fluor-488 anti-mouse IgG (1:500, Invitrogen) or Alexa Fluor-488 anti-rabbit IgG (1:200, Invitrogen) were used as secondary antibodies. Fluorescence images were captured using an A1R confocal imaging system (NIKON SOLUTIONS CO., LTD.). To quantify the nuclear localization of MRLC, the MRLC intensity was calculated as the nuclear/cytoplasm ratio using ImageJ software.

**Small interfering RNA (siRNA) transfection**

Cells were transfected with the 6 mL of 5 mM appropriate siRNA or non-targeting RNA as a negative control using Lipofectamine RNAiMAX reagent (Thermo Fisher Scientific, Waltham, MA, USA). The siRNAs were generated with in vitro transcription T7 kit (Takara Bio Inc., Shiga, Japan) by using the following sequences: MYL9 (human): 5’-TGATAAGAAAGGCAACTTCAACCCTATAG-3’ (sense sequence), MYL9 (mouse): 5’-GGCCTTCAACATGATTGATCACCCTATAG-3’ (sense sequence), MYL12A (human): 5’-ACCATGTTTGGTGAGAAGTTACCCTATAG-3’ (sense sequence), MYL12A (mouse): 5’-TTCTGTCATTGTGATGAGAAACCCTATAG-3’ (sense sequence), MYL12B (human): 5’- TTCCAGTTACATTGTCTTACTCCCTATAG-3’ (sense sequence), MYL12B (mouse): 5’- CACTTAGCATGTGCATAATCACCCTATAG-3’ (sense sequence), DAPK1 (human): 5’- TAGCTGAAAAGGAATCTTTAACCCTATAG-3’ (sense sequence), MafB (human): 5’-ACCAATGCATTGCGTTTCTTTCCCTATAG-3’ (sense sequence), non-targeting RNA: 5’-AAACTACATGTCACATCACGG CCCTATAG-3’. ZIPK siRNA was purchased from Dharmacon (Lafayette, CO, USA). We prepared siMRLC as a mixture of 2 mL of 5 mM siMYL9, siMYL12A, and siMYL12B.

**Vector transfection**

Cells were transfected with GFP-encoding vector (632484, Takara Bio Inc.) or GFP-MafB-encoding vector (MG204681, ORIGENE) with Xfect transfection reagent (Takara Bio Inc.).

**Western blotting**

Cells were washed once with cold PBS, then lysed in sodium dodecyl sulfate (SDS) buffer (0.125 M Tris–HCl, 0.2 M dithiothreitol, 4% SDS, 20% glycerol, and 0.01% bromophenol blue, pH 6.8). The cell lysates were heated at 95 °C for 5 min. Equal volumes of cell lysateswere separated on 8, 10, 12, or 14% SDS-polyacrylamide gels and then transferred to polyvinylidene fluoride membranes(Merck Millipore, Billerica, MA, USA). The membrane was blocked with 1% skim milk in Tris-buffered saline-Tween solution (TBS-T; 10 mM Tris–HCl containing 150 mM NaCl and 0.05% Tween 20, pH 7.5). The blots were incubated with primary antibodies (Caspase3: 1/1,000, GAPDH: 1/200,000, Lamin A/C: 1/10,000, MafB: 1/5,000, 2P-MRLC: 1/1,000 Total-MRLC: 1/1,000, -tubulin 1/20,000, ZIPK: 1/5,000) diluted in TBS-T or Can Get Signal Immunoreaction Enhancer Solution 1 (Toyobo, Osaka, Japan) at 4°C overnight. Membranes were then washed three times with TBS-T and incubated with the same dilution rate as the primary antibody of secondary antibody (HRP anti-rabbit IgG for Caspase3, MafB, 2P-MRLC, Total-MRLC and ZIPK; HRP anti-mouse IgG for GAPDH, LaminA/C and -tubulin) in TBS-T or Can Get Signal Immunoreaction Enhancer Solution 2 (Toyobo) for 1 h at room temperature. Protein signals were detected using Immobilon Western Chemiluminescent HRP substrate (Merck Millipore). GAPDH immunocomplexes were used as the internal standards for equal loading. The levels of LaminA/C immunocomplexes were used as the nuclear internal standard for equal loading of nuclear extraction. Levels of a-tubulin or GAPDH immunocomplexes were used as cytosol internal standards for equal loading of cytosol extraction. Images of the blots were captured using the ChemiDoc Touch Imaging System (Bio-Rad Laboratories Inc., Hercules, CA, USA). The signal intensity was quantified using Image Lab software (Bio-Rad) and normalized to the control value (Relative expression). Full unedited blots were showed in Fig. S2.

**Nuclear/Cytosolic fraction**

Nuclear and cytosolic extracts were isolated using the nuclear/cytosolic fractionation kit (AKR-171, Cell Biolabs, Inc. San Diego, CA, USA). Lamin A/C was used as a loading control for nuclear extracts. α-Tubulin and GAPDH were used as loading controls for the nuclear and cytosolic extracts, respectively.

**Quantitative PCR (qPCR)**

RNA extraction was performed using a FastGeneTM RNA Basic Kit (FG-80250, Nippon Genetics Co., Ltd., Tokyo, Japan). Reverse transcription was performed using the ReverTra Ace qPCR RT Kit (FSQ-201, TOYOBO). qRT-PCR was performed using the KAPA SYBR Fast qPCR kit (Kapa Biosystems, Inc., Woburn, MA, USA) and the Applied Biosystems StepOnePlus™ Real-Time PCR System (Thermo Scientific). Primer sequences are listed in Table.S2.

**Chromatin Immunoprecipitation (ChIP)**

ChIP was performed using the SimpleChIPR Plus Enzymatic Chromatin IP Kit (Magnetic Beads) (9005S, Cell Signaling Technology). For PCR, 2 μL of the eluted DNA and 40 cycles of amplification were used with the CTNNB1 core promotor-specific primers used in previous studies[21] with the KAPA SYBR Fast qPCR kit (Kapa Biosystems, Inc.). PCR products were subjected to agarose gel electrophoresis and stained with Midori Green Advance (NE-MG04, NIPPON Genetics).

**Death cell rate measurement**

For measuring the dead cell rate, cells were stained with 0.2% Trypan Blue stain (T10282, Thermo Scientific) for 5 min at room temperature. At least 500 cells were counted in a counting chamber and the percentage of cells stained with trypan blue was measured.

**Immunohistochemistry**

Formalin-fixed paraffin-embedded tissue sections obtained from tumor samples developed in the KPC autochthonous mouse model of pancreatic ductal adenocarcinoma[26] were deparaffinized, followed by antigen retrieval by boiling the samples in Target-Retrieval Solution (Dako An Agilent Technologies Company, Carpinteria, CA, USA) at pH 9 for 30 min and conventional staining procedures, as described previously[26], using an anti-phospho-myosin light chain 2 (Thr18/Ser19) (3674S, Cell Signaling Technology) antibody. Mouse spinal cord tissues were fixed in 4% (w/v) paraformaldehyde (PFA) overnight, dehydrated in serial methanol washes, and embedded in paraffin. Serial sections (6 μm) embedded in paraffin were prepared for immunohistochemical analysis. Primary antibody against 2P-MRLC (3674S, Cell Signaling Technology) was used with 1% goat serum in PBS. Antigen retrieval was performed using citrate buffer, incubated at 121 °C for 1 min. Primary antibodies were detected using Alexa Fluor 546-conjugated IgG (Molecular Probes, Oregon, USA) and counterstained with Hoechst 33342 (Sigma-Aldrich). The fluorescent signal was visualized using an Olympus BX51 fluorescence microscope, and images were captured using cellSens Standard 1.6 (Olympus Corporation, Tokyo, Japan) software.

**Results**

**Stiff substrates promote the nuclear localization of 2P-MRLC**

Previous studies have shown that 2P-MRLC exists in the nucleus [20]. MRLC acts as a transcription factor[20-22]. It is phosphorylated in response to stiff substrates[14]. However, whether substrate stiffness controls the nuclear localization of MRLC remains unknown. Therefore, we determined 2P-MRLC localization in HeLa cells on stiff or soft substrates. For these substrates, we used collagen-I-coated polyacrylamide hydrogels; the stiffness of soft substrates was 0.4 kPa and that of stiff substrates was 271 kPa. We found that 2P-MRLC tended to localize to the nuclei of cells on stiff substrates (Fig. 1A and D). Total-MRLC also localized to the nuclei in cells on stiff substrates, although the rate of change was smaller than that of 2P-MRLC (Fig. 1C and D). In contrast, nuclear localization of 1P-MRLC was not affected by stiff substrates (Fig. 1B–D). In addition, western blotting with nuclear and cytosolic extracts showed that both nuclear and cytosolic 2P-MRLC levels were increased on stiff substrates (Fig. 1E and F). Moreover, 2P-MRLC expression increased significantly in the nucleus than in the cytoplasm in response to stiff substrates. The results suggest that the nuclear localization of MRLC in response to substrate stiffness is regulated by the diphosphorylation state of MRLC at Thr18 and Ser19. Furthermore, we found that stiff substrates significantly localized 2P-MRLC to the nucleus in A431 human epidermoid cancer cells, A549 human lung cancer cells, human umbilical vein endothelial cells (HUVECs), and mouse mesenchymal stem cells (MSCs) (Fig. 1G; Fig. S3A–D). The results suggest that substrate stiffness enhances the nuclear localization of 2P-MRLC. Finally, we confirmed the changes in the nuclear localization of 2P-MRLC on substrates of various elasticities between 0.4 kPa and 271 kPa. The results showed that the nuclear localization of 2P-MRLC was significantly promoted as the substrate stiffened from 1.2 to 61 kPa (Fig 1H and I). In contrast, the nuclear localization of 2P-MRLC did not change significantly in the range of 61 to 134 kPa (Fig 1H and I). These results suggest that cells respond to differences in substrate stiffness between 1.2 and 61 kPa, causing changes in the nuclear localization of 2P-MRLC.

**Nuclear localization of ZIPK regulated by substrate stiffness increases the localization of 2P-MRLC to the nucleus**

Next, we focused on ZIPK (DAPK3) to identify the mechanism by which stiff substrates localize 2P-MRLC to the nucleus. ZIPK promotes the di-phosphorylation of MRLC[27]. To investigate whether ZIPK regulated the nuclear localization of 2P-MRLC, we downregulated ZIPK expression using specific siRNAs (Fig. S4A and B). We evaluated the nuclear localization of 2P-MRLC in ZIPK-knockdown (KD) and negative control (NC) cells on stiff glass substrates. ZIPK-KD cells exhibited suppressed nuclear localization of 2P-MRLC (Fig. 2A and B). Next, we confirmed whether inhibition of ZIPK activity affected the nuclear localization of 2P-MRLC. The cells treated with a ZIPK inhibitor exhibited suppressed nuclear localization of 2P-MRLC (Fig. 2C and D). ZIPK inhibitors also inhibited the activity of death-associated protein kinase 1 (DAPK1). To confirm the effect of DAPK1 on the nuclear localization of MRLC, we downregulated DAPK1 expression using specific siRNAs (Fig. S5A). Nuclear localization of 2P-MRLC was not affected in DAPK1-KD cells (Fig. S5B and C). The results indicate that ZIPK, but not DAPK1, enhances the nuclear localization of 2P-MRLC.

We further investigated whether the activity of other typical kinases of MRLC, myosin light-chain kinase (MLCK) and Rho-associated protein kinase (ROCK), enhanced the nuclear localization of 2P-MRLC. In particular, we confirmed whether the nuclear localization of 2P-MRLC changes in cells treated with ML-7, an MLCK inhibitor, or Y-27632, a ROCK inhibitor. Both inhibitors did not affect the nuclear localization of 2P-MRLC (Fig. S6A and B). These results indicate that ZIPK is a specific kinase for nuclear MRLC.

Next, we focused on ZIPK regulation by substrate stiffness. ZIPK has a nuclear localization signal in its amino acid sequence [28]. Because ZIPK diphosphorylates nuclear MRLC (Fig. 2C), we hypothesized that stiff substrates enhance the nuclear localization of ZIPK. We conducted western blotting for nuclear or cytosolic extracts from HeLa cells on soft (0.4 kPa) or stiff (1 MPa<) substrates and compared the ZIPK levels in these extracts. We found that ZIPK levels were significantly increased in the nucleus and reduced in the cytosol of cells on stiff substrates (Fig. 2E and F). As shown in Fig. 2E, the molecular weights of ZIPK in the nuclear and cytosolic extracts differed. We verified that the bands corresponding to both molecular weights were decreased in intensity by the knockdown of ZIPK using siRNA (Fig. S4B). In addition, we found that treatment with a ZIPK inhibitor decreased nuclear ZIPK and increased cytosolic ZIPK (Fig. S7). Therefore, ZIPK in the nucleus and cytosol may represent activated and inactivated forms of ZIPK, respectively. These results indicate that the nuclear localization of ZIPK, which is regulated by substrate stiffness, enhances the nuclear localization of 2P-MRLC.

**Actin fiber regulates the nuclear localization of 2P-MRLC and ZIPK**

Next, we aimed to clarify the detailed mechanisms underlying the stiffness-dependent nuclear localization of 2P-MRLC via ZIPK. The actin cytoskeleton, which generates, transmits, and responds to mechanical stress, appears to be a central player in mechanotransduction[29]. In fact, stabilization of the actin cytoskeleton by mechanical stimulation localizes the transcription factor YAP to the nucleus[30]. Moreover, it is also reported that actin fiber disintegration by inhibition of actin polymerization suppresses the nuclear localization of YAP in response to stiffness[16]. From these perspectives, we hypothesized that stabilization of actin filaments in response to substrate stiffness regulates the nuclear localization of 2P-MRLC and ZIPK. First, we investigated whether treatment with jasplakinolide, an inhibitor of actin depolymerization, affected the nuclear localization of 2P-MRLC. Cells treated with jasplakinolide exhibited promoted-nuclear localization of 2P-MRLC on soft substrates (Fig. 3A and B). Next, we investigated whether treatment with latrunculin A, an inhibitor of actin polymerization, regulated the nuclear localization of 2P-MRLC. Cells treated with latrunculin A exhibited suppressed nuclear localization of 2P-MRLC (Fig. 3C and D). To confirm whether actin fiber stabilization promotes the nuclear localization of ZIPK, we performed western blotting of ZIPK for nuclear or cytosolic extracts from HeLa cells on stiff (1 MPa<) substrates treated with or without latrunculin A. This result showed that ZIPK levels were reduced in the nucleus and increased in the cytosol after treatment with latrunculin A (Fig. 3E and F). These results indicate that actin fibers promoted the nuclear localization of ZIPK and 2P-MRLC.

Actin fiber disintegration disturbs actomyosin contraction and actin fiber stabilization. It has also been reported that actomyosin contraction induced by substrate stiffness activates transcription factors that mediate the mechanotransduction signaling pathway[14,16]. Thus, in order to investigate the effects of contraction force on nuclear localization of 2P-MRLC, we investigated whether the nuclear localization of 2P-MRLC is affected in cells by treatment with blebbistatin, a myosin II inhibitor, or Y-27632, a ROCK inhibitor; both blebbistatin and Y-27632 inhibit actomyosin contraction[31,32]. The results showed that these inhibitors did not affect the nuclear localization of 2P-MRLC (Fig. S6B and C). The data suggest that the substrate stiffness-mediated actin fiber stabilization enhances nuclear localization of 2P-MRLC, whereas actomyosin contraction does not affect nuclear localization of 2P-MRLC.

**Nuclear localization of 2P-MRLC suppresses MafB expression**

Next, we examined whether the nuclear localization of 2P-MRLC, in response to substrate stiffness, showed specific functions. Nuclear MRLC has been reported to function as a transcription factor[20-22]. In a gastric cancer cell line, MRLC binds to the CTNNB1 core promoter region[21]. Therefore, we first investigated whether nuclear 2P-MRLC in cells on the stiff substrate actually binds to the CTNNB1 core promoter region by ChIP-PCR. We found that 2P-MRLC bound to the CTNNB1 promoter region in cells on stiff substrates (Fig. 4A). Based on these results, we hypothesized that nuclear 2P-MRLC functions as a transcription factor and regulates specific gene expression in response to substrate stiffness. Therefore, we performed qPCR screening and explored genes which is 1.15 times up- or downregulated by stiff substrates (Fig. S8A). For these genes, we performed qPCR screening and explored the genes which is 1.15 times up- or downregulated by ZIPK activation (Fig. S8B). Finally, for these genes, we performed qPCR screening and explored genes which is 1.15 times up- or down-regulated by MRLC expression (Fig. S8C). Next, we confirmed whether the initial screening results were correct. First, to examine whether MRLC regulates MafB expression, we downregulated MRLC expression with specific siRNAs (Fig. S4C–G). This result showed MRLC-KD enhanced MafB mRNA expression in HeLa, A431, A549, and MSC cells (Fig. 4B). Next, we investigated MafB mRNA expression in HeLa cells on soft (0.4 kPa) or stiff (271 kPa) substrates. This result showed that MafB expression was suppressed on stiff substrates (Fig. 4C). We also examined MafB mRNA expression in cells treated with a ZIPK inhibitor that suppressed the nuclear localization of 2P-MRLC. This result showed that the ZIPK inhibitor increased MafB expression (Fig. 4D). In addition, we confirmed that the inhibition of ZIPK promoted MafB protein expression by western blotting (Fig. 4E). These results indicate that the nuclear localization of 2P-MRLC triggered by ZIPK in response to substrate stiffness suppressed MafB expression.

**Nuclear localization of 2P-MRLC suppresses apoptosis via downregulation of MafB expression**

Next, we examined the specific cellular response caused by the suppression of MafB expression mediated by nuclear 2P-MRLC in cells on stiff substrates. MafB promotes apoptosis during limb morphogenesis[33]. Thus, we hypothesized that suppression of MafB expression, which is induced by the nuclear localization of 2P-MRLC in response to substrate stiffness, prevents apoptosis. We first evaluated the cell death rates and levels of cleaved-caspase3, which is a typical apoptosis marker, in HeLa cells on stiff (0.4 kPa) or soft (271 kPa) substrates. The results showed that cells on stiff substrates exhibited reduced cell death rates and cleaved-caspase3 levels (Fig. 5A and B). Second, we examined the cell death rates and cleaved-caspase3 levels in NC, MRLC-KD, MRLC, and MafB double KD cells. The results showed that MRLC-KD cells exhibited increased cell death rates and cleaved-caspase3 levels, whereas MafB KD rescued this phenomenon in MRLC-KD cells (Fig. 5C and D). Finally, we confirmed that MafB overexpression promoted cell death (Fig. 5E). These results indicate that substrate stiffness drives the suppression of apoptosis via the downregulation of MafB expression and ZIPK-dependent nuclear localization of 2P-MRLC.

**2P-MRLC localizes to the nucleus in various tissues**

Finally, we examined whether 2P-MRLC was present in the nucleus *in* *vivo*. We found that 2P-MRLC was weakly present in the nuclei of some stromal and inflammatory cells that constitute tumors developed in the KPC autochthonous mouse model of pancreatic ductal adenocarcinoma (Fig. S9A). It was also found to be present in the nuclei of cells in the P1 male mouse spinal cord (Fig. S9B). These results indicate that 2P-MRLC is present in the nuclei of cells *in vivo.*

**Discussion**

Our results suggest a new mechanotransduction pathway mainly based on the nuclear localization of 2P-MRLC (Fig. 6). We found that substrate stiffness enhanced the nuclear localization of 2P-MRLC. Furthermore, localization is promoted by nuclear localization of ZIPK in response to substrate stiffness. In addition, actin fiber formation in response to substrate stiffness activates the nuclear localization of 2P-MRLC via ZIPK. Finally, we found that nuclear localization of 2P-MRLC downregulated MafB expression and suppressed apoptosis. Our findings provide a novel mechanism for restricting apoptosis via the nuclear localization of 2P-MRLC, which is regulated by substrate stiffness.

In this study, we revealed that ZIPK localizes to the nucleus in response to substrate stiffness via actin polymerization, resulting in the nuclear localization of 2P-MRLC. However, the detailed mechanism remains unknown. Regarding ZIPK nuclear localization, previous research showed that dephosphorylation of T299 or inactivation of the leucine zipper (LZ) domain in ZIPK promotes the nuclear localization of ZIPK[34,35]. Actin polymerization in response to substrate stiffness may regulate the dephosphorylation of T299 or inactivation of the LZ domain in ZIPK to promote its nuclear localization. Regarding the means by which ZIPK regulates the nuclear localization of 2P-MRLC, since ZIPK has a nuclear localization sequence that mediates active transport to the nucleus[28], but MRLC does not have such a sequence, it is possible that ZIPK actively transports MRLC to the nucleus in response to substrate stiffness. Another possibility is that MRLC passively diffuses into the nucleus. Molecules of 40 kDa or less have been reported to passively diffuse into the nucleus of HeLa cells[36]. It has also been reported that substrate stiffness promotes the passive diffusion of molecules into the nucleus by nuclear stretching and opening of nuclear membrane pores [37]. Substrate stiffness may passively localize MRLC to the nucleus, followed by its phosphorylation by nuclear ZIPK.

We confirmed that the signals for 2P-MRLC were found in the nuclei of some cells that constitute tumors that developed in the KPC mouse model of pancreatic cancer (Fig. S9A). We revealed that nuclear 2P-MRLC suppressed MafB expression and apoptosis in response to stiffness. It has been reported that pancreatic cancer tissue is stiff than normal pancreatic tissue[38]. In addition, pancreatic cancer cells on stiff substrates have been shown to exhibit a phenotype that promotes cancer malignancy[38,39]. Increased expression of lysyl oxidase-like 2, which promotes substrate stiffening, increases the number of 2P-MRLC-positive cells in pancreatic ductal adenocarcinoma[40]. These reports suggest that nuclear 2P-MRLC is an important therapeutic target for the malignant transformation of cancer associated with pancreatic tumor stiffening. Of note, we found that 2P-MRLC existed in the nuclei of stromal cells in KPC mouse pancreatic tumors (Fig. S9A). Stromal cells, such as immune cells and fibroblasts, in the cancer microenvironment, which have recently attracted attention as targets for cancer therapy, have been reported to respond to substrate stiffness[41]. Nuclear 2P-MRLC may indirectly contribute to cancer progression by affecting the phenotype of stromal cells within the tumor stroma. Nuclear 2P-MRLC may be a potential therapeutic target for inhibiting cancer progression caused by tumor stiffening.

We found that 2P-MRLC bound to the CTNNB1 (-catenin gene) promoter region in cells on the stiff substrate (Fig. 4A). It has been reported that MRLC bound to the CTNNB1 promoter region and promote CTNNB1 expression in gastric cancer cells[21]. Therefore, 2P-MRLC interaction with the promoter region of CTNNB1 may also increase CTNNB1 expression. Previous studies have shown that -catenin activation due to substrate stiffness promotes tumor growth and intravasation of cancer cells[39,42]. 2P-MRLC may be an upstream factor that regulates substrate-stiffness-dependent -catenin-mediated cancer malignancy.

In conclusion, our findings have revealed the substrate-stiffness-dependent nuclear localization of 2P-MRLC and a part of its localization mechanism. These findings provide new insights into the mechanism by which cells respond to substrate stiffening.

**Conflict of interest**

The authors report no conflict of interest regarding this study.

**Author Contributions**

K.O., S.I., M.T., A.S., A.E., K.S., and H.H. conceived and designed the experiments. K.O., M.T., A.S., A.E., and K.S. prepared the experimental materials. K.O., S.I., A.S., A.E., and K.S. performed the experiments. K.O., S.I., A.S., A.E., and K.S. interpreted and analyzed the data. K.O., S.I., A.E., K.S., and H. H. wrote the manuscript.

**Acknowledgements**

This work was supported by JSPS KAKENHI (Grant Numbers 21K07142 to H.H., 21K07141 to S.I.); Advanced Research and Development Programs for Medical Innovation by Japan Agency for Medical Research and Development to H.H. and A.E.; the cancer research grant of SGH Foundation to S.I.; Grants-in-Aid for Regional R&D Proposal-Based Program from Northern Advancement Center for Science & Technology of Hokkaido Japan to S.I.; the research grant of Astellas Foundation for Research on Metabolic Disorders to S.I.; the research grant from The Uehara Memorial Foundation to S.I.; donations from MD Mariko Takamura to H. H.; and a grant from JST SPRING (Grant Number JPMJSP2119) to K.O.

**Data Availability**

The data that supports the findings of this study are available in Figs 1-6 and the supplementary material of this article.

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**Figures and Figure Legends**

**Fig. 1│Stiff substrates promote the nuclear localization of 2P-MRLC.**

(A–C) Representative immunofluorescent images of 2P-MRLC (A), 1P-MRLC (B), or total-MRLC (C) and nucleus in HeLa cells on soft (0.4 kPa) or stiff (271 kPa) polyacrylamide hydrogel substrates coated with collagen I. (D) Quantification of the fluorescent intensities of 2P-MRLC, 1P-MRLC, and total-MRLC in the nucleus relative to those in the cytosol (A–C). n = at least 60 cells in three independent experiments. (E) Representative western blots of the nuclear or cytosolic extracts of HeLa cells on soft (0.4 kPa) or stiff (271 kPa) polyacrylamide hydrogel substrates coated with collagen-I using anti-2P-MRLC, anti--tubulin, and anti-LaminA/C antibodies. (F) Relative 2P-MRLC expression of (E). Ratio of 2P-MRLC to internal control is shown. LaminA/C and -tubulin were used as the internal controls for nuclear and cytosolic extracts, respectively. n = 3 experiments. (G) Quantification of the fluorescent intensity of 2P-MRLC in the nucleus relative to that in the cytosol in several cell lines and primary cells of Fig. S3A–D. n= at least 60 cells in three independent experiments. (H) Representative immunofluorescent images of 2P-MRLC and the nucleus in HeLa cells on 1.2, 2.3, 25, 61, 83, or 134 kPa polyacrylamide hydrogel substrates coated with collagen I. (I) Quantification of the fluorescence intensities of 2P-MRLC in the nucleus relative to those in the cytosol (H). Scale bars in (A–C) and (H) are 20 m. Bars represent mean ± standard error of the mean (SEM). n.s., not significant. Statistical significance was determined using an Student’s *t*-test in (D) and an Welch’s *t*-test in (G) and (I). The individual values were tested for significant differences using the Bonferroni correction in (I). \*Statistical significance was determined with a 95% confidence interval in (F).

**Fig. 2│Nuclear localization of ZIPK induced by substrate stiffness localizes 2P-MRLC to the nucleus.**

(A) Representative immunofluorescent images of 2P-MRLC and nucleus in HeLa cells on stiff glass substrates coated with collagen-I after transfection with the negative control of short interfering RNA (siRNA) (control) or siRNA targeting ZIPK (siZIPK). (B) Quantification of the fluorescent intensity of 2P-MRLC in the nucleus relative to that in the cytosol of (A). n = at least 60 cells in three independent experiments. (C) Representative immunofluorescent images of 2P-MRLC and nucleus in HeLa cells on stiff glass substrates coated with collagen-I after treatment with dimethyl sulfoxide (DMSO) (control) or ZIPK inhibitor. (D) Quantification of the fluorescent intensity of ZIPK in the nucleus relative to that in the cytosol of (C). n = at least 60 cells in three independent experiments. (E) Representative western blots of the nuclear or cytosolic extracts of HeLa cells on soft (0.4 kPa) polyacrylamide hydrogel or stiff plastic (>1MPa) substrates coated with collagen-I using anti-ZIPK, anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and anti-LaminA/C antibodies. (F) Relative ZIPK expression of (E). Ratio of ZIPK to internal control is shown. LaminA/C and GAPDH were used as the internal controls for nuclear and cytosolic extracts, respectively. n = at least three independent experiments. Scale bars in (A and C) are 20 m. Bars represent the mean ± SEM. Statistical significance was determined using an Student’s *t*-test.

**Fig. 3│Actin polymerization regulates the nuclear localization of 2P-MRLC and ZIPK.**

(A) Representative immunofluorescent images of 2P-MRLC and nucleus in HeLa cells on soft (0.4 kPa) polyacrylamide hydrogel substrates coated with collagen-I after treatment DMSO or jasplakinolide (Jasp). (B) Quantification of the fluorescent intensity of 2P-MRLC in the nucleus relative to that in the cytosol of (A). n = at least 60 cells in three independent experiments. (C) Representative immunofluorescent images of 2P-MRLC and nucleus in HeLa cells on stiff glass substrates coated with collagen-I after treatment with DMSO or latrunculin A (LatA). (D) Quantification of the fluorescent intensity of 2P-MRLC in the nucleus relative to that in the cytosol of (C). n= at least 105 cells in three independent experiments. (E) Representative western blots of ZIPK in the nuclear or cytosolic extracts from HeLa cells on stiff plastic (1MPa<) substrates coated with collagen-I after treatment with DMSO (Control) or LatA. (F) Relative ZIPK expression of (E). Ratio of ZIPK to internal control is shown. LaminA/C and -tubulin were used as the internal controls for nuclear and cytosolic extracts, respectively. n = 3 experiments. Scale bars in (A and C) are 20 m. Bars represent the mean ± SEM. Statistical significance was determined using an student’s *t*-test except (F). \*Statistical significance was determined with a 95% confidence interval in (F).

**Fig. 4│Nuclear localization of 2P-MRLC suppresses the** **MAF bZIP transcription factor B (MafB) expression.**

(A) Chromatin immunoprecipitation (ChIP)-polymerase chain reaction (PCR) amplification using primers against the catenin beta 1 (CTNNB1) core promoter region. PCR products were separated via agarose gel electrophoresis; 2P-MRLC bound to the CTNNB1 core promoter. IgG served as a negative control. (B) Relative mRNA expression of MafB/endogenous control detected via quantitative PCR (qPCR) in HeLa cells, A431 cells, A549 cells, and mesenchymal stem cells (MSCs) on stiff plastic substrates after transfection with the negative control of siRNA (control) or siRNA targeting MRLC (siMRLC). Endogenous control gene, *GAPDH*, in HeLa, A431, and A549 cells or *s18* in MSCs. (C) Relative mRNA expression of MafB/GAPDH determined via qPCR in HeLa cells on soft (0.4 kPa) or stiff (271 kPa) polyacrylamide hydrogel substrates. (D) Relative mRNA expression of MafB/GAPDH determined via qPCR in HeLa cells on stiff plastic substrates after treatment with or without ZIPK inhibitor. (E) Representative western blots (left) and quantification (right) of MafB levels in HeLa cells on stiff plastic substrates after treatment with DMSO (control) or ZIPK inhibitor. n = 3 independent experiments. Bars represent the mean ± SEM. Statistical significance was determined using an Welch’s *t*-test except (E). \*Statistical significance was determined with a 95% confidence interval in (E).

**Fig. 5│Nuclear localization of 2P-MRLC suppresses apoptosis via downregulation of MafB expression.**

(A) Cell death rate evaluated via trypan blue staining in HeLa cells on soft (0.4 kPa) or stiff (271 kPa) substrates. (B) Representative western blots (left) and quantification (right) of cleaved caspase3 levels in HeLa cells on soft (0.4 kPa) or stiff (271 kPa) polyacrylamide hydrogel substrates. (C) Cell death rate evaluated via trypan blue staining in HeLa cells on stiff plastic substrates after transfection with the negative control of siRNA (control), siRNA targeting MRLC (siMRLC), or siRNA targeting MRLC and MafB (siMRLC + siMafB). (D) Representative western blots (left) and quantification (right) of cleaved caspase3 levels in HeLa cells after transfection with the negative control of siRNA (control), siRNA targeting MRLC (siMRLC), or siRNA targeting MRLC and MafB (siMRLC + siMafB). (E) Cell death rate evaluated via trypan blue staining in HeLa cells on stiff plastic substrates after transfection with GFP or MafB-GFP vectors. n = 3 experiments except (E). n = 4 experiments in (E). Bars represent the mean ± SEM. Statistical significance was determined using an Student’s *t*-test in (A-C) and Welch’s *t*-test in (F). The individual values were tested for significant differences using the Bonferroni correction in (C) \*Statistical significance was determined with a 95% confidence interval in (D).

**Fig. 6│Nuclear localization of 2P-MRLC in response to substrate stiffness suppresses apoptosis.**

Actin fiber formation responded to substrate stiffness activates the nuclear localization of 2P-MRLC via ZIPK. Then, nuclear localization of 2P-MRLC downregulate MafB expression and suppress apoptosis.