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Title	Substrate stiffness regulates temporary NF-kappa B activation via actomyosin contractions
Author(s)	Ishihara, Seiichiro; Yasuda, Motoaki; Mizutani, Takeomi et al.
Citation	Experimental cell research, 319(19), 2916-2927 https://doi.org/10.1016/j.yexcr.2013.09.018
Issue Date	2013-11-15
Doc URL	https://hdl.handle.net/2115/57311
Type	journal article
File Information	Ishihara_ECR_2014_final-1.pdf



1 **Title:** Substrate stiffness regulates temporary NF- κ B activation via actomyosin contractions

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Abstract

Physical properties of the extracellular matrix (ECM) can control cellular phenotypes via mechanotransduction, which is the process of translation of mechanical stresses into biochemical signals. While current research is clarifying the relationship between mechanotransduction and cytoskeleton or adhesion complexes, the contribution of transcription factors to mechanotransduction is not well understood. The results of this study revealed that the transcription factor NF- κ B, a major regulator for immunoreaction and cancer progression, is responsive to substrate stiffness. NF- κ B activation was temporarily induced in H1299 lung adenocarcinoma cells grown on a stiff substrate but not in cells grown on a soft substrate. Although the activation of NF- κ B was independent of the activity of integrin β 1, an ECM-binding protein, the activation was dependent on actomyosin contractions induced by phosphorylation of myosin regulatory light chain (MRLC). Additionally, the inhibition of MRLC phosphorylation by Rho kinase inhibitor Y27632 reduced the activity of NF- κ B. We also observed substrate-specific morphology of the cells, with cells grown on the soft substrate appearing more rounded and cells grown on the stiff substrate appearing more spread out. Inhibiting NF- κ B activation caused a reversal of these morphologies on both substrates. These results suggest that substrate stiffness regulates NF- κ B activity via actomyosin contractions, resulting in morphological changes.

1 **Keywords**

2 actomyosin; lung adenocarcinoma cell; NF- κ B; mechanotransduction; substrate stiffness

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Introduction

Mechanical cues, which include mechanical forces and physical properties of the surrounding environment, modulate cellular phenotypes. For example, substrate stiffness affects cell adhesion [1], direction of migration [2], and collectiveness of migration [3] in various cell types. External deformation of an elastic substrate induces cellular stiffness responses in fibroblasts [4]. In addition, mechanical cues regulate the differentiation state of mesenchymal stem cells *in vitro* [5] and morphogenesis *in vivo* [6]. Mechanical cues are also related to specific diseases [7]. In breast cancer, a stiffer extracellular matrix can induce more malignant phenotypes [8]. In fibrosarcoma, mechanical stimuli increase the proportion of invading cells in type I collagen/fibronectin matrices [9]. In blood vessels, extraordinary shear stress induces atherosclerosis [10]. Thus, aberrant mechanical cues can affect cellular phenotypes and promote certain types of diseases.

When cells sense mechanical cues, they convert them into biochemical signals. This conversion is called “mechanotransduction.” Previous studies have identified several molecules whose primary responsibility is to sense mechanical stresses in cells. These molecules are called “mechanosensors” and include molecules such as p130Cas [11] and filamin [12], which undergo structural deformation in response to mechanical stresses in cells. Cells alter the protein expression levels of adhesion complexes and reorganize their cytoskeletons, as a consequence

1 of sensing mechanical stresses [13]. Following this process, adhesion complexes and
2 cytoskeletons regulate cellular phenotypes such as migratory activity and morphology [1, 2].
3 Several primary and terminal mechanotransduction molecules have recently been identified [11,
4 12, 13], but the transcriptional molecules that regulate gene expression are not well understood.

5 The transcription factor NF- κ B is a well-known regulator of immunoreaction and cancer
6 progression and responds to chemical stimuli, DNA damage, and reactive oxygen species.
7 Activated NF- κ B induces the expression of cytokines (IL-1 β , IL-8, etc.) and cancer regulatory
8 genes (TNF α , MMP9, etc.) [14, 15, 16, 17]. NF- κ B consists of 5 subunits (RelA/p65, p105/p50,
9 p100/p52, RelB, and c-Rel), which form homo- or heterodimers [18]. Once the stimuli are
10 received, NF- κ B dimers are translocated from the cytoplasm to the nucleus, where they function
11 as transcriptional activators. NF- κ B activation is also induced by mechanical stresses such as
12 uniaxial stretch [19, 20] and shear stress [21].

13 Previous studies have suggested that NF- κ B activation is regulated by various stimuli via
14 cytoskeleton and adhesion molecules. Actomyosin is one of these cytoskeletal complexes,
15 consisting of actin and myosin, which generates tensional force in cells. The tensional force in
16 actomyosin is regulated by phosphorylation of MRLC. Di-phosphorylated MRLC provokes
17 much stronger tensional force in actomyosin than mono- or non-phosphorylated MRLC does
18 [22]. Previous research has revealed that mechanical cues induce MRLC phosphorylation via

1 the RhoA signaling cascade [23]. Furthermore, MRLC regulates NF- κ B activity by
2 TNF α -induced RhoA activation [24]. Integrins are heterodimeric glycoproteins that directly
3 bind extracellular matrices (ECM) and activate intracellular signaling pathways when they
4 connect to ECMs [25]. The heterodimers of integrins are composed of α and β chains. Integrin
5 β 1 is a major component of the collagen receptor and has been reported to regulate NF- κ B
6 activity [26, 27]. Thus, MRLC and integrin β 1 are potential regulators of NF- κ B activation by
7 mechanical stimuli.

8 Based on these previous studies, we proposed the hypothesis that substrate stiffness
9 regulates NF- κ B activation and changes the expression of NF- κ B-related genes. In this study,
10 we showed that stiff substrates induce temporary NF- κ B activation and up-regulate IL-1 β , IL-8,
11 and MMP9 mRNA expression in H1299 lung adenocarcinoma cells. Furthermore, NF- κ B
12 activation does not require cell-substrate adhesion by integrin β 1, but does require actomyosin
13 contraction depending on MRLC phosphorylation. Finally, NF- κ B activity regulates cell
14 morphology, and high NF- κ B activity induces cells to exhibit a spreading shape. These data
15 collectively suggest that NF- κ B activation is triggered by a stiff substrate via cytoskeletal
16 tensions, leading to inflammatory reactions and spreading cell shape.

Materials and Methods

Cell culture, reagents, antibodies, plasmids

Human lung adenocarcinoma cell line H1299 cells were cultured in DMEM (Sigma, St. Louis, MO) containing 10% FBS (BIST TECH) and 1% antibiotic/antimycotic solution (Sigma). Monoclonal antibody AIIB2 (Developmental Studies Hybridoma Bank at the University of Iowa, Iowa City, IA) was used for inhibition of integrin $\beta 1$ activity. Y27632 (Sigma), blebbistatin (TRC, Toronto, Canada), and 1-oleoyl-lysophosphatidic acid (LPA, Cayman Chemical, Ann Arbor, MI) were used for Rho-associated protein kinase (ROCK) inhibition, myosin motor activity inhibition, and Rho activation, respectively. Anti-NF- κ B p65 (IBL, 18667), Alexa Fluor 594 goat anti-rabbit IgG (Invitrogen, Carlsbad, CA, A-11012), anti-paxillin monoclonal antibody (BD Biosciences, San Jose, CA, 610052), phospho-myosin light chain 2 (Ser19/Thr18) antibody (anti PP-MRLC; Cell Signaling Tech, Danvers, MA, #3674), Alexa Fluor 546 goat anti-mouse IgG (Invitrogen, A-11003), and Alexa Fluor 546 goat anti-rabbit IgG (Invitrogen, A-11010) were used for immunofluorescent staining and western blotting. Myosin light chain 2 antibody (anti-MRLC; Cell Signaling Tech, #3672), phospho-myosin light chain 2 (Ser19) antibody (anti-P-MRLC; Cell Signaling Tech, #3671), anti-GAPDH (Ambion, Foster City, CA, 1103016), HRP anti-rabbit IgG (Cell Signaling Tech, #7074), and HRP anti-mouse IgG (Bio-Rad Hercules, CA, 70-6516) were used for western blotting. MFP 488-phalloidin (Mo Bi

Tee, Göttingen, Germany) or Alexa Fluor 546 phalloidin (Invitrogen, A22283) was used for F-actin staining. Rat NF- κ B (p65) gene was obtained by RT-PCR from a cDNA library and cloned into pDsRed-Monomer-N1 or pEGFP-N1 (BD Biosciences). For luciferase reporter assay, pNF- κ B-Luc, pTK-RL, and pcDNA3 plasmids were purchased from Stratagene, Promega, and Invitrogen, respectively. Transfection of plasmids was performed with Lipofectamine 2000 (Invitrogen) or polyethyleneimine. For selection of transgenic cells, G418 (Promega, Madison, WI) reagent was used at a concentration of 0.6–0.8 mg/mL. H-NLS or H1299-p65-1/p65-2 cells were established by selecting the transgenic cells from H1299 cells transfected with NLS-venus or Rat-p65wt-EGFP plasmid. Human TNF α (Cell Signaling Technology) and BAY-11-7082 (Sigma) were used for NF- κ B activation and NF- κ B inhibition, respectively.

ECM coating and collagen gel substrates

For stiff substrates, we prepared silanized cover glass (>1 GPa [8]) coated with type-I collagen (Cell matrix I-C; Nitta Gelatin, Osaka, Japan). ECM-coated silanized cover glass is a more stable and uniform ECM substrate than non-treated cover glass coated with the same ECM. To prepare silanized cover glasses, we soaked cover glasses in saturated KOH in isopropanol solution and incubated them overnight at room temperature. The cover glasses were washed 3 times with distilled water and dried. We soaked the cover glasses in 0.33%

(3-aminopropyl)triethoxysilane in toluene solution and incubated them overnight at room temperature. The cover glasses were washed 3 times with toluene and dried. For stiff substrates, we also prepared collagen type-I-coated plastic dishes or 24-well plates (>1 GPa [8]). For soft substrates, we prepared 1.6 mg/mL type-I collagen gels (approximately 0.6 kPa, based on previously reported methods [28]; Cell matrix I-P, Nitta Gelatin). Plastic 24-well plates were coated with fibronectin (Roche, Mannheim, Germany), laminin (Trevigen, Gaithersburg, MD), or poly-L-lysine (Sigma), and used for ECM-coated stiff substrates.

Polyacrylamide gel substrate

Polyacrylamide gels were prepared as substrates of different stiffness [29]. The stiffness of each gel was measured as previously reported [30]. Gels of 30.1 kPa (containing 0.16% N,N'-methylenebisacrylamide (BIS), 7.5% acrylamide, 115 mM N-acryloyl-6-aminocaproic acid (ACA)), 2.0 kPa (containing 0.01% BIS, 7.5% acrylamide, 240 mM ACA), 13.8 kPa (containing 0.08% BIS, 7.5% acrylamide, 150 mM ACA), and 67.2 kPa (containing 0.32% BIS, 7.5% acrylamide, 90 mM ACA) were polymerized for 1 h at room temperature. The gels were fully hydrated in PBS and transferred to MES buffer (0.1 M MES, 0.5 M NaCl; pH, 6.1). Carboxyl groups of the ACA gels were activated with 0.2 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and 0.5 M

1 *N*-hydroxysuccinimide in MES buffer for 15 min at room temperature. The gels were then
2 washed with cold 70% methanol diluted with PBS before they were reacted with 0.2 mg/mL
3 type-I collagen (AteroCell IAC-50; KOKEN, Tokyo, Japan) in HEPES buffer (0.5 M HEPES;
4 pH 9.0) overnight at 4°C. Finally, the gels were transferred to 0.5 M ethanolamine in HEPES
5 buffer for reaction with any remaining active carboxyl groups. The gels were washed with
6 HEPES buffer and then 3 times with PBS. All gels were sterilized with UV light in a sterile
7 hood and equilibrated in culture medium before cell plating.

9 **Immunofluorescence staining**

10 H1299 and H1299-p65-2 cells ($1-2 \times 10^4$) were seeded on collagen-coated cover
11 glasses or collagen gels and cultured for 0.5, 2, 4, or 6 h. After culturing, the cells were fixed
12 with PFA (4%, room temperature, 10 min) and washed 3 times. Next, Triton-X100 (0.5%)
13 permeabilization was performed for 10 min at room temperature. The samples were washed 3
14 times. Blocking treatment with 0.5% BSA (for p65 and paxillin staining) or 0.5% skim milk
15 (PP-MRLC staining) in PBS was performed for 1 h at room temperature. Primary antibody
16 solution (p65 1:200 in PBS, paxillin 1:200 in PBS, PP-MRLC 1:150 with 0.5% skim milk in
17 PBS) was added for incubation overnight at 4°C. After 3 washes, secondary antibody solution
18 (Alexa Fluor 594 goat anti rabbit IgG 1:200 for p65 staining, Alexa Fluor 546 goat anti mouse

IgG, 1:500 for paxillin staining, Alexa Fluor 546 goat anti rabbit IgG, 1:500 for PP-MRLC staining) with or without F-actin staining solution (MFP 488-phalloidin, 1:1000 or Alexa Fluor 546 phalloidin, 1:500) was added, and the mixtures were incubated for 1 h at room temperature. After 3 washes, anti-fading solution (2.5% DABCO, 90% glycerol, 6% PBS; pH 8.0) was added. The resulting images were captured with Nikon C1 or A1R confocal imaging system (Nikon Instech., Tokyo, Japan) and edited with IMARIS software (Carl Zeiss, Oberkochen, Germany). The proportion of NF- κ B localization in the nucleus was calculated using Image J software as the p65 intensity in the nucleus divided by the p65 intensity in the cytoplasm. The relative number of the adhesion sites was calculated using Image-Pro software (Media Cybernetics Inc., Silver Spring, MD) as the number of paxillin dots in 1 cell divided by the cell area, evaluated from F-actin or p65-EGFP fluorescent images. Statistical analysis was performed using Student's t-test.

Luciferase assay

Luciferase plasmids (225 ng/mL pNF- κ B-Luc, 2.25 ng/mL pTK-RL, and 900 ng/mL pcDNA3) were transfected to semi-confluent H1299 cells on a plastic dish with Lipofectamine 2000. The medium was exchanged after 6 h, and after 24 h, it was changed to non-serum DMEM, and the samples were incubated for 2 h. Cells were suspended and re-seeded and

luciferase activity was detected 6 h later by using Dual Luciferase Reporter Assay System (Promega). Statistical analysis was performed using Student's t-test.

Time-lapse fluorescent live imaging

H1299-p65-1 cells were pre-incubated with non-serum DMEM for 2 h and resuspended using serum-free medium. The cells were seeded with DMEM containing 10% FBS on collagen-coated cover glasses or collagen gels. The samples were filled with the DMEM and sealed with silicone grease to avoid changes in the pH of the medium. p65-EGFP fluorescent images were observed with Nikon A1R confocal imaging system (Nikon Instech.). Images were captured every 10 min, and three-dimensional images and movies were edited with IMARIS software. The fluorescent intensity of p65-EGFP in the nucleus was calculated using Image J software. The relative intensities of PP-MRLC were evaluated from the fluorescent intensity ratio of PP-MRLC/GAPDH calculated by Image-Pro software (Media Cybernetics Inc.). Statistical analysis was performed using Student's t-test.

Western blotting

Cell lysates were prepared in SDS sample buffer (0.25 M Tris-HCl, 5% dithiothreitol, 2.3% sodium dodecyl sulfate, 10% glycerol, 0.01% bromophenol blue, pH 8.8) and separated on

polyacrylamide gels. For the detection of MRLC, P-MRLC, and PP-MRLC, the cells were fixed with 10% trichloroacetic acid in PBS and washed 3 times before lysis. When we prepared the lysates from the cells on a collagen gel, we treated the cells with 0.5% collagenase (Nitta Gelatin) in PBS for 1 h at 37°C to remove the collagen gel before lysis. Lysates were treated with supersonic wave treatment and boiled for 5 min. Polyacrylamide gels (10% for p65 and GAPDH, 12.5% for MRLC, P-MRLC, PP-MRLC, and GAPDH) were prepared for SDS-PAGE (20 mA per gel, 1 h). Following SDS-PAGE, blotting to PVDF membranes was performed (92 mA per gel, 30 min for p65 and GAPDH, 1 h for MRLC, P-MRLC, PP-MRLC, and GAPDH). After blotting, the membranes were incubated in 5% skim milk in TBS-Tween solution for 1 h at room temperature. Membranes were incubated with primary antibodies (p65 1:1000, MRLC 1:1000, P-MRLC 1:300, PP-MRLC 1:300–1:600, and GAPDH 1:1000000–1:10000000) overnight at 4°C. After 3 washes, membranes were incubated with secondary antibodies (HRP anti-rabbit IgG 1:10000 for p65, MRLC, P-MRLC, and PP-MRLC; HRP anti-mouse IgG 1:200000 for GAPDH) for 1 h at room temperature. Signals were detected with Immobilon Western Chemiluminescent HRP substrate (Millipore). The relative expressions of the proteins were evaluated from intensity of the proteins/GAPDH ratios calculated by Image J software. Statistical analysis was performed using Student's t-test.

RT-PCR

H1299 cells (3×10^5) were seeded onto each substrate and incubated at 37°C. RNAs were isolated 6 h later using TriPure isolation Reagent (Roche). The isolating manipulation was performed twice to ensure removal of any contaminating DNA or protein. cDNA were generated with RevTra Ace kit (TOYOBO, Osaka, Japan), and PCR was performed with Taq polymerase with ThermoPol Buffer (NEB, Ipswich, MA). The following primers were used:

GAPDH, 5'-ACCACAGTCCATGCCATCAC-3' (upper) and 5'-TCCACCACCCTGTTGCTGTA-3' (lower); IL-1 β , 5'-TGCACGCTCCGGGACTCACA-3' (upper) and 5'-AGGCAGGCAGTTGGGCATTGG-3' (lower); IL-8, 5'-GTGGACCACACTGCGCCAACA-3' (upper) and 5'-GGCCCTTGGCCTCAATTTTGCT-3' (lower); and MMP9, 5'-GACACCTCTGCCCTCACCATGAG-3' (upper) and 5'-AGAAGCCAAACCGGTCGTCGG-3' (lower). The relative expressions of inflammatory genes were evaluated from intensity of the genes/GAPDH ratios calculated by Image J software. Statistical analysis was performed using Student's t-test.

Adhesion area assay

For assay of H1299, H-NLS, and H1299-p65-2 cells, 1×10^5 cells were seeded onto plastic dishes coated with collagen or collagen gels, or 1×10^4 cells were seeded onto

collagen-coated cover glass. For assay of the cells expressing DsRed or p65DN plasmids, the plasmids were transfected into semi-confluent H1299 cells with polyethyleneimine. After 1-day incubation, cells were resuspended and re-seeded (1×10^4 cells) on collagen gels or plastic 24-well plates coated with collagen. Following incubation, phase-contrast images and fluorescent images were captured by a TE300 phase-contrast microscope with a 10× objective (Nikon Instech.) or an EVOS microscope with a 20× objective (AMG, Bothell, WA). Cell adhesion areas were calculated by ImageJ software. Statistical analysis was performed using Student's t-test.

Adhesion ability assay

H-NLS and H1299-p65-2 cells (1.5×10^5) were seeded onto a plastic 96-well plate coated with collagen or onto a 0.6-kPa collagen gel. Plates were then centrifuged at 2000 rpm for 35 s at room temperature and incubated at 37°C. After 30 min, the wells were washed 3 times with PBS. The images of adhering cells on the well were captured by a TE300 phase-contrast microscope with a 10× objective (Nikon Instech.). The relative number of adhering cells was evaluated by calculating the number of cells per field of the images.

Results

Substrate stiffness regulates temporary activation of NF- κ B

Because NF- κ B is translocated from the cytoplasm to the nucleus and functions as a transcription factor when cells receive certain stimuli [18], we observed NF- κ B localization in NLS-venus transgenic H1299 (H-NLS) cells on stiff or soft substrates. For the stiff substrate, we used a cover glass coated with type-I collagen (>1 GPa). For the soft substrate, we prepared 1.6 mg/mL type-I collagen gel (0.6 kPa). Cells exhibited a higher rate of NF- κ B localization to the nucleus 2–6 h after seeding on the stiff substrate than on the soft substrate (Fig. 1A, B). The transcriptional activity of NF- κ B detected by luciferase reporter assay was also higher in the cells grown on the stiff substrate than on the soft substrate (Fig. 1C). For the luciferase reporter assay, we used 30.1-kPa polyacrylamide gels containing 0.16% *N,N'*-methylenebisacrylamide (BIS) as the stiff substrate and 2.0-kPa polyacrylamide gels containing 0.01% BIS as the soft substrate. The differences in NF- κ B localization detected by immunofluorescence staining between cells on stiff and soft substrates were smaller than those in NF- κ B activity detected by luciferase reporter assay, but this may be due to differences in experimental design. The differences in NF- κ B activity detected by luciferase reporter assay reflected the transcriptional activity at 6 h after seeding, while conversely, NF- κ B localization was observed at a single time point by immunofluorescence staining. Thus, we suggest that the differences in NF- κ B activity

1 detected by luciferase assay indicated total differences for 6 h and indicated larger differences
2 than NF- κ B localization did at a single time point.

3 To investigate the dose responsivity of NF- κ B activity to substrate stiffness, we
4 performed luciferase reporter assay using polyacrylamide gels of 2.0, 13.8, 30.1, 67.2 kPa
5 stiffness. The NF- κ B activities of H1299 cells on 30.1- and 67.2-kPa gels were higher than
6 those on 2.0- and 13.8-kPa gels (Fig. S1). This result indicates that substrate stiffness between
7 13.8 and 30.1 kPa is critical to activate NF- κ B for H1299 cells.

8 In addition, to observing the time-course of NF- κ B localization in living cells, we
9 performed time-lapsed fluorescent live imaging. To observe the localization of NF- κ B in living
10 cells, we established 2 cell lines, H1299-p65-1 and H1299-p65-2, which resulted from the
11 sub-cloning of transgenic H1299 cells expressing NF- κ B (p65)-EGFP. We used H1299-p65-1
12 cells for the fluorescent live imaging, because these cells showed more normal NF- κ B activity
13 than H1299-p65-2 cells (Fig. S2A). On the stiff (>1 GPa) substrate, p65-EGFP fluorescent
14 intensity in the nucleus was increased from 0 to 1.5 h after seeding. From 4.5 to 6 h after
15 seeding, the intensity began to decrease (Fig. 2A, B, Video S1). On the soft (0.6 kPa) substrate,
16 p65-EGFP intensity in the nucleus exhibited almost no change after seeding (Fig. 2A, B, Video
17 S2).

18 To investigate whether the changes in NF- κ B nuclear localization depended on the

amount of NF- κ B protein in the cells, we examined protein expression by western blotting and found no significant differences between cells grown on stiff (30.1 kPa) versus soft (2.0 kPa) substrates (Fig. S3). These data suggest that NF- κ B is temporarily activated in H1299 cells grown on the stiff substrate.

Substrate stiffness and NF- κ B activity regulate the expression of inflammatory genes

Next, we investigated gene expression regulated by substrate stiffness and NF- κ B activity. Because IL-1 β [31], IL-8 [32], and MMP9 [33] are known to be upregulated by NF- κ B activation, RT-PCR was performed to detect the mRNA expression of these genes. IL-1 β , IL-8, and MMP9 expressions were upregulated to a greater extent in H1299 cells grown on a stiff (>1 GPa) substrate compared with cells grown on a soft (0.6 kPa) substrate 6 h after seeding, although the differences for IL-8 were not significant (Fig. 3A, B). Furthermore, H1299-p65-2 cells, which showed higher NF- κ B activity than H-NLS cells (Fig. S2B), expressed higher levels of IL-1 β , IL-8, and MMP9 mRNA than did H-NLS cells grown on a soft (0.6 kPa) substrate, although the differences for IL-1 β were not significant (Fig. 3C, D). These results indicate that substrate stiffness regulates the NF- κ B-dependent mRNA expressions of IL-1 β , IL-8, and MMP9 in H1299 cells.

NF- κ B activation by stiff substrates is independent of adhesion molecules

Next, we examined which molecules are regulated by substrate stiffness, thus inducing NF- κ B activation. To adhere to the substrates, cells form a linkage between ECM proteins and adhesion molecules [25]. Integrin β 1 plays a major role in the connection between cells and type-I collagen, which is an abundant component of connective tissues. Integrin β 1 is also reported to induce NF- κ B activation in response to mechanical stimuli [26, 27]. Because of this property of integrin β 1, we evaluated NF- κ B activity in H1299 cells cultured with or without monoclonal antibody AIIB2, which inhibits integrin β 1 activity [34]. AIIB2 treatment did not reduce the proportion of cells adhered to the substrate, but did cause significant changes in cell morphology compared with non-treated cells (Fig. S4A, B). However, there was no significant difference in NF- κ B activity between the cells treated with AIIB2 and the non-treated cells (Fig. S4C). In addition, H1299 cells cultured on a plastic substrate coated with poly-L-lysine, fibronectin, laminin, or collagen showed no remarkable differences in NF- κ B activity (Fig. S4D). These results suggest that NF- κ B activation in H1299 cells grown on a stiff substrate may not be regulated by adhesion molecules such as integrin β 1 and ECM molecules.

Actomyosins are regulated by substrate stiffness and induce NF- κ B activation

Next, we focused on actomyosin, a cytoskeletal complex generating cellular contractile forces. Treatment with Y27632—a ROCK inhibitor—or blebbistatin (a myosin II inhibitor) inhibited actomyosin contractions and significantly decreased NF- κ B activity in cells grown on a stiff (>1 GPa) substrate (Fig. 4A). Furthermore, treatment with lysophosphatidic acid (LPA) (an inducer of RhoA activation) increased NF- κ B activity on a soft (0.6 kPa) substrate (Fig. 4A). These results indicate that actomyosin contractions lead to NF- κ B activation.

For observation of actomyosin contractile forces, we performed di-phosphorylated MRLC (PP-MRLC) immunofluorescent staining in the cells 45 min after seeding on stiff (>1 GPa) or soft (0.6 kPa) substrates. PP-MRLC localization indicates local contractile sites, generated by actomyosins [22]. On a soft (0.6 kPa) substrate, localization of PP-MRLC was not observed (Fig. 4B). In contrast, on a stiff (>1 GPa) substrate, PP-MRLC was localized at the bottom of the cell periphery (Fig. 4B). The relative fluorescent intensity of PP-MRLC per F-actin was higher in the cells on a stiff (>1 GPa) substrate than in the cells on a soft (0.6 kPa) substrate (Fig. 4C). Western blotting analysis also indicated that MRLC was significantly more di- or mono-phosphorylated on a stiff (30.1 kPa) substrate than on a soft (2.0 kPa) substrate 45 min after seeding (Fig. 4D, E). In addition, western blotting analysis indicated that Y27632

1 treatment reduced PP-MRLC on both stiff (>1 GPa) and soft (0.6 kPa) substrates whereas LPA
2 treatment increased PP-MRLC on only a soft (0.6 kPa) substrate (Fig. S5). Taken together, these
3 data suggest that stiff substrates induce actomyosin contractions generated by MRLC
4 phosphorylation, leading to NF- κ B activation in H1299 cells.

5 In contrast, NF- κ B activation did not induce reconstruction of F-actin and MRLC
6 di-phosphorylation. F-actin localization detected by fluorescence staining was similar in H1299
7 and H1299-p65-2 cells, which showed higher NF- κ B activity than H1299 cells (Fig. S2A) on
8 both stiff (>1 GPa) and soft (0.6 kPa) substrates (Fig. S6A). Conversely, H1299-p65-2 cells
9 exhibited higher PP-MRLC signal on a stiff (>1 GPa) substrate than on a soft (0.6 kPa) substrate,
10 as well as in H1299 cells (Fig. S6A). Western blotting analysis indicated that H1299-p65-2 cells
11 showed lower PP-MRLC expression than H-NLS cells on a soft (2.0 kPa) substrate (Fig. S6B).
12 These results suggest that NF- κ B activation does not induce MRLC di-phosphorylation in
13 H1299 cells.

14 **NF- κ B activation induces cell spreading in H1299 cells**

15 Next, we investigated the cellular phenotype affected by NF- κ B activation in H1299
16 cells grown on stiff or soft substrates. On a stiff (>1 GPa and 30.1 kPa) substrate, H1299 cells
17 exhibited a higher degree of spreading than they did on a soft (2.0 and 0.6 kPa) substrate (Fig.

5A, B and Fig. S7, 8). To examine the contribution of NF- κ B activity on cell morphology, we transfected dominant-negative mutant p65 conjugated with DsRed (p65DN) or DsRed backbone plasmids into H1299 cells and analyzed the morphology of the cells. Cells transfected with p65DN exhibited lower NF- κ B activity (Fig. S9) and less spreading compared to DsRed transfected cells when grown on a stiff (>1 GPa) substrate (Fig. 5C, D and Fig. S7). In contrast, H1299-p65-1 and H1299-p65-2 cells, which showed higher activity of NF- κ B than H-NLS cells on a soft (0.6 kPa) substrate (Fig. S2B), showed more cell spreading than H-NLS cells on a soft (0.6 kPa) substrate (Fig. 5E, F and Fig. S7). We also treated cells with TNF α , and found that TNF α induced NF- κ B activation and spreading shape in cells grown on a soft (0.6 kPa) substrate (Fig. S10A, B, C; S11A, B). In addition, we treated cells with BAY-11-7082, which inhibited NF- κ B activity induced by TNF α (Fig. S10D, E). The effect of TNF α on cellular morphology was inhibited by BAY-11-7082 treatment (Fig. S11C). In contrast to NF- κ B activation and cell spreading, TNF α did not affect F-actin localization or MRLC di-phosphorylation on soft substrates (Fig. S12). Thus, TNF α may induce NF- κ B activation and cell spreading, independent of actomyosin contractions. Collectively these data suggest that NF- κ B activation induces morphological changes.

Adhesion ability in H1299 cells is promoted by NF- κ B activity

Next, we evaluated adhesion ability in H-NLS and H1299-p65-2 cells, which exhibited higher NF- κ B activity than H-NLS cells (Fig. S2B) with both stiff and soft substrates. H1299-p65-2 showed greater adhesion ability than H-NLS cells on stiff (>1 GPa) and soft (0.6 kPa) substrates (Fig. S13). This result indicates that NF- κ B activity promotes both spreading and adhesion ability in H1299 cells. Conversely, in contrast to cell spreading, adhesion abilities of H-NLS and H1299-p65-2 were higher on a soft (0.6 kPa) substrate compared than on a stiff (>1 GPa) substrate (Fig. S13). Taken together, these results indicate that stiff substrates increase cell spreading and decrease cell adhesion ability in H1299 cells, and that cell spreading is regulated by a mechanism other than molecular signaling, as evidenced by the strength of cell-substrate adhesion.

Actomyosin contractions regulate the formation of focal adhesions independent on NF- κ B activity

Because the formation of focal adhesions is upregulated by stiff substrate and actomyosin contractions [13, 35], we investigated whether focal adhesions were also regulated by substrate stiffness and actomyosin contractions in our experimental system. Focal adhesions were observed by immunofluorescence staining of paxillin, which is a component protein of

focal adhesion [36]. H1299 cells exhibited more focal adhesions (paxillin dots) on a stiff (>1 GPa) substrate than on a soft (0.6 kPa) substrate (Fig. S14A, B). Furthermore, Y27632 and blebbistatin treatment reduced the number of focal adhesions in H1299 cells on stiff (>1 GPa) and/or soft (0.6 kPa) substrates (Fig. S14A, B). Several H1299 cells treated with LPA on a soft (0.6 kPa) substrate increased focal adhesions, although the difference was not significant (Fig. S14A, B).

Next, we investigated whether the formation of focal adhesions was regulated by NF- κ B activity. H1299-p65-2 cells, which exhibited higher NF- κ B activity than H1299 cells (Fig. S2A) on a soft (0.6 kPa) substrate, produced fewer focal adhesions than on a stiff (>1 GPa) substrate, and this was also observed with H1299 cells (Fig. S15A, B). These results suggest that substrate stiffness regulates the formation of focal adhesions via actomyosin contraction in H1299 cells, independent on NF- κ B activity.

Actomyosin contractions regulate cell spreading in H1299 cells

To evaluate the effects of actomyosin on cell spreading in H1299 cells, we calculated cell area in H1299 cells treated with Y27632, blebbistatin, and LPA. F-actin distributions, which are important for maintenance of cell shape, were similar in H1299 cells (both treated and non-treated with these drugs) on stiff (>1 GPa) and soft (0.6 kPa) substrates (Fig. S14A).

1 Blebbistatin treatment slightly decreased cell area in H1299 cells on a stiff (>1 GPa) substrate,
2 although the difference was not comparatively significant (Fig. S14C). This result suggests that
3 actomyosin plays an important role for maintaining cell spreading in H1299 cells on a stiff
4 substrate. In addition, blebbistatin and LPA treatment increased the cell area in H1299 cells on a
5 soft (0.6 kPa) substrate (Fig. S14C), and these results further suggest that actomyosin
6 contractions regulate cell spreading in H1299 cells.

Discussion

The results of this study showed that substrate stiffness regulates NF- κ B activation in H1299 cells. When grown on a stiff (>1 GPa, 67.2 kPa, and 30.1 kPa) substrate, the cells activated NF- κ B, while on a soft (13.8, 2.0, and 0.6 kPa) substrate, the cells exhibited only low levels of NF- κ B activity. While biochemical stimuli [18] and mechanical forces [19, 20, 21] have been shown to induce NF- κ B activation, the effects of substrate stiffness on NF- κ B activation have not been studied well. To our knowledge, this is the first study to reveal that substrate stiffness regulates NF- κ B activation.

The results of a previous study have indicated that stiffness of normal lung tissue is approximately 2.0 kPa [37], while another study showed that tissue stiffening promotes malignancy in breast cancer [38]. We demonstrated in this study that H1299 lung cancer cells exhibit lower NF- κ B activity on the soft substrates (~ 2.0 kPa) than on the stiff substrates (30.1 kPa). Since it is known that NF- κ B plays an important role in cancer progression [15], our results suggest that an abnormally stiff environment in lung tissue promotes malignancy in lung cancer cells via NF- κ B activation.

Additionally, we demonstrated that the NF- κ B activation regulated by substrate stiffness is a temporary event, which is consistent with the findings from a previous study indicating that NF- κ B activation in response to various stimuli is typically temporary [39]. Similarly, we

1 observed temporary NF- κ B activation by stiff substrates 2–6 h after seeding. A previous study
2 showed that YAP/TAZ, which is the first reported transcription factor regulated by substrate
3 stiffness, was activated 48 h after the cells were seeded on a stiff substrate [40]. In this study,
4 NF- κ B activation did not significantly differ between the cells grown on stiff versus soft
5 substrates at 48 h after seeding. Thus, while both YAP/TAZ and NF- κ B act as
6 mechanotransductive transcription factors, the time courses of their activation are entirely
7 different.

8 In this study, we further demonstrated that NF- κ B activation is induced by substrate
9 stiffness. However, this activation was weaker than the NF- κ B activation by TNF- α treatment.
10 This may be because NF- κ B activation by mechanical stress is weaker than that by biochemical
11 stimuli. A previous study showed that the activation of NF- κ B by cyclic stretch, a mechanical
12 stress, is weaker than the activation by IL-1, a biochemical stimulus [20]. Thus, the activation of
13 NF- κ B by substrate stiffness, a mechanical stress, may be weaker than that by TNF- α , a
14 biochemical stimulus.

15 Stiff substrates activate NF- κ B, and as a result, induce the expression of inflammatory
16 genes such as IL-1 β , IL-8, and MMP9. These genes are known to facilitate migration, invasion,
17 and metastasis in cancer cells [33, 39, 41], suggesting that substrate stiffness may regulate
18 cancer malignancy via the expression of these genes.

1 To better understand the possible relationship between NF- κ B activation and the
2 migratory and invasive potential of cells, we examined several mechanisms controlling cell
3 adhesion and movement. Interestingly, we found that NF- κ B activation in H1299 cells was not
4 dependent on adhesion molecules such as integrin β 1 and ECM molecules. The inhibition of
5 integrin β 1 activity by AIIB2 treatment induced round morphology of H1299 cells grown on a
6 stiff substrate. Thus, activation of integrin β 1 may induce a spreading morphology of H1299
7 cells via cell-substrate adhesion. On the other hand, integrin β 1 inhibition by AIIB2 did not
8 suppress NF- κ B activation. Furthermore, H1299 cells grown on poly-L-lysine, fibronectin,
9 laminin, or collagen substrates exhibited no remarkable differences in NF- κ B activity. These
10 results indicate that the extension of the cells and the interactions of integrin β 1 with ECM may
11 not play important roles in NF- κ B activation. Thus, H1299 cells may sense substrate stiffness
12 through a mechanism independent of cell-substrate adhesion to regulate NF- κ B activity.

13 Stiff substrates induce actomyosin contractile forces, which, in turn, trigger NF- κ B
14 activation. Previous research has shown that stiff substrates increase the traction forces
15 generated by actomyosins [42], further enhancing NF- κ B activation. Here, we showed that stiff
16 substrates induced MRLC di-phosphorylation at the bottom of the cell periphery 45 min after
17 seeding H1299 cells. Furthermore, we showed that actomyosin contractile forces triggered
18 NF- κ B activation by using Y27632 as a ROCK inhibitor, blebbistatin as a myosin II inhibitor, or

1 LPA as a Rho activator. However, the differences in NF- κ B activity among H1299 cells treated
2 with Y27632, blebbistatin, and LPA were small. This may be because the drug treatments are
3 not sufficient to regulate the entire activation of NF- κ B by a stiff substrate. It is therefore
4 possible that there may be an actomyosin-independent signaling cascade to regulate NF- κ B
5 activity by substrate stiffness. On the other hand, previous study reported that NF- κ B activation
6 induced by TNF α stimuli requires MRLC phosphorylation in renal endothelial cells [24].
7 However, in this study, TNF α stimuli did not induce MRLC di-phosphorylation in H1299 cells.
8 MRLC may regulate NF- κ B activation induced by mechanical stimuli whereas MRLC may not
9 induce NF- κ B activation by chemical stimuli in H1299 cells.

10 Stiff substrates appear to induce cell spreading via NF- κ B activation. A previous study
11 revealed that substrate stiffness regulates cell adhesion area by modulating adhesion and
12 cytoskeletal molecules in MCF10A breast epithelial cells [42]. Therefore, spreading of H1299
13 cells regulated by NF- κ B may depend on the molecules that induce cell extension, such as
14 molecules related to cytoskeletons or adhesion complexes. However, in this study, we
15 demonstrated that NF- κ B activation did not increase the formation of focal adhesions, whereas
16 Y27632, blebbistatin, and LPA treatment did affect the formation of NF- κ B in H1299 cells.
17 Thus, substrate stiffness regulates the formation of focal adhesions via actomyosin contractions,
18 independent of NF- κ B activation. Furthermore, NF- κ B activation did not induce MRLC

1 di-phosphorylation and reconstruction of actin filament in H1299 cells. Cell spreading in H1299
2 cells induced by NF- κ B activation may therefore be triggered by another adhesion molecule
3 and/or cytoskeleton.

4 *In vivo*, many types of cells, including cancer cells, adhere to their substrates, which
5 include collagen-rich connective tissues such as basement membrane. A previous study showed
6 that stiffened substrates induce cancer progression *in vivo* and the substrates are stiffened by
7 expression of lysyl oxidase (LOX), which involves crosslinking of collagen fibers [38]. Thus,
8 LOX may be a good target for cancer therapies, although there have been no reports on the use
9 of LOX-targeting therapies in clinical practice. Our study indicated that stiff substrates trigger
10 activation of NF- κ B in H1299 lung adenocarcinoma cells. In addition, previous studies have
11 shown that inhibition of NF- κ B is effective in cancer therapies [43]. Thus, combined therapies
12 targeting LOX with NF- κ B inhibitors may be more effective than stand-alone therapy.

13 In summary, we showed that substrate stiffness regulated temporary NF- κ B activation via
14 actomyosin contractions in H1299 cells. The activation of NF- κ B appeared to trigger the
15 expression of inflammatory genes. Furthermore, NF- κ B activation induced a spreading
16 morphology, which may be caused by inflammatory genes. These results suggest that when lung
17 cancer cells are exposed to stiff substrates, the cells may induce NF- κ B activation and, as a
18 result, modulate inflammatory reactions and cancer progression. Therefore, substrate stiffness

1 and NF- κ B may be good targets to explore for cancer therapy.

2

1 **Acknowledgments**

2 This study was supported by Grants-in-Aid for JSPS Fellows (11J06280) to I.S., Scientific
3 Research (A) (21249065) to H.H., Scientific Research on Innovative Areas (24106502) to T.M.,
4 and Exploratory Research (23651099) to K.K. from the Ministry of Education, Culture, Sports,
5 Science and Technology, Japan. I.H. is currently affiliated with the Laboratory for Mechanical
6 Medicine, Locomotive Syndrome Research Institute, Nadogaya Hospital. 687-4 Nadogaya,
7 Kashiwa-City, Chiba 277-0032, Japan. The authors thank Hiro-taka Masuda and Sawa
8 Kobayashi for technical assistance.

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Figure legends

Fig. 1. Stiff substrates induced temporary activation of NF- κ B. (A) Immunofluorescent images of nucleus (NLS-venus) and NF- κ B (p65) in H-NLS cells on a stiff substrate (>1 GPa, a cover glass coated with collagen) and on a soft substrate (0.6 kPa, a collagen gel) 2 h after seeding. (B) The proportion of NF- κ B localization in the nucleus in H-NLS cells 0.5, 2, 4, 6 h after seeding evaluated by immunofluorescent intensity. *, $P < 0.05$. Error bar = SE. $n =$ at least 30 cells. (C) NF- κ B activity detected by luciferase reporter assay of H1299 cells on a stiff substrate (30.1 kPa, polyacrylamide gel containing 0.16% BIS) and on a soft substrate (2.0 kPa, polyacrylamide gel containing 0.01% BIS) 6 h after seeding. *, $P < 0.05$. Error bar = SE. $n = 3$ independent experiments.

1 Fig. 2. Stiff substrates induced the temporary nuclear localization of NF- κ B (p65-EGFP) in
2 living cells. (A) p65-EGFP images of H1299-p65-1 cells 2.5 h after seeding and (B) time-course
3 changes of NF- κ B intensity in the nucleus of H1299-p65-1 cells 0–5 h after seeding on a stiff
4 substrate (>1 GPa, a cover glass coated with collagen) and on a soft substrate (0.6 kPa, a
5 collagen gel). Error Bar = SE. n = 3 independent experiments.
6

Fig. 3. Stiff substrates induced the expression of inflammatory genes via NF- κ B activation. (A) Inflammatory genes and GAPDH mRNA expression detected by RT-PCR in H1299 cells on a stiff substrate (>1 GPa, a plastic dish coated with collagen) or on a soft substrate (0.6 kPa, a collagen gel) 6 h after seeding. (B) The relative expression of the inflammatory genes/GAPDH ratio evaluated from RT-PCR (A). *, $P < 0.05$. Error bar = SE. $n = 3$ independent experiments. (C) Inflammatory genes and GAPDH mRNA expressions detected by RT-PCR in H-NLS (NLS) cells and H1299-p65-2 (p65-2) cells on a soft substrate (0.6 kPa, a collagen gel) 6 h after seeding. H1299-p65-2 exhibited higher NF- κ B activity than H-NLS did. (D) The relative expression of the inflammatory genes/GAPDH ratio evaluated from RT-PCR (C). *, $P < 0.05$. Error bar = SE. $n = 3$ independent experiments.

Fig. 4. Stiff substrates induced NF- κ B activation via actomyosin contractions. (A) The NF- κ B activity in H1299 cells on a stiff substrate (>1 GPa, a plastic dish coated with collagen) and on a soft substrate (0.6 kPa, a collagen gel) 6 h after seeding. NT, non-treated; Y, 10 μ M Y27632 as ROCK inhibitor; Bleb, 100 μ M blebbistatin as myosin II inhibitor; LPA, 20 μ M LPA as Rho activator; DMSO, negative control for blebbistatin and LPA. *, $P < 0.05$. Error bar = SE. $n = 5$ independent experiments. (B) F-actin and di-phosphorylated MRLC (PP-MRLC) immunofluorescent images of H1299 cells 45 min after seeding on a stiff substrate (>1 GPa, a cover glass coated with collagen) and on a soft substrate (0.6 kPa, a collagen gel). Cross-sectional views of XZ and YZ directions are shown together. The dotted line shows the surface of the collagen gel. Bar = 10 μ m. (C) The relative fluorescent intensity of PP-MRLC/F-actin ratio evaluated from immunofluorescence images (B). *, $P < 0.05$. Error bar = SE. $n =$ at least 10 cells. (D) The protein expression of PP-MRLC, mono-phosphorylated MRLC (P-MRLC), total MRLC (MRLC), and GAPDH in H1299 cells grown on a stiff substrate (30.1 kPa, a polyacrylamide gel containing 0.16% BIS) and on a soft substrate (2.0 kPa, a polyacrylamide gel containing 0.01% BIS) 45 min after seeding. (E) The relative expression of the proteins/GAPDH ratio evaluated from western blotting (D). *, $P < 0.05$. Error bar = SE. $n = 3$ independent experiments.

Fig. 5. Stiff substrates induced spreading cellular morphology via NF- κ B activation. (A) Phase-contrast images of H1299 cells 24 h after seeding. (B) The cell adhesion area of H1299 cells 0.5, 1.5, 3.0, 12, and 20 h after seeding on a stiff substrate (>1 GPa, a plastic dish coated with collagen) and on a soft substrate (0.6 kPa, a collagen gel). *, $P < 0.05$, Error bar = SE. $n =$ at least 10 cells. (C) Phase-contrast (Phase) and fluorescent images of DsRed and p65DN cells on a stiff substrate (>1 GPa, a plastic dish coated with collagen) 24 h after seeding. DsRed, H1299 cells transfected with DsRed backbone; p65DN, H1299 cells transfected with DsRed conjugated with p65 dominant-negative mutant. (D) The cell area on a stiff substrate (>1 GPa, a plastic dish coated with collagen) 24 h after seeding. *, $P < 0.05$, Error bar = SE. $n =$ at least 20 cells. (E) Phase-contrast (Phase) and fluorescent images of NLS-venus in H-NLS or p65-EGFP (p65) in H1299-p65-2 cells 24 h after seeding on a soft substrate (0.6 kPa, a collagen gel). (F) The cell area of H-NLS (NLS), H1299-p65-1 (p65-1), and p65-2 (p65-2) 24 h after seeding on a soft substrate (0.6 kPa, a collagen gel). *, $P < 0.05$, Error bar = SE. $n =$ at least 52 cells.

Supplementary data

Fig. S1. Substrate stiffness between 13.8 and 30.1 kPa is critical to the activation of NF- κ B in H1299 cells. NF- κ B activity detected by luciferase reporter assay in H1299 cells on substrates of different stiffness (2.0, 13.8, 30.1, and 67.2 kPa) 6 h after seeding. Error bar = SE. n = 3 samples.

1 Fig. S2. H1299-p65-1 and H1299-p65-2 cells exhibited higher NF- κ B activity than H1299. (A)
2 NF- κ B activity of H1299 (WT), H1299-p65-1 (p65-1), and H1299-p65-2 (p65-2) cells on a stiff
3 substrate (>1 GPa, a plastic dish coated with collagen) 6 h after seeding, detected by luciferase
4 reporter assay. $n = 3$ samples. Error bar = SE. (B) NF- κ B activity of H-NLS cells (NLS) and
5 H1299-p65-2 cells on a soft substrate (0.6 kPa, a collagen gel) 6 h after seeding, detected by
6 luciferase reporter assay. $n = 3$ independent experiments. Error bar = SE.

7

1 Fig. S3. Substrate stiffness did not affect protein expression of NF- κ B. NF- κ B (p65) and
2 GAPDH protein expressions of H1299 cells 2, 4, 6 h after seeding on a stiff substrate (30.1 kPa,
3 a polyacrylamide gel containing 0.16% BIS) or on a soft substrate (2.0 kPa, a polyacrylamide
4 gel containing 0.01% BIS) as detected by western blotting.

5

Fig. S4. Integrin $\beta 1$ and ECM molecules did not affect NF- κ B activation. (A) The phase-contrast images of H1299 cells on a stiff substrate (>1 GPa, a collagen-coated plastic dish) non-treated or treated with 400 ng/mL monoclonal antibody AIIB2, which inhibited integrin $\beta 1$ activity, 6 h after seeding. (B) The cell area of H1299 cells on a stiff substrate (>1 GPa, a plastic dish coated with collagen) 6 h after seeding. Error bar = SE. n = at least 34 cells. (C) NF- κ B activity of non-treated or AIIB2-treated H1299 cells on a stiff substrate (>1 GPa, a plastic 24-well plate coated with collagen) 6 h after seeding, as detected by luciferase reporter assay. n = 3 independent experiments. (D) NF- κ B activity of H1299 cells on a plastic 24-well plate coated with ECMs 6 h after seeding, as detected by luciferase reporter assay. PLL, poly-L-lysine as non ECM condition; FN, fibronectin; Lam, laminin; Col, collagen. n = 3 samples. Error bar = SE.

1 Fig. S5. Y27632 treatment reduced di-phosphorylated myosin regulatory right chain
2 (PP-MRLC) whereas LPA treatment increased PP-MRLC. The protein expressions of
3 PP-MRLC and GAPDH in H1299 cells grown on a stiff substrate (>1 GPa, a plastic dish coated
4 with collagen) compared with a soft substrate (0.6 kPa, a collagen gel) 6 h after seeding. NT,
5 non-treated; Y, 10 μ M Y27632 as ROCK inhibitor; LPA, 20 μ M LPA as Rho activator; DMSO,
6 negative control for LPA.

1 Fig. S6. NF- κ B activity did not increase di-phosphorylated myosin regulatory light chain
2 (PP-MRLC). (A) F-actin, p65-EGFP, and PP-MRLC fluorescent images of H1299 and
3 H1299-p65-2 (p65-2) cells 24 h after seeding on a stiff substrate (>1 GPa, a cover glass coated
4 with collagen) or on a soft substrate (0.6 kPa, a collagen gel). Cross-sectional views of XZ and
5 YZ directions are shown together. Bar = 20 μ m. (B) Protein expression of PP-MRLC and
6 GAPDH in H-NLS (NLS) and H1299-p65-2 cells grown on a stiff substrate (30.1 kPa, a
7 polyacrylamide gel containing 0.16% BIS) and on a soft substrate (2.0 kPa, a polyacrylamide
8 gel containing 0.01% BIS) 24 h after seeding, detected by western blotting.

9

1 Fig. S7. Stiff substrates induce spread cellular morphology via NF- κ B activation. (A, B)
2 Representative phase-contrast images of H1299 cells 24 h after seeding on stiff (>1 GPa, a
3 plastic dish coated with collagen) and soft (0.6 kPa, a collagen gel) substrates. (C, D)
4 Representative phase-contrast (Phase) and fluorescent images of DsRed and p65DN cells on a
5 stiff substrate (>1 GPa, a plastic dish coated with collagen) 24 h after seeding. DsRed, H1299
6 cells transfected with DsRed backbone; p65DN, H1299 cells transfected with DsRed conjugated
7 with p65 dominant-negative mutant. (E, F, G) Representative phase-contrast (Phase) and
8 fluorescent images of NLS-venus in H-NLS (E) or p65-EGFP (p65) in H1299-p65-1 (F) and
9 H1299-p65-2 (G) cells 24 h after seeding on a soft substrate (0.6 kPa, a collagen gel).

1 Fig. S8. Stiff polyacrylamide gel substrates induced spreading cellular morphology. Stiff (30.1
2 kPa), polyacrylamide gels containing 0.16% BIS; Soft (2.0 kPa), polyacrylamide gels
3 containing 0.01% BIS. (A) The phase-contrast images of H1299 cells on stiff and soft substrates
4 12 h after seeding. Bar = 100 μ m. (B) The cell area on stiff and soft polyacrylamide gels 2, 4, 6,
5 12, and 24 h after seeding. Error bar = SE. n = at least 16 cells at each time of observation.

6

7

1 Fig. S9. H1299 cells transfected with p65 dominant-negative plasmid (p65DN) exhibited
2 decreased NF- κ B activity. The NF- κ B activity of H1299 cells transfected with or without
3 p65DN detected by luciferase reporter assay. Cells were seeded on a stiff substrate (>1 GPa, a
4 plastic 24-well plate coated with collagen) and harvested 6 h after seeding. $n = 3$ samples. Error
5 bar = SE.

6

Fig. S10. TNF α induced NF- κ B activation in H1299 cells grown on a soft substrate (0.6 kPa, a collagen gel). (A) The immunofluorescence images of H1299 cells treated with 15 ng/mL TNF α or of non-treated (NT) H1299 cells 1.5 h after seeding in a serum-free medium. Bar = 20 μ m. (B) The proportion of NF- κ B localization in the nucleus evaluated from intensity of immunofluorescence images. n = 10 cells. Error bar = SE. (C) NF- κ B activity measured by luciferase reporter assay in H1299 cells 6 h after seeding. n = 3 samples. Error bar = SE. (D) The immunofluorescence images of H1299 cells treated with TNF α and DMSO (TNF α +DMSO) or cells treated with TNF α and 1 μ M BAY-11-7082 (TNF α +BAY) 1.5 h after seeding in a serum-free medium. Bar = 20 μ m. (E) The proportion of NF- κ B localization in the nucleus was evaluated from the intensity of immunofluorescence images. n = at least 9 cells. Error bar = SE.

1 Fig. S11. TNF α treatment induced spreading morphology in H1299 cells grown on a soft
2 substrate (0.6 kPa, a collagen gel). (A) The phase-contrast images and (B) areas of H1299 cells
3 treated with 15 ng/mL TNF α or non-treated (NT) 24 h after seeding in a serum-free medium.
4 Bar = 100 μ m. n = at least 130 cells. Error bar = SE. (C) Area of H1299 cells treated with TNF α
5 and DMSO (TNF α +DMSO) or treated with TNF α and 1 μ M BAY-11-7982 (TNF α +BAY) 24 h
6 after seeding in a serum-free medium. n = at least 214 cells. Error bar = SE.

7

Fig. S12. TNF α treatment did not increase di-phosphorylated myosin regulatory light chain (PP-MRLC). (A) F-actin and PP-MRLC fluorescent images of H1299 cells non-treated or treated with 15 ng/mL TNF α 24 h after seeding on a soft substrate (0.6 kPa, a collagen gel). Cross-sectional views of XZ and YZ directions are shown together. Bar = 20 μ m. (B) Protein expression of PP-MRLC and GAPDH in H-NLS (NLS) cells grown on a stiff substrate (30.1 kPa, a polyacrylamide gel containing 0.16% BIS) and H1299 cells non-treated (NT) or treated with 15 ng/mL TNF α grown on a soft substrate (2.0 kPa, a polyacrylamide gel containing 0.01% BIS) 24 h after seeding, detected by western blotting. Non-treated or TNF α -treated H1299 cells were cultured with serum-free media.

1 Fig. S13. NF- κ B activity increased adhesion ability in H1299 cells. The relative number of
2 adhering cells in H-NLS (NLS) and H1299-p65-2 (p65-2) on a stiff substrate (>1 GPa, a plastic
3 dish coated with collagen) and on a soft substrate (0.6 kPa, a collagen gel) 30 min after seeding.
4 *; $P < 0.05$, Error bar = SE. n = 3 samples.

5

Fig. S14. Substrate stiffness regulated the formation of focal adhesions via actomyosin contractions. (A) F-actin and paxillin immunofluorescence images of H1299 cells 24 h after seeding on a stiff substrate (>1 GPa, a cover glass coated with collagen) or on a soft substrate (0.6 kPa, a collagen gel). White arrows indicate the adhesion sites (focal adhesions) observed as paxillin dots. Cross-sectional views of XZ and YZ directions are shown together. Bar = 20 μ m. NT, non-treated; Y, 10 μ M Y27632 as ROCK inhibitor; Bleb, 100 μ M blebbistatin as myosin II inhibitor; LPA, 20 μ M LPA as Rho activator; DMSO, negative control for blebbistatin and LPA. (B) The relative number of adhesion sites (focal adhesions) evaluated from immunofluorescence images (A). *, $P < 0.05$. Error bar = SE. $n = 10$ cells. (C) The cell area evaluated from F-actin images in immunofluorescence staining (A). *, $P < 0.05$. Error bar = SE. $n = 10$ cells.

Fig. S15. NF- κ B activity did not regulate the formation of focal adhesions. (A) p65-EGFP and paxillin immunofluorescence images of H1299-p65-2 (p65-2) cells 24 h after seeding on a stiff substrate (>1 GPa, a cover glass coated with collagen) or on a soft substrate (0.6 kPa, a collagen gel). White arrows indicate the adhesion sites (focal adhesions) observed as paxillin dots. Cross-sectional views of XZ and YZ directions are shown together. Bar = 20 μ m. (B) The relative number of the adhesion sites (focal adhesions) evaluated from immunofluorescence images (A). *, $P < 0.05$. Error bar = SE. n = 10 cells.

1 Video S1. Time-lapse observations of H1299-p65-1 cells on a stiff substrate (a glass coated with
2 collagen) after seeding. Cross-sectional views of XZ (horizontal) and YZ (vertical) directions
3 (axes) are shown together. Green: p65-EGFP, Video time 1 second = real time 60 min, Bar = 20
4 μm .
5

1 Video S2. Time-lapse observations of H1299-p65-1 cells on a soft substrate (a collagen gel)
2 after seeding. Cross-sectional views of XZ (horizontal) and YZ (vertical) directions (axes) are
3 shown together. Green: p65-EGFP, Video time 1 second = real time 60 min, Bar = 20 μm .
4

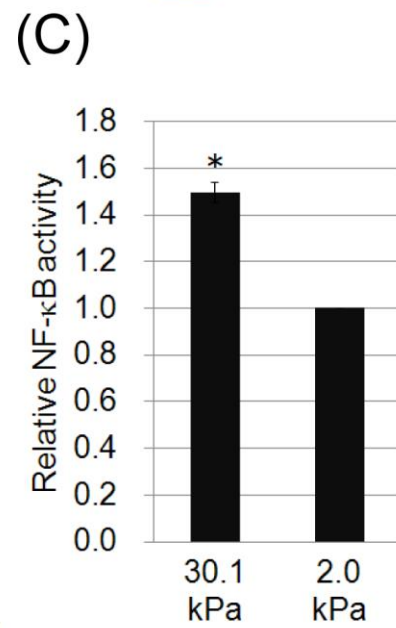
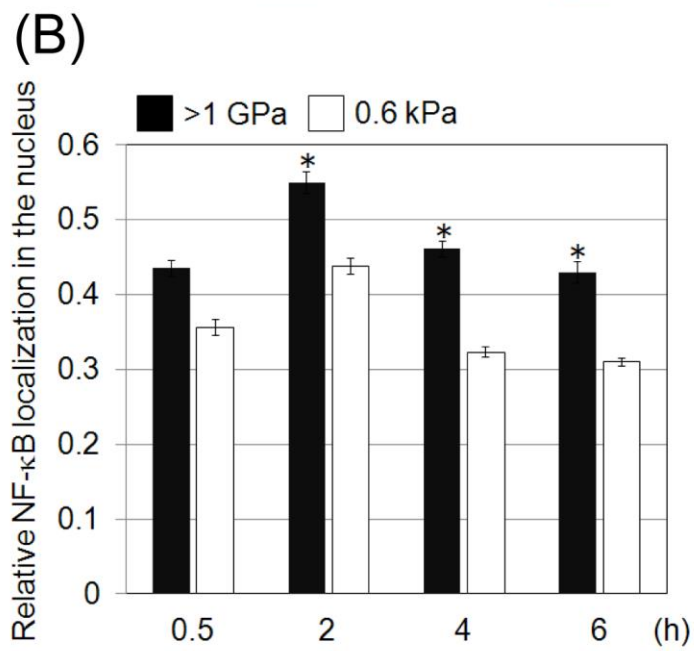
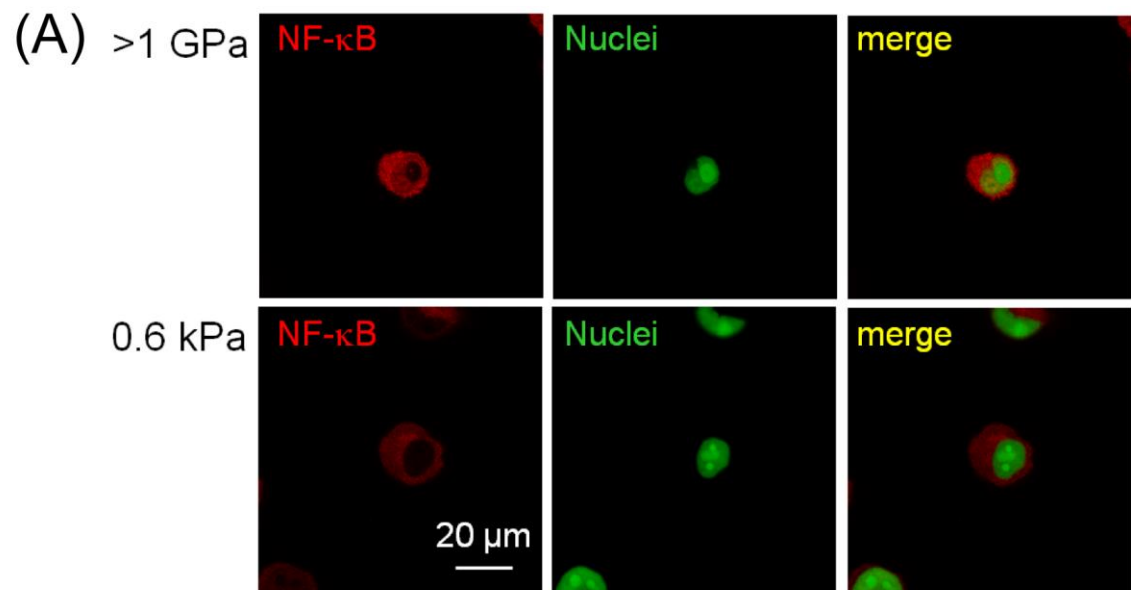


Figure 1

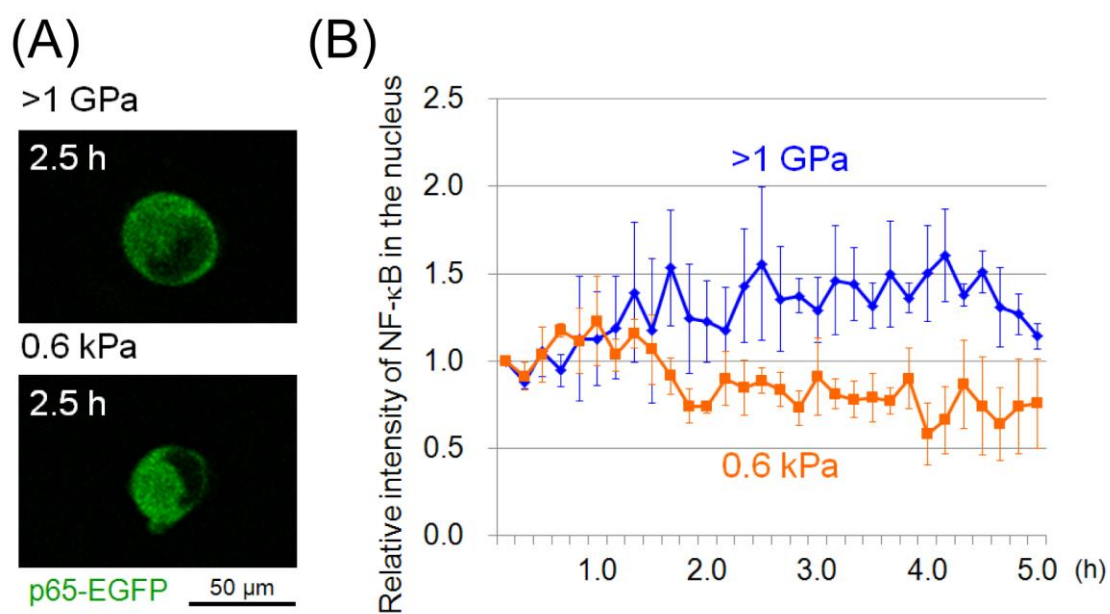


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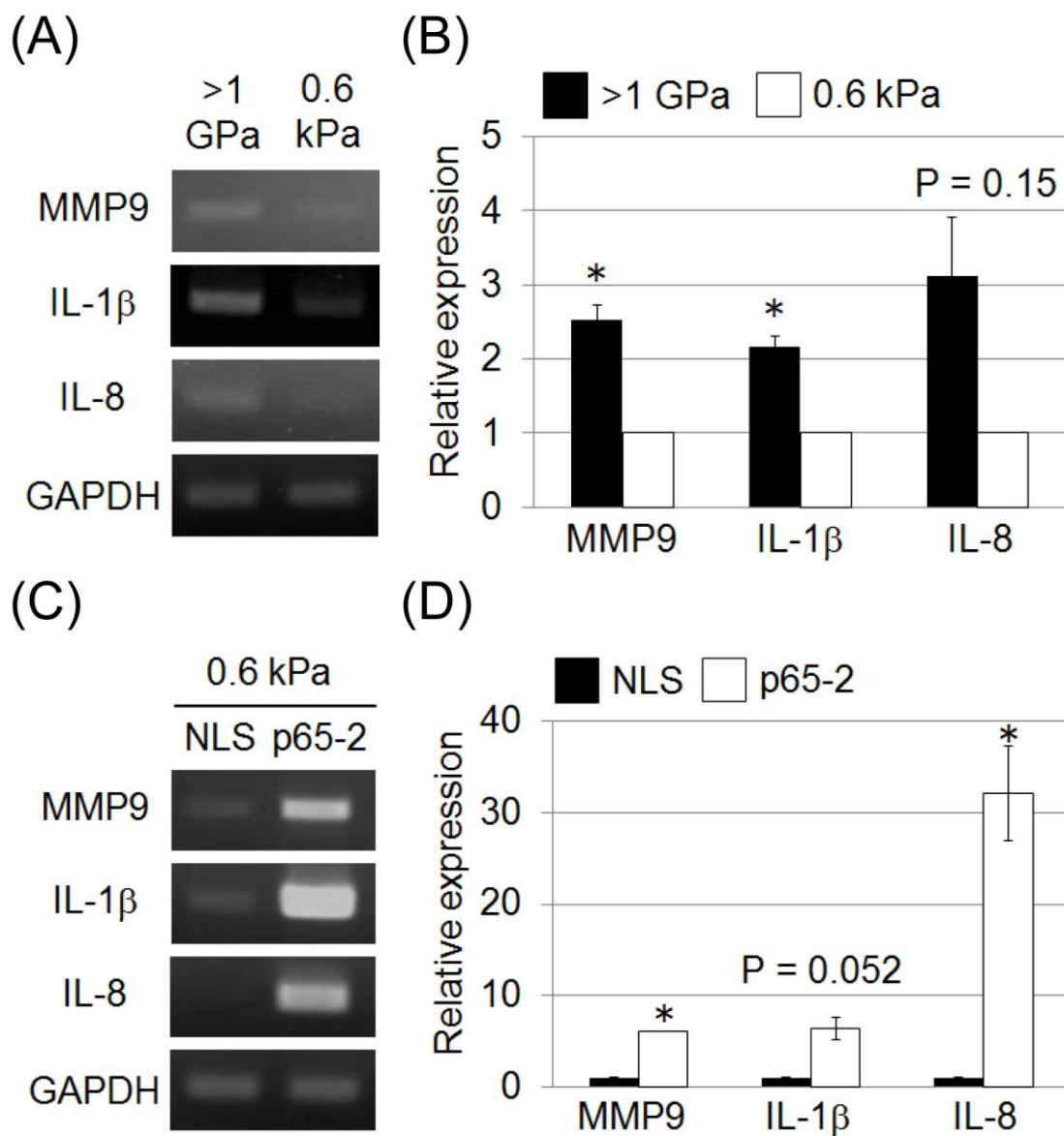


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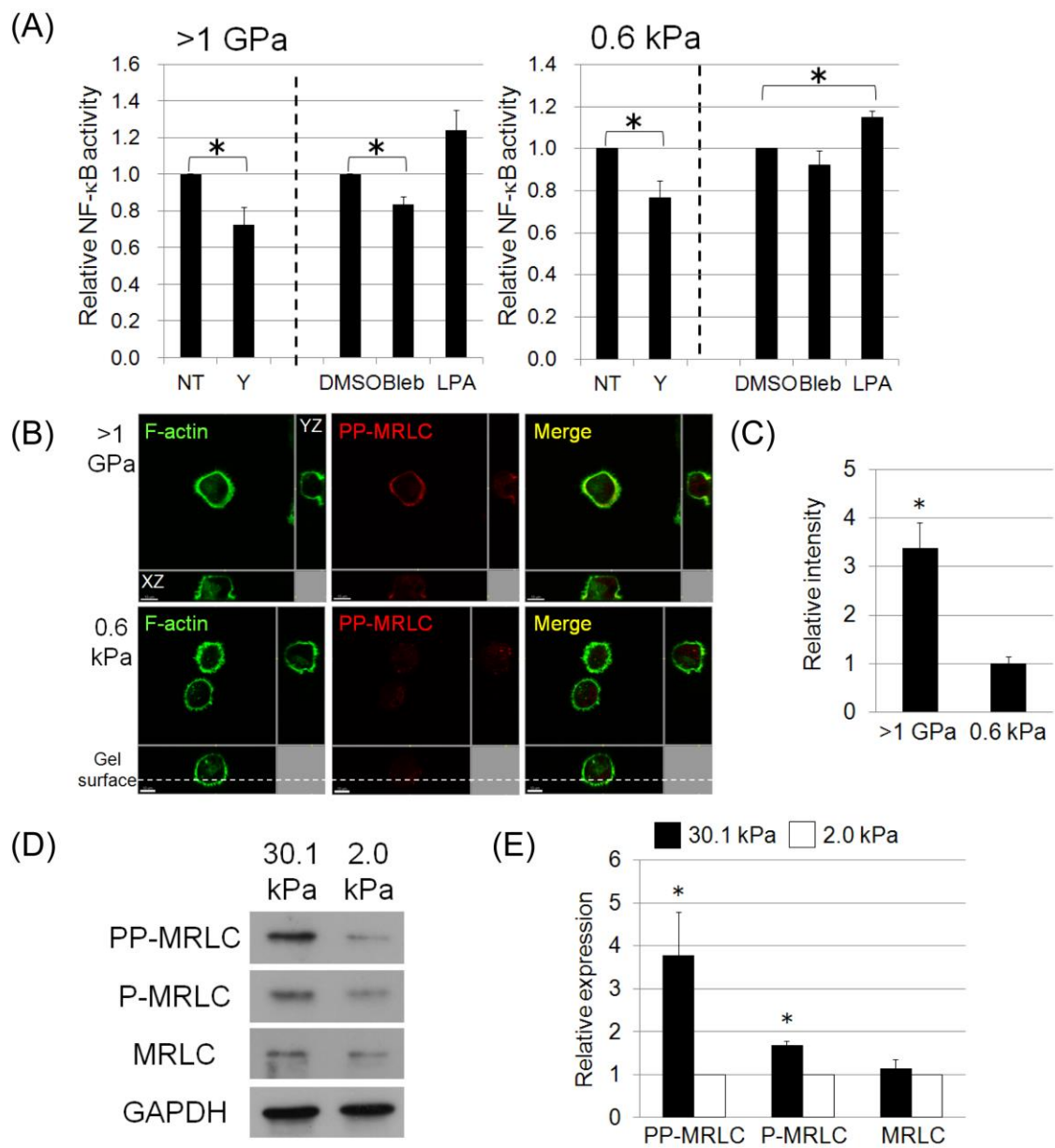


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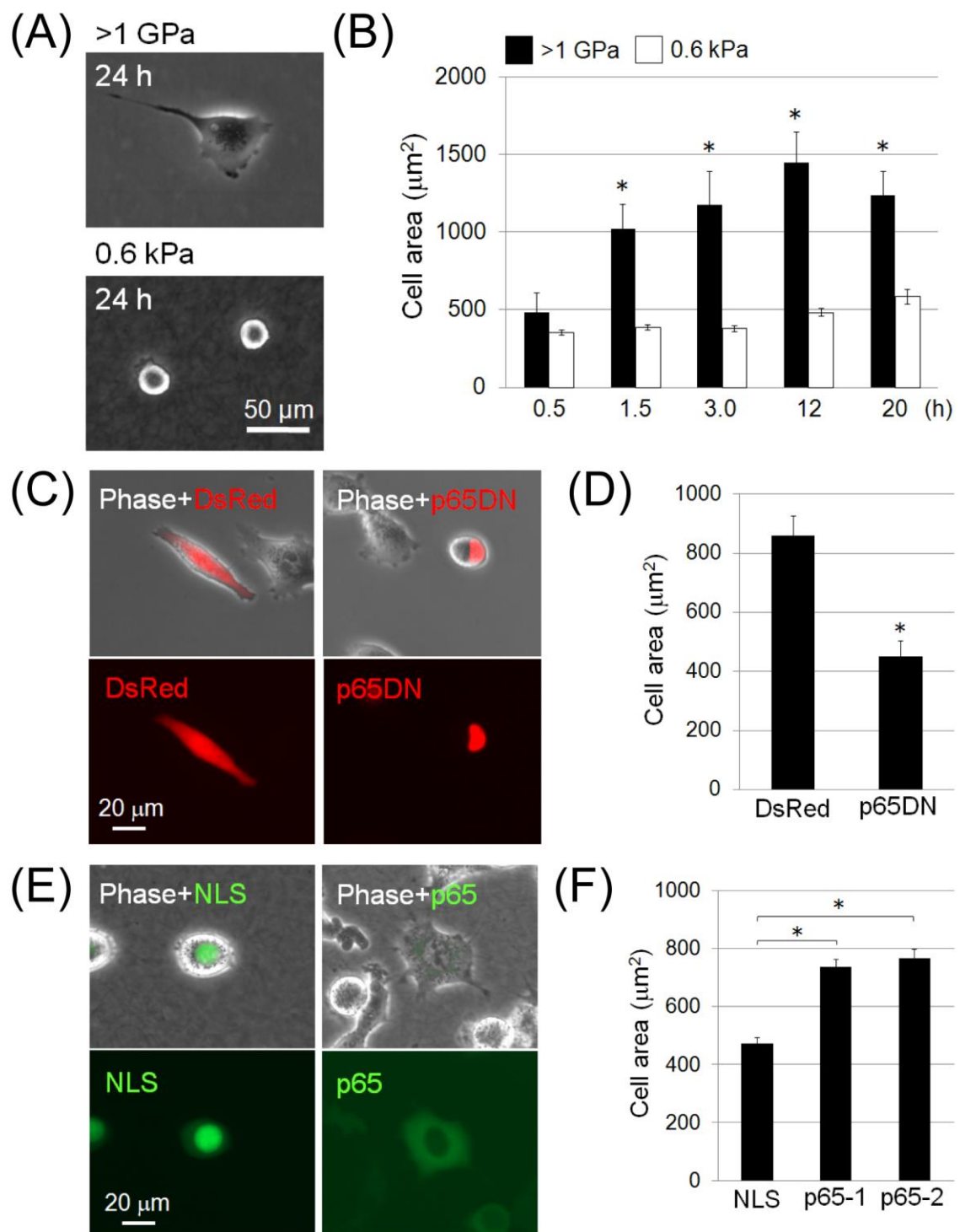
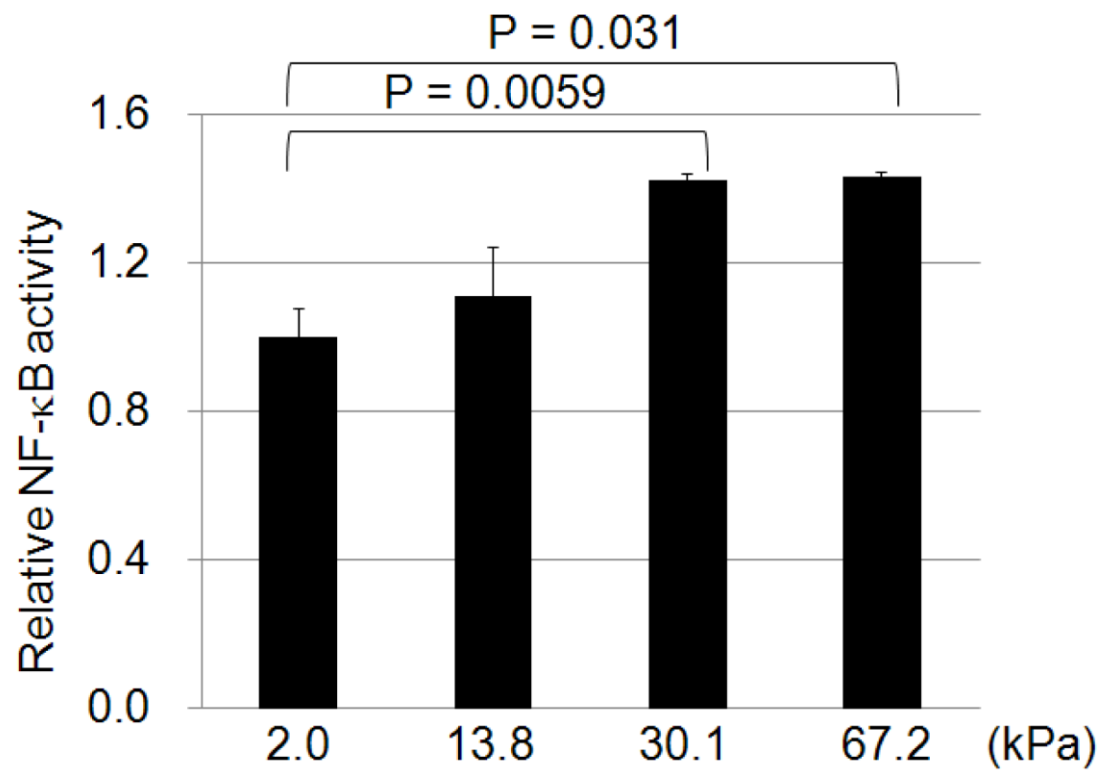


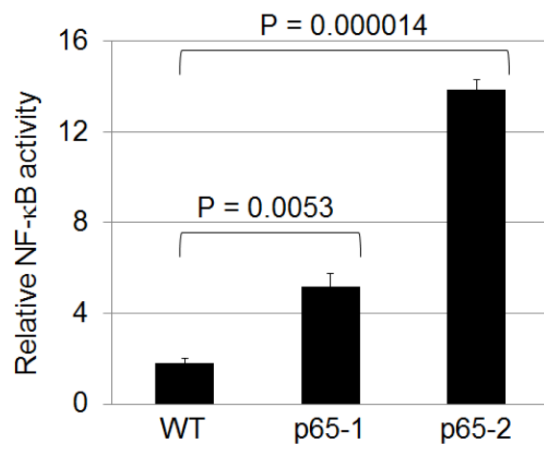
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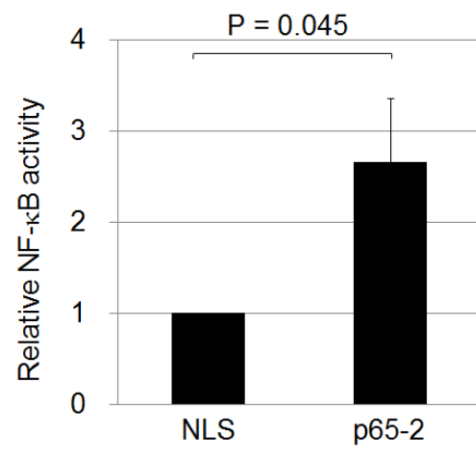
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2 **Figure S1**

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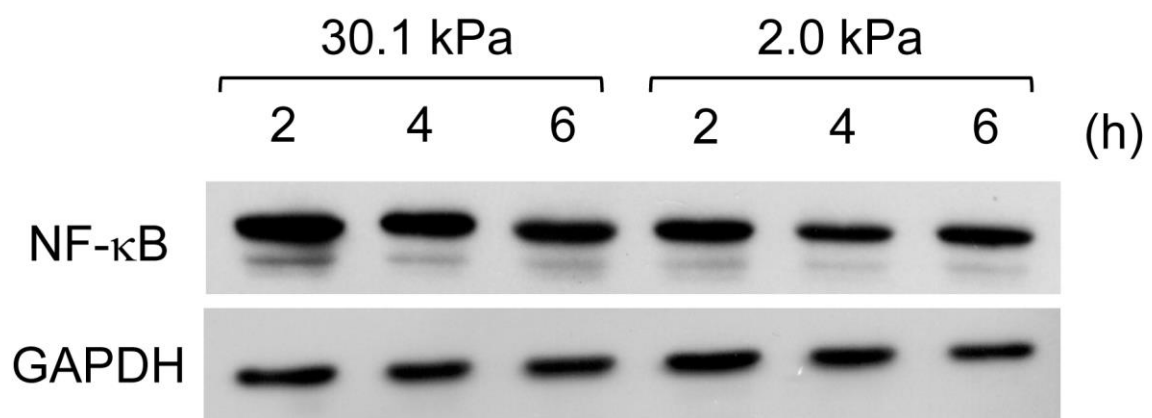


(B)



1

2 **Figure S2**



1

2 **Figure S3**

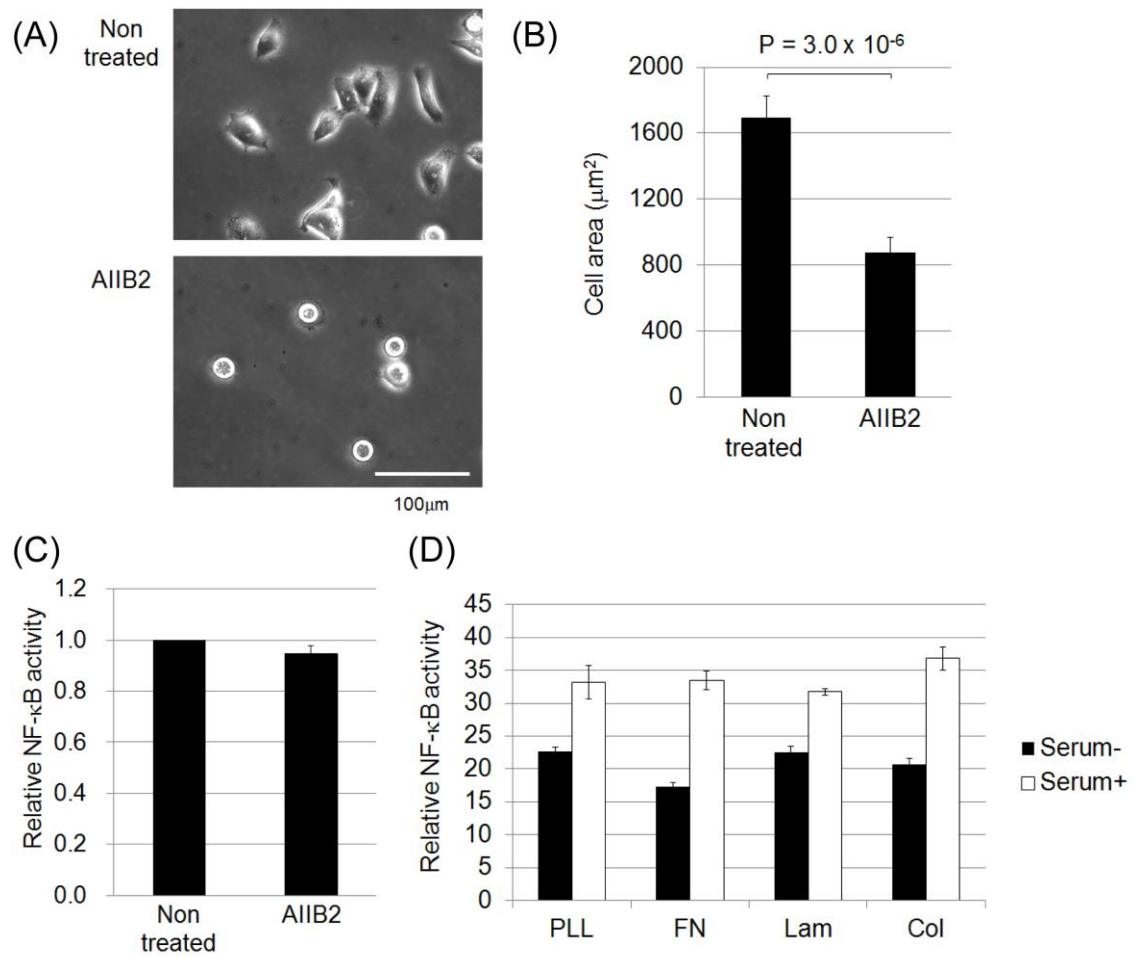
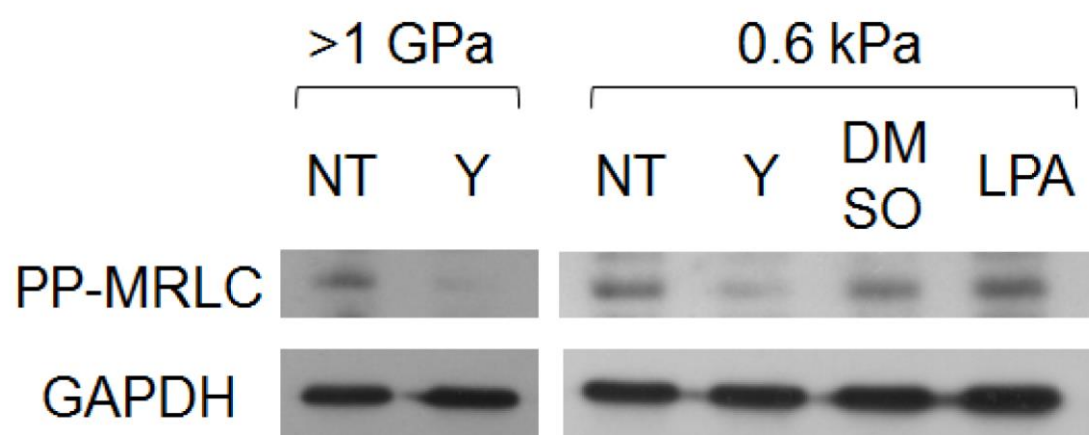


Figure S4



1

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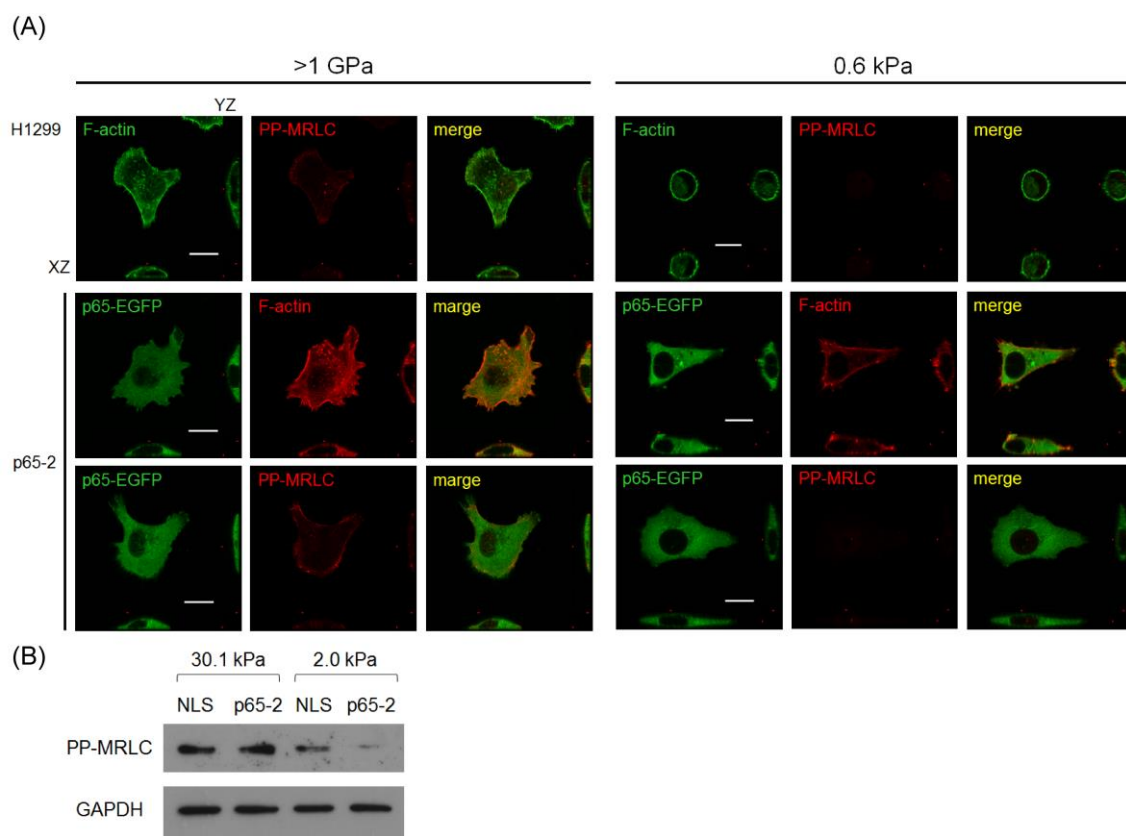
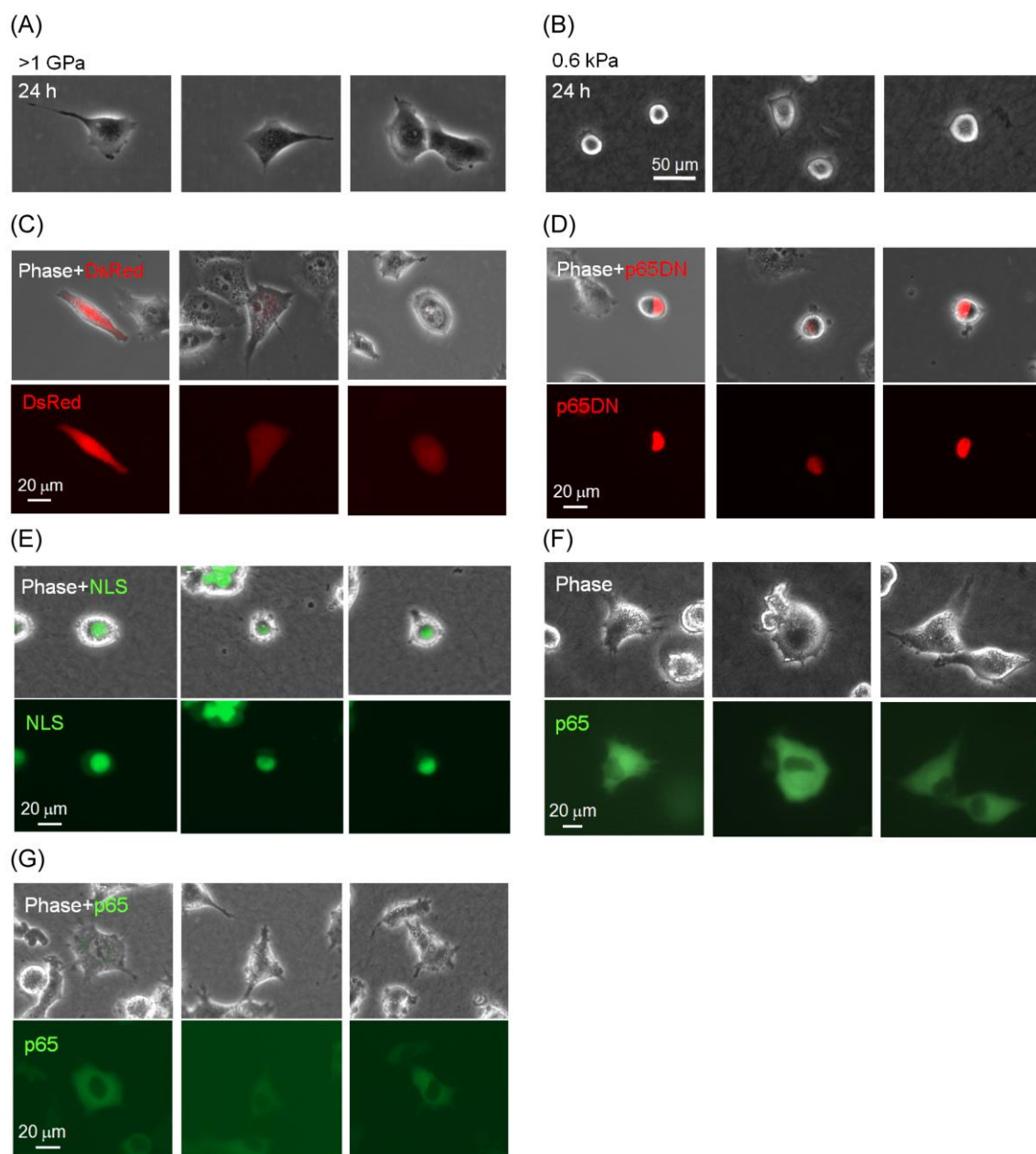


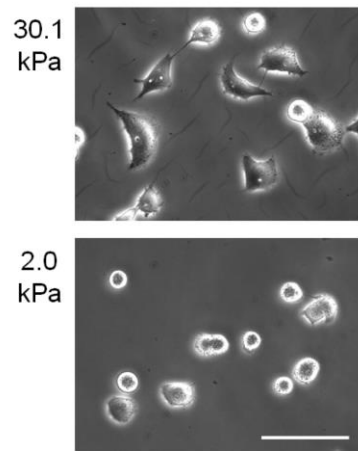
Figure S6



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2 **Figure S7**

(A)



(B)

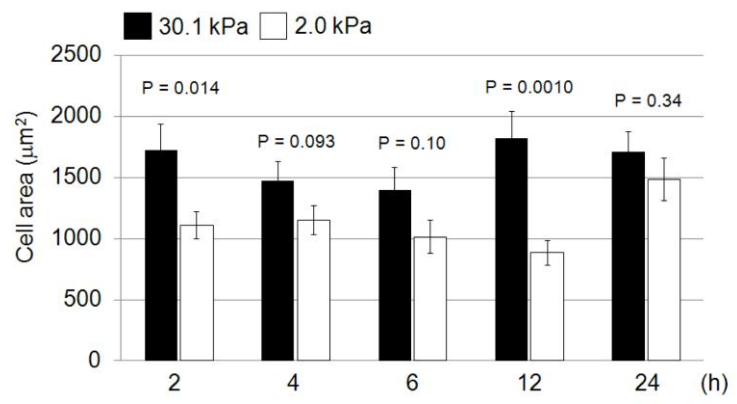
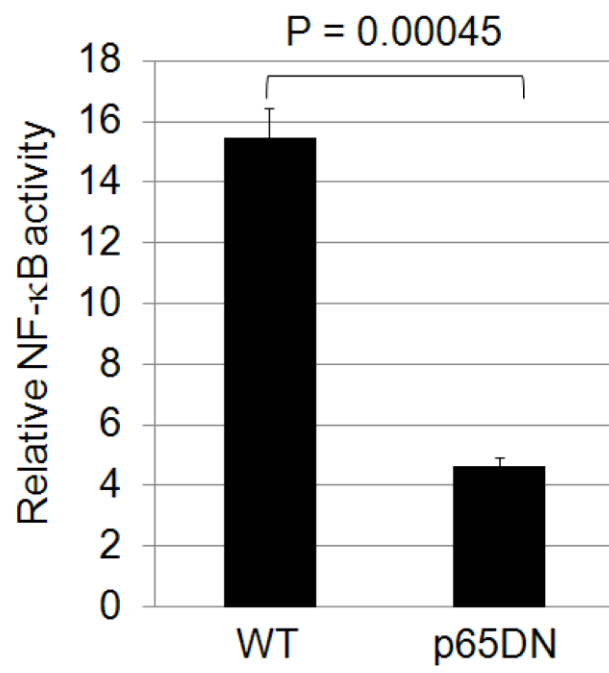
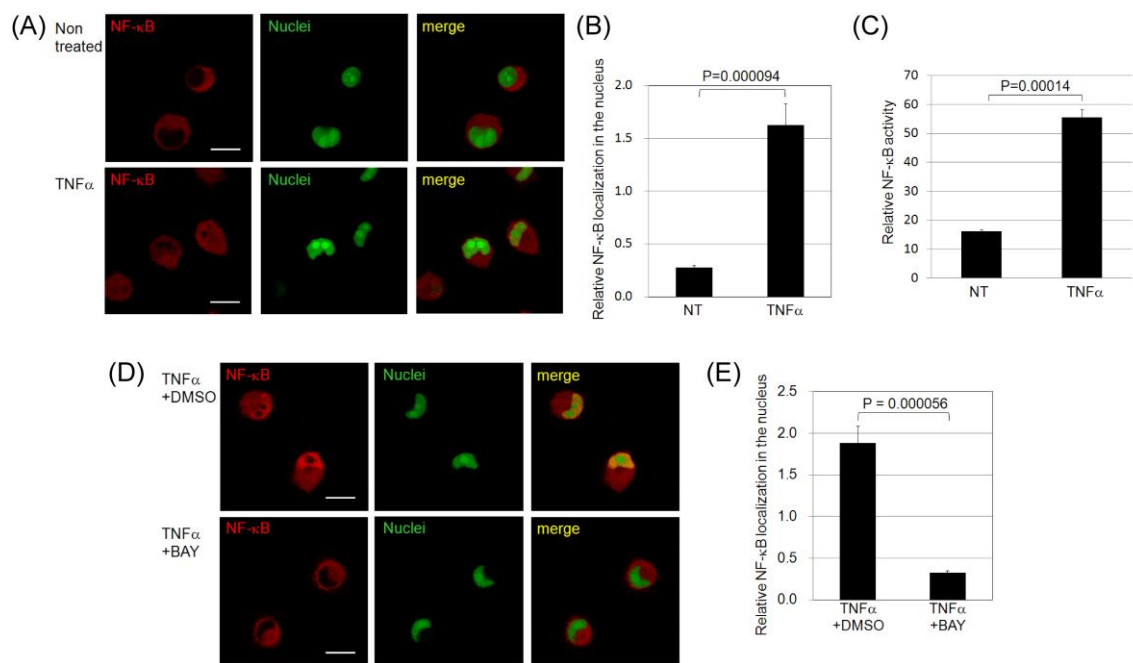


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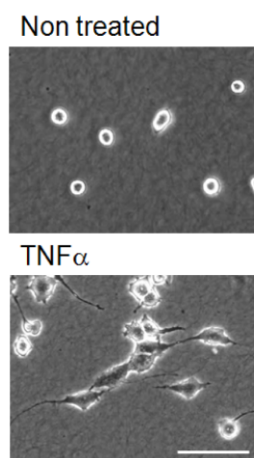
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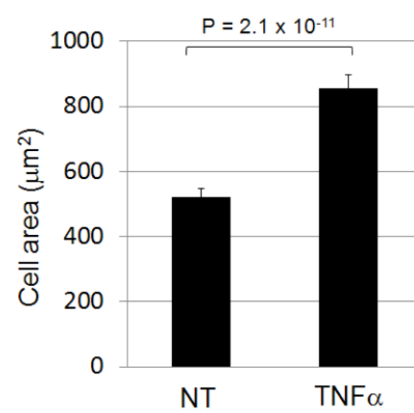
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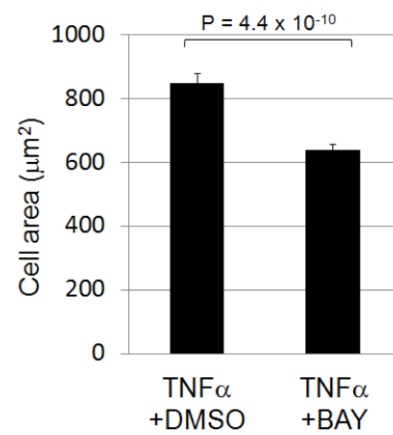
(A)



(B)



(C)



1

2 **Figure S11**

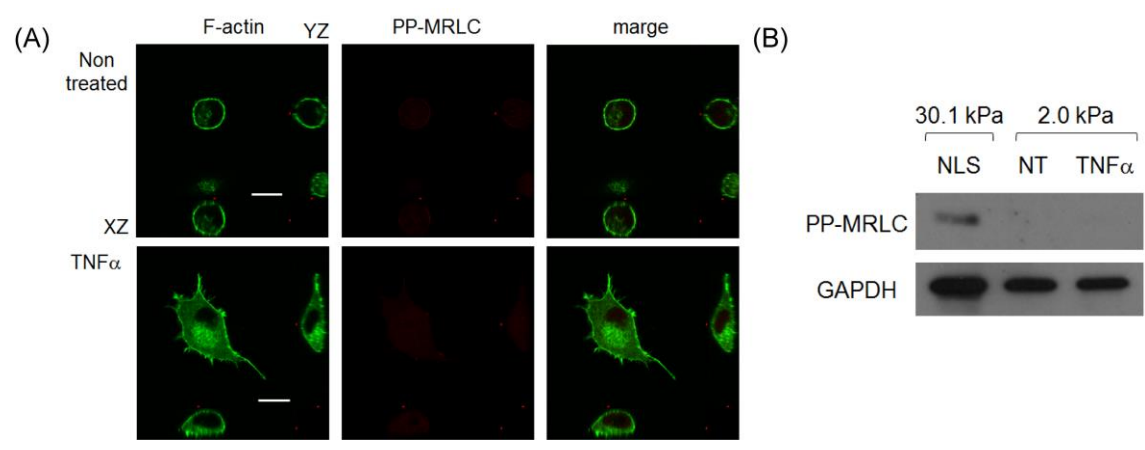
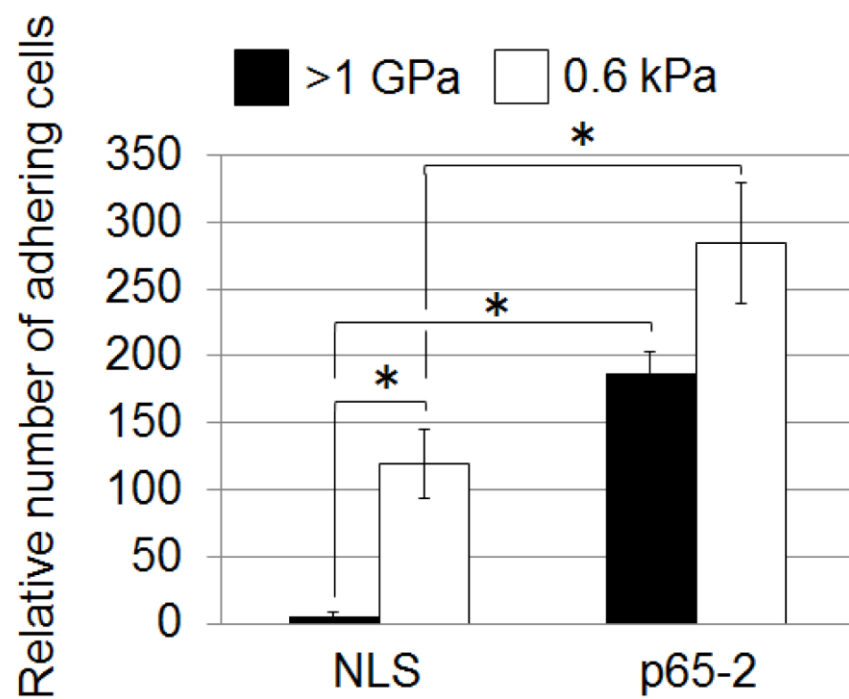


Figure S12



1

2 **Figure S13**

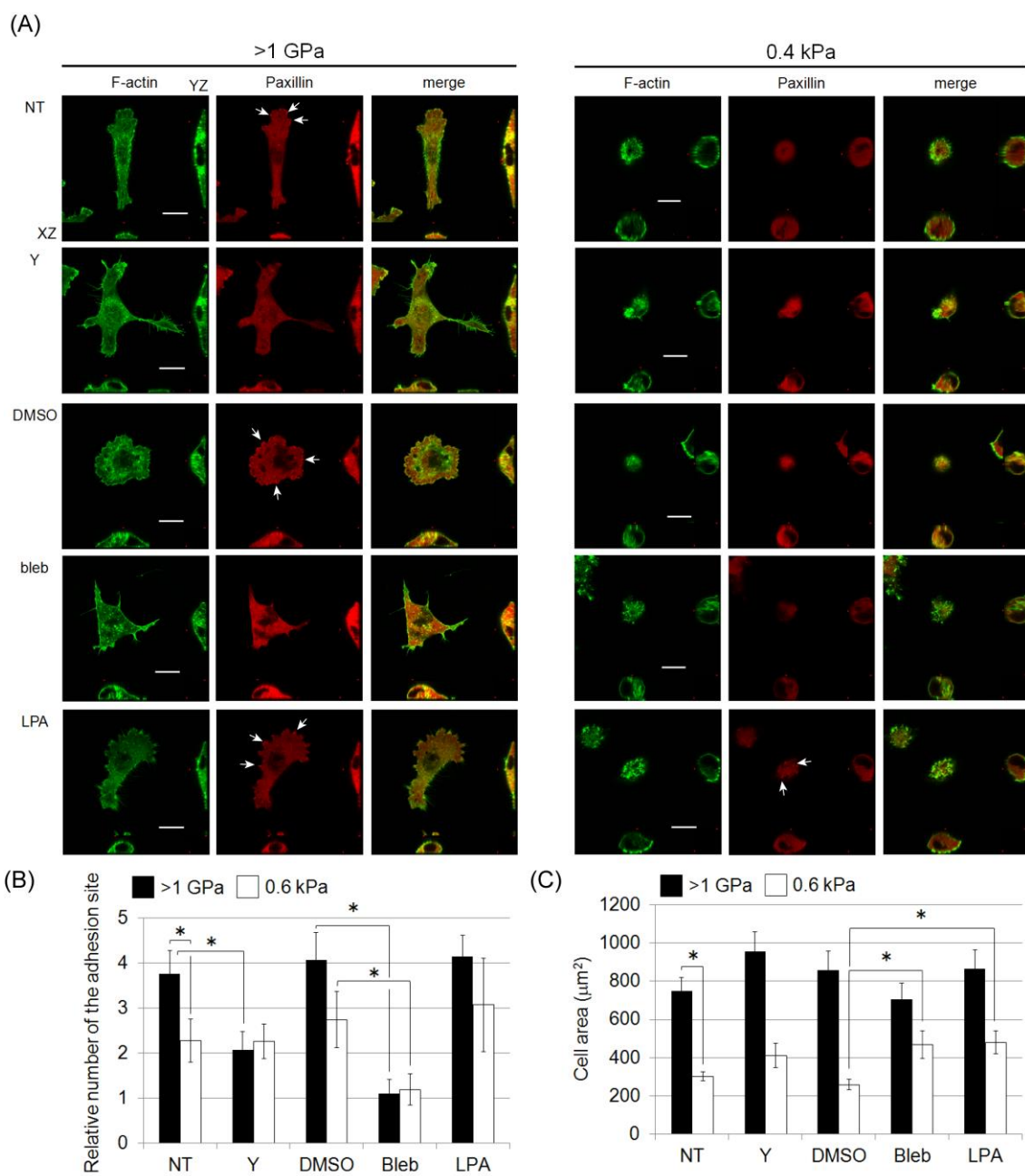


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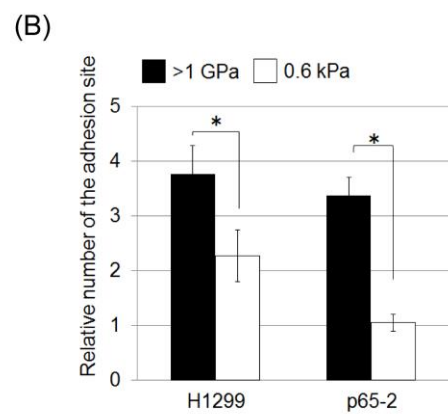
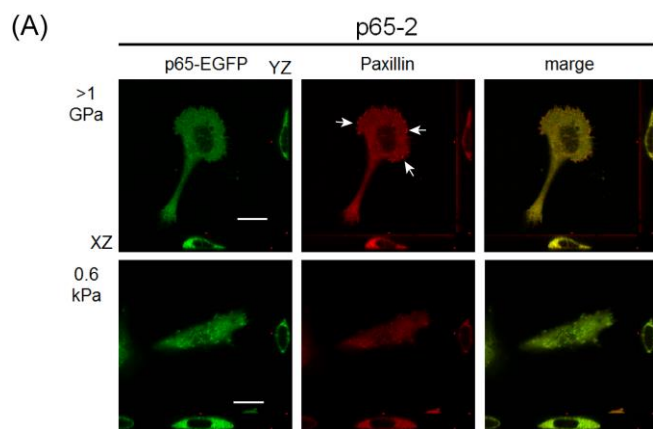


Figure S15