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Capsaicin partially mimics heat in mouse fibroblast cells in vitro

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Abstract

Purpose: Capsaicin activates transient receptor potential vanilloid 1 (TRPV1), a cation channel in the transient receptor potential family, resulting in the transient entry of Ca^{2+} and Mg^{2+} and a warm sensation. However, the effects of capsaicin on cells have not fully elucidated in fibroblasts. In this study, we investigated whether capsaicin could induce signal transduction in mouse fibroblast cells and compared the effect with that of heat-induced signal transduction.

Methods: The activation of the mitogen-activated protein kinases (MAPKs) ERK and p38 MAPK, expression levels of heat shock protein 70 (HSP70) and HSP90, actin assembly, and cell proliferation were analyzed in NIH3T3 mouse fibroblast cells.

Results: A 15-min stimulation with capsaicin (~100 μM) phosphorylated ERK and p38 MAPK and induced actin assembly. A 2-d stimulation with capsaicin increased the level of HSP70, but not HSP90, and the 2-d stimulation with capsaicin (~100 μM) did not affect cell proliferation. A 15-min exposure to moderate heat (39.5°C) phosphorylated both ERK and p38 MAPK and induced actin assembly to similar degrees as stimulation with capsaicin. A 2-d exposure to moderate heat increased the levels of both HSP70 and HSP90 and prevented cell proliferation. However, 2-d stimulation with capsaicin (100 μM) failed to prevent heat shock-induced cell death.

Conclusions: Thus, our results suggest that the effects of capsaicin on fibroblast cells partially differ from those of heat. Notably, a 2-d stimulation with capsaicin was not sufficient to develop heat tolerance in fibroblast cells.

Keywords

capsaicin, heat, temperature sense, TRP family, tolerance

Introduction

Capsaicin is found in the most frequently consumed chili peppers in the genus *Capsicum*. Many who live in warm regions frequently eat foods containing capsaicin. After eating those foods, individuals feel warm, experience an increase in body temperature, and begin to sweat (Szolcsányi 2015). On the other hand, when in ambient warm conditions, the body temperature increases, and sweat is exuded. Thus, eating capsaicin and exposure to heat might have many similar effects on the body.

Transient receptor potential (TRP) cationic channels are non-selective channels that perform various cellular functions (Venkatachalam and Montell 2007; Bandell et al. 2007). Chemical and temperature sensing by cells is performed largely by the direct activation of TRP cationic channels such as the TRPV subfamily, TRPV1, TRPV2, TRPV3, and TRPV4. TRPV1 and TRPV2 are activated by invasive moderate heat (>42°C) and noxious heat (>51°C), respectively (Caterina et al. 1997, 1999). TRPV3 and TRPV4 are activated at temperatures ranging from 33–39°C and 27–34°C, respectively (Peier et al. 2002; Güler et al. 2002; Montell 2005). TRPV1, TRPV2, and TRPV4 are the most abundantly expressed TRP channels (Yang et al. 2006). Capsaicin activates TRPV1, resulting in the transient entry of Ca²⁺ and Mg²⁺ and a warm and burning sensation (Szolcsányi 2015).

Recently, we demonstrated that moderate heat (39.5°C) upregulates the expression of heat shock proteins (HSPs), induces morphological changes, and causes cells to develop heat tolerance (Sugimoto et al. 2012, 2014a). If capsaicin affects cells in a similar manner as heat by activating TRPV1, it is postulated that capsaicin can regulate HSPs, cell morphology, and heat tolerance. However, the effects of capsaicin on cells have not been fully elucidated.

In this study, to determine whether capsaicin mimics heat, we conducted various in vitro experiments with cultured cells. We investigated the effects of capsaicin and heat on cellular responses, i.e., activation of mitogen-activated protein kinases (MAPKs), cell morphology, levels of HSPs, and heat tolerance, in mouse fibroblast cells.

Materials and Methods

Chemicals

Capsaicin and Dulbecco's modified Eagle's medium (DMEM) were obtained from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Fetal bovine serum (FBS) was obtained from Invitrogen Corporation (Carlsbad, CA). Anti-phospho-specific p38 mitogen-activated protein kinase (p38 MAPK) (Thr180/Tyr182) rabbit antibody (#9211), anti-p38 MAPK rabbit antibody (#9212), anti-phospho-specific extracellular signal-regulated kinase (ERK1/2) (Thr202/Tyr204) (20G11) rabbit antibody (#4376), anti-ERK1/2 rabbit antibody (#9102), anti-HSP90 (E289)

rabbit antibody (#4875), anti-HSP70 rabbit antibody (#4872), anti- β -actin rabbit antibody (#4967), and horseradish peroxidase-conjugated anti-rabbit IgG (#7074) were purchased from Cell Signaling Technology, Inc. (Danvers, MA).

Cell culture

NIH3T3 mouse fibroblast cells were provided by Dr. Komine (Kanazawa University). The cells were maintained in DMEM containing 10% FBS at 37°C in a 5% CO₂ incubator.

Reverse transcription-mediated polymerase chain reaction analysis

To evaluate the expression pattern of transient receptor potential vanilloid 1 (TRPV1) mRNA in the cells, reverse transcription-mediated polymerase chain reaction (RT-PCR) was performed as follows. Briefly, RNA was extracted from the cells and reverse transcribed using the reverse transcriptase ReverTra Ace (TOYOBO, Tokyo, Japan). PCR-based subtype-specific gene amplification for HSP70, HSP90, and β -actin was performed with LA Taq (TAKARA, Tokyo, Japan) using the following sets of primers: 5'-AACGTGCTGCGGATCATCAAC-3' and 5'-GCTTGTCTGGCTGATGTCCT-3' for HSP70, 5'-TGAGGCAGAGGAAGAGAAAGG-3' and 5'-AGTGCTTGACTGCCAAGTGGT-3' for HSP90, and 5'-ATGGTGGGTATGGGTCAGAAG-3' and 5'-CTGGGGTGTTGAAGGTCTCAA-3' for β -actin. The PCR conditions were 32 cycles of amplification (30 s at 94°C; 30 s at 55°C; 30 s at 72°C) or 25 cycles of amplification (30 s at 94°C; 30 s at 60°C; 30 s at 72°C) for HSP70 and HSP90 or β -actin, respectively.

Exposure to moderate heat

Two CO₂ incubators were maintained. One was kept at 37°C, and the other was kept at 39.5°C. NIH3T3 cells were seeded in 35-mm culture dishes or 96-well plates. After 24 h of incubation at 37°C, cells were incubated at 37°C or 39.5°C in 5% CO₂ for 15 min or 2 d.

Western blotting analysis

Western blotting was performed as described previously (Sugimoto et al. 2016). Briefly, proteins were extracted from cells, and protein concentrations were determined by a protein assay. Equal amounts of protein (30 μ g) were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The resolved proteins were transferred onto polyvinylidene fluoride (PVDF) membranes, which were incubated with primary antibodies (1:1000), followed by incubation with HRP-linked secondary antibodies (1:2000).

Actin filament staining

To evaluate the actin cytoskeletons, the cells were fixed in 3.7% (v/v) formaldehyde in Dulbecco's phosphate buffered saline and processed as described previously (Sugimoto et al, 2016). F-actin was visualized with TRITC-labeled phalloidin under an inverted EVOS fluorescence microscope (Life Technologies Japan, Tokyo, Japan).

Cell proliferation and viability assay

Cell proliferation and viability were analyzed using the Cell Counting Kit 8 (Wako, Japan) as described previously (Leu et al. 2016). NIH3T3 cells were seeded in 96-well plates at a density of 1×10^3 cells/well. After a 24-h incubation, the cells were divided into five groups: no stimulation at 37°C (control), 2-d stimulation with capsaicin (10, 100, and 1000 μ M) at 37°C, and 2-d exposure to 39.5°C. At the end of 2 d of continuous treatment, cells were allowed to recover at the standard culture temperature (37°C) until their medium temperature returned to 37°C, which required approximately 1 h. Then, the cells were incubated with 10 μ L WST-8 for 3 h at 37°C. The absorbance of the colored formazan product produced by mitochondrial dehydrogenases in metabolically active cells was recorded at 450 nm as the background value. Cell proliferation was expressed as a ratio of the absorbance obtained in treated wells relative to that in untreated (control) wells.

Severe heat shock treatment

The cells were exposed at 45°C for 30 min following the 2-d treatment with capsaicin at 37°C or without capsaicin at 39.5°C in 5% CO₂. One day after the heat shock treatment, cell viability was analyzed using the Cell Counting Kit 8 as described above.

Statistical analysis

Data are presented as the mean \pm SEM from at least three independent experiments. Statistical analysis was performed using a Student's unpaired t-test or a Kruskal Wallis non-parametric ANOVA followed by a Bonferroni test, and results were considered statistically significant when $p < 0.05$ or $p < 0.01$.

Results

Capsaicin activates ERK, p38 MAPK, and actin assembly in NIH3T3 cells

First, we examined the expression of TRPV1 mRNAs in NIH3T3 cells using reverse transcription-PCR. TRPV1 expression was detectable in these cells (Supplemental Fig. 1). We then tested the effects of the TRPV1 agonist capsaicin on ERK, p38 MAPK, and cellular morphology in these cells. Although all MAPKs play crucial roles in the transduction from

extracellular stimuli to intracellular signaling, p38 MAPK in particular is a key kinase that is activated by several forms of stress. A 15-min treatment with capsaicin increased the phosphorylation of ERK and p38 MAPK in a dose-dependent manner (Fig. 1a), indicating the activation of ERK and p38 MAPK. A 15-min treatment with capsaicin also induced stress fiber formation, indicating the activation of actin assembly (Fig. 1b). Interestingly, ERK inhibitor prevented capsaicin-induced stress fiber formation (Supplemental Fig. 2). A 60-min treatment with capsaicin increased the phosphorylation of ERK as well as a 15-min treatment with capsaicin (Fig. 1c).

Moderate heat activates ERK, p38 MAPK, and actin assembly in NIH3T3 cells

In order to compare the capsaicin and heat treatments, we examined the effects of moderate heat (39.5°C), which can induce cellular heat adaptation (Sugimoto et al. 2012, 2014a), on ERK, p38 MAPK, and cellular morphology in these cells. A 15-min or 60-min exposure to moderate heat phosphorylated ERK (Fig. 2a and 2c) and p38 MAPK (Fig. 2a) and increased stress fiber formation (Fig. 2b).

These results indicate that the effects of capsaicin on ERK, p38 MAPK, and actin assembly are similar to the effects of moderate heat in mouse fibroblast cells.

Capsaicin does not affect cell proliferation in NIH3T3 cells

We previously showed that a 5-d exposure to moderate heat inhibits cell proliferation in mouse fibroblast cells (Sugimoto et al. 2012). Next, we investigated the effect of a 2-d treatment with capsaicin or 2-d exposure to heat on cell proliferation in NIH3T3 cells. Cell proliferation was not significantly altered by capsaicin (~100 μ M), although capsaicin at 1 mM and heat significantly prevented cell growth after 2 d (Fig. 3). These results indicate that a continuous 2-d treatment with low doses of capsaicin (~100 μ M), unlike a high dose of capsaicin (1 mM) and heat, does not alter cell proliferation.

Continuous 2-d treatment with capsaicin induces HSP70 expression, but not HSP90 expression, in NIH3T3 cells

The continuous exposure for more than 24 h to moderate heat induces the up-regulation of heat shock protein 70 (HSP70) and HSP90 (Sugimoto et al. 2012). Indeed, the intensity of the bands corresponding to HSP70 and HSP90 increased after continuous 2-d exposure to moderate heat in mouse fibroblast cells (Fig. 4b and 5). As mentioned above, the effects of capsaicin on cells may be similar to effects of heat. We therefore examined whether a continuous 2-d treatment with capsaicin up-regulates HSPs in NIH3T3 cells. The expression of HSP70, but not HSP90, was increased after a 2-d treatment with capsaicin in NIH3T3 cells (Fig. 4a and 5).

Continuous 2-d treatment with capsaicin does not attenuate severe heat shock-induced cell death in NIH3T3 cells

HSPs play an important role in protecting cells from environmental stressors such as heat shock (Morotomi et al. 2014; Sugimoto et al. 2012, 2014a, b). Next, we examined whether a continuous 2-d treatment with capsaicin can improve heat tolerance in NIH3T3 cells by measuring cell viability after severe heat shock in mouse fibroblast cells. The cells were incubated at 45°C for 30 min (severe heat shock) after an initial 2-d treatment with capsaicin at 37°C or without capsaicin at 39.5°C and were then returned to incubation at 37°C. Cell proliferation was analyzed 1 d after severe heat shock, and cell viability was examined with a Cell Counting Kit 8 (Wako, Japan) as described in the Materials and Methods. Cell viability after the capsaicin treatment was not significantly different from that with no treatment (Fig. 6). However, 2-d incubation at 39.5°C significantly prevented heat-shock induced cell death (Fig. 6). This result suggested that the continuous 2-d treatment with capsaicin does not attenuate severe heat shock-induced cell death and may not improve heat tolerance in NIH3T3 cells.

Discussion

Capsaicin activates TRPV1, a temperature sensing channel, resulting in a warm sensation. However, the effects of capsaicin on cells have not fully elucidated in fibroblasts. The results of our investigation provide evidence that capsaicin activates MAPKs and induces HSP70 in a similar fashion as heat but fails to upregulate HSP90 and prevent heat shock-induced cell death *in vitro*, indicating that capsaicin only partially mimics heat.

TRPV1 mediates heat sensation by allowing the transient entry of Ca^{2+} and Mg^{2+} . Intracellular Ca^{2+} is a major second messenger and plays crucial roles in the transduction from extracellular stimuli to intracellular signaling, especially that involving MAPKs and actin assembly. Zhang et al. (2007) has shown that capsaicin induces signal transduction in a TRPV1-dependent manner. Therefore, the present results indicate that capsaicin might regulate ERK, p38 MAPK, and actin assembly via TRPV1 activation in mouse fibroblast cells (Fig. 1).

Several investigators have performed extensive studies to elucidate the molecular mechanisms underlying the cellular response to invasive moderate heat (Richter et al. 2011). However, body, tissue, or cellular temperature increases by only a few degrees Celsius during physiological events, i.e., heat acclimation and febrile disease. Moderate heat (39.5°C) unlike invasive moderate heat (>42°C) does not induce apoptosis (Park et al. 2005; Sugimoto et al. 2012, 2014a). Therefore, the physiological cellular response to moderate heat (febrile range temperature) rather than invasive moderate heat (>42°C) is likely to be more important.

Although TRPV1 is usually activated by invasive moderate heat (>42°C) (Caterina et al. 1997), temperatures lower than 42°C partially activate TRPV1 (El Kouhen et al, 2005; Sun and Zakharian 2015). We selected a temperature of 39.5°C to examine cells under the same conditions as in our previous studies (Sugimoto et al. 2012, 2014a).

Moderate heat (39.5°C) activates MAPKs and actin assembly (Fig. 2) to similar degrees as capsaicin (Fig. 1). However, moderate heat (39.5°C) might be able to partially activate TRPV1 (El Kouhen et al, 2005; Sun and Zakharian 2015). Thus, moderate heat (39.5°C) may activate MAPKs and actin assembly in a TRPV1-dependent and/or TRPV1-independent manner.

p38 MAPK is generally known as the principal stress-activated protein kinase, and the modulation of the p38 MAPK pathway regulates HSP transcription (Gong et al. 2012) through the activation of heat shock factor-1 (HSF-1) (Westerheide et al. 2012). In this study, capsaicin elevated the level of HSP70 but failed to upregulate HSP90 expression (Fig. 4 and 5). On the other hand, our present and previous studies (Sugimoto et al. 2012, 2014a) indicate that moderate heat increases the levels of both HSP70 and HSP90 (Fig. 4 and 5). It is not known why capsaicin failed to increase the level of HSP90 following the activation of p38 MAPK in fibroblast cells. Further experiments are required to reveal the reason, but capsaicin did partially mimic heat in terms of the regulation of HSP expression *in vitro*.

Cells activate various mechanisms to counteract stress and maintain cellular homeostasis when challenged with acute changes in their physical, cellular, or intracellular environment. This stress response helps the cell to avoid apoptotic cell death and survive. HSPs have been demonstrated to play an essential chaperoning role that helps the cell to maintain cellular protein homeostasis and to escape apoptosis under diverse forms of stress (Creagh et al. 2000; Morimoto et al. 1998; Horowitz and Assadi, 2010; Morotomi et al. 2014). The upregulation of HSP70 and HSP90 has been shown to cause the development of heat tolerance in NIH3T3 cells (Sugimoto et al. 2012, 2014a). In the present study, we found that capsaicin did not upregulate HSP90 levels in NIH3T3 cells (Fig. 4 and 5). Hence, capsaicin failed to improve heat shock-induced cell death in this study (Fig. 6).

In this study, treatment with capsaicin (~100 µM) did not affect cell proliferation (Fig. 3) or caspase-3 activation (data not shown), indicating that a low dose of capsaicin is non-toxic in mouse fibroblast cells. However, capsaicin at 1 mM reduced cell viability in NIH3T3 cells, indicating that a high dose of capsaicin is cytotoxic in mouse fibroblast cells. Namely, capsaicin and its analogs have dose-dependent cytotoxic effects, resulting in apoptotic cell death (Clark and Lee 2016; Lee et al. 2016). However, moderate heat prevented cell proliferation (Fig. 3) without caspase-3 activation (data not shown), suggesting that moderate heat-induced inhibition of cell growth is independent from cell apoptosis (Kühl et al. 2000; Kühl and Rensing 2000;

Morotomi et al. 2014; Sugimoto et al. 2012). Based on our previous data and that from other studies, moderate heat is thought to elicit cell cycle arrest (Kühl et al. 2000; Kühl and Rensing 2000; Morotomi et al. 2014). In this regard also, capsaicin differs from heat.

Conclusions

The current study showed that capsaicin upregulated HSP70 but failed to prevent heat shock-induced cell death in vitro. These results suggest that capsaicin cannot fully mimic heat (physiological temperature range).

Potential conflicts of interest

The authors do not have any conflicts of interest to declare.

Authors' contributions

NS designed the study and prepared the manuscript. NS, MK, and KM conducted the experiments. MK and HN analyzed the data. NS obtained funding. AY and OS edited the manuscript. All authors read and approved the final version of this manuscript.

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Figure Legends

Fig. 1 Capsaicin phosphorylates ERK and p38 MAPK, and alters in actin assembly in NIH3T3 mouse fibroblast cells.

(a) Effect of capsaicin on ERK and p38 MAPK phosphorylation. The cells were stimulated with capsaicin (10, 100 μ M) for 15 min. Total cell extracts were analyzed for ERK or p38 MAPK phosphorylation by Western blotting. The densities of bands corresponding to phosphorylated ERK, phosphorylated p38 MAPK, total ERK, and total p38 were quantified by densitometry. Results are means \pm SEM of three independent experiments. Statistical analysis was conducted using a Kruskal Wallis non-parametric ANOVA followed by a Bonferroni test. Statistically significant at the level of * $P < 0.05$ and ** $P < 0.01$ compared with untreated controls.

(b) Effect of capsaicin on actin assembly. The cells were treated with capsaicin (100 μ M) for 15 min and stained for visualizing F-actin with TRITC-phalloidin. Capsaicin induced stress fiber formation. Results are representative of three independent experiments.

(c) Effect of capsaicin on ERK phosphorylation. The cells were stimulated with capsaicin (100 μ M) for 15 or 60 min. Total cell extracts were analyzed for ERK phosphorylation by Western blotting. The densities of bands corresponding to phosphorylated ERK and total ERK were quantified by densitometry. Results are means \pm SEM of three independent experiments. Statistical analysis was conducted using a Kruskal Wallis non-parametric ANOVA followed by a Bonferroni test. Statistically significant at the level of * $P < 0.05$ compared with untreated controls.

Fig. 2 Moderate heat phosphorylates ERK and p38 MAPK, and alters in actin assembly in NIH3T3 mouse fibroblast cells.

(a) Effect of moderate heat on ERK and p38 MAPK phosphorylation. The cells were exposed to moderate heat (39.5°C) for 15 min. Total cell extracts were analyzed for ERK or p38 MAPK phosphorylation by Western blotting. The densities of bands corresponding to phosphorylated ERK, phosphorylated p38 MAPK, total ERK, and total p38 were quantified by densitometry. Results are means \pm SEM of three independent experiments. Statistical analysis was conducted using a Student's unpaired t-test. Statistically significant at the level of * $P < 0.05$ compared with untreated controls.

(b) Effect of moderate heat on actin assembly. The cells were exposed to moderate heat (39.5°C) for 15 min and stained for visualizing F-actin with TRITC-phalloidin. Moderate heat induced stress fiber formation. Results are representative of three independent experiments.

(c) Effect of moderate heat on ERK phosphorylation. The cells were exposed to moderate heat (39.5°C) for 15 or 60 min. Total cell extracts were analyzed for ERK phosphorylation by Western blotting. The densities of bands corresponding to phosphorylated ERK and total ERK

were quantified by densitometry. Results are means \pm SEM of three independent experiments. Statistical analysis was conducted using a Student's unpaired t-test. Statistically significant at the level of *P < 0.05 and **P < 0.01 compared with untreated controls.

Fig. 3 Change in cell growth in NIH3T3 mouse fibroblast cells by capsaicin or moderate heat. The cells were stimulated with capsaicin (10-1000 μ M) or exposed to moderate heat (39.5°C) for 2 d. Cell proliferation and viability were analyzed using the Cell Counting Kit 8 (Wako, Japan). Each column represents the mean \pm SEM (n=12). Statistical analysis was conducted using a Kruskal Wallis non-parametric ANOVA followed by a Bonferroni test. Statistically significant at the level of **P < 0.01 compared with untreated controls (37°C).

Fig. 4 Capsaicin and moderate heat induce HSPs expressions in NIH3T3 mouse fibroblast cells. (a) Effect of capsaicin on the levels of HSP70 and HSP90 proteins. The cells were stimulated with capsaicin (100 μ M) for 2 d. Total cell extracts were analyzed for HSP70 and HSP90 expressions by Western blotting. The densities of bands corresponding to HSP70 and HSP90 were quantified by densitometry. Results are means \pm SEM of three independent experiments. Statistical analysis was conducted using a Student's unpaired t-test. Statistically significant at the level of *P < 0.05 compared with untreated controls. (b) Effect of moderate heat on the levels of HSP70 and HSP90 proteins. The cells were exposed to moderate heat (39.5°C) for 2 d. Total cell extracts were analyzed for HSP70 and HSP90 expressions by Western blotting. The densities of bands corresponding to HSP70 and HSP90 were quantified by densitometry. Results are means \pm SEM of three independent experiments. Statistical analysis was conducted using a Student's unpaired t-test. Statistically significant at the level of *P < 0.05 compared with untreated controls.

Fig. 5 Capsaicin and moderate heat induce HSPs expressions in NIH3T3 mouse fibroblast cells. Effect of capsaicin and moderate heat on the levels of HSP70 and HSP90 mRNA. The cells were stimulated with capsaicin (100 μ M) or exposed to moderate heat (39.5°C) for 2 d. Total cell extracts were analyzed for HSP70 and HSP90 expressions by RT-PCR. The densities of bands corresponding to HSP70 and HSP90 were quantified by densitometry. Results are means \pm SEM of three independent experiments. Statistical analysis was conducted using a Kruskal Wallis non-parametric ANOVA followed by a Bonferroni test. Statistically significant at the level of *P < 0.05.

Fig. 6 Change in cell growth 1 d after heat shock following a 2-d treatment with capsaicin (100 μ M) or moderate heat (39.5°C) in NIH3T3 mouse fibroblast cells.

The cells were stimulated with capsaicin (1000 μ M) or exposed to moderate heat (39.5°C) for 2 d. The cells were incubated at 45°C for 30 min (severe heat shock) and were then returned to incubation at 37°C. Cell proliferation was analyzed 1 d after severe heat shock, and cell viability was examined with a Cell Counting Kit 8 (Wako, Japan). Each column represents the mean \pm SEM (n=8). Statistical analysis was conducted using a Kruskal Wallis non-parametric ANOVA followed by a Bonferroni test. Statistically significant at the level of *P < 0.05.

Suppl. Fig. 1 Expression of TRPV1 in NIH3T3 cells

Suppl. Fig. 2 ERK inhibitor prevents capsaicin-induced stress fiber formation in NIH3T3 cells

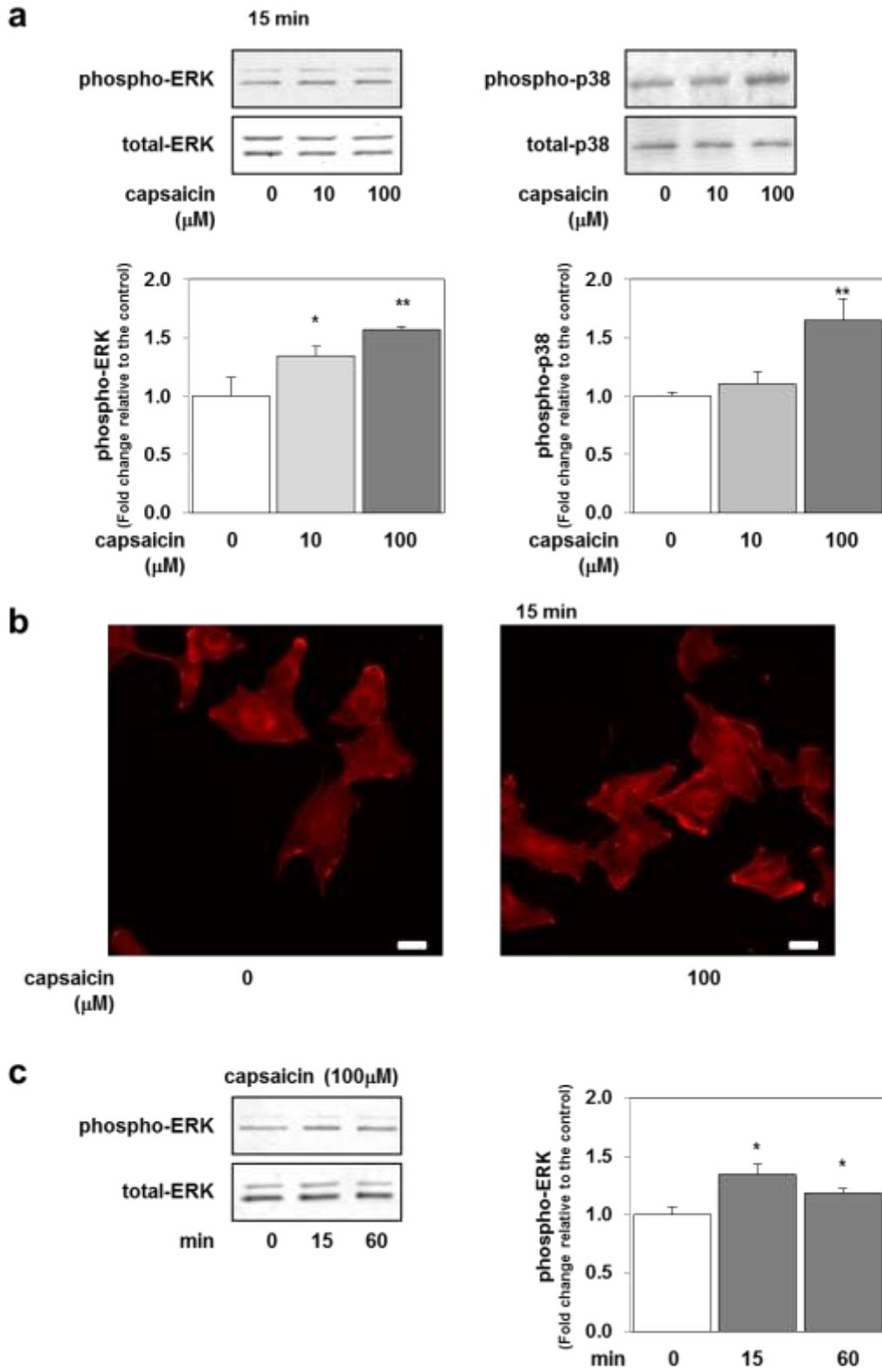


Figure 1

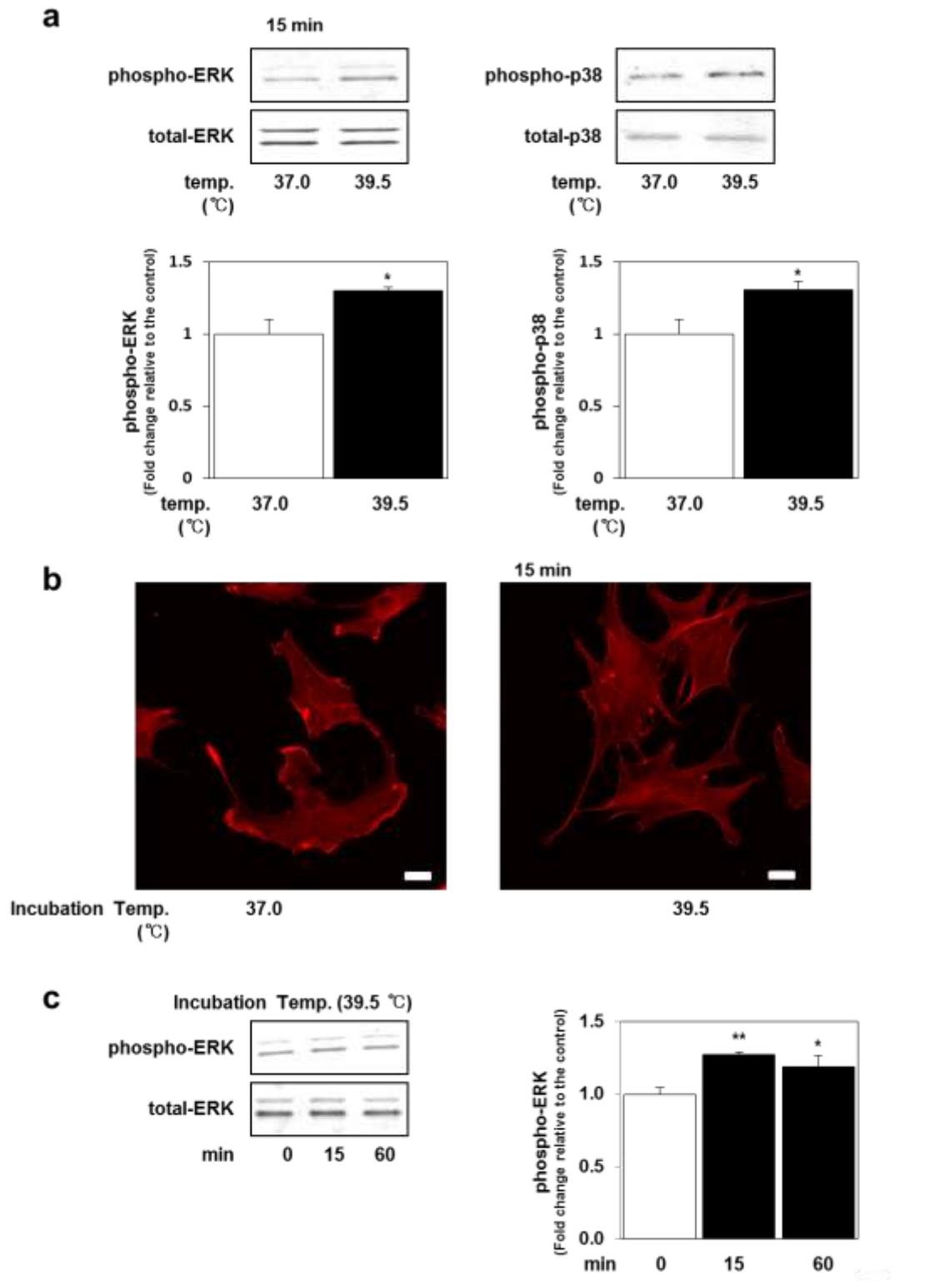


Figure 2

2 days

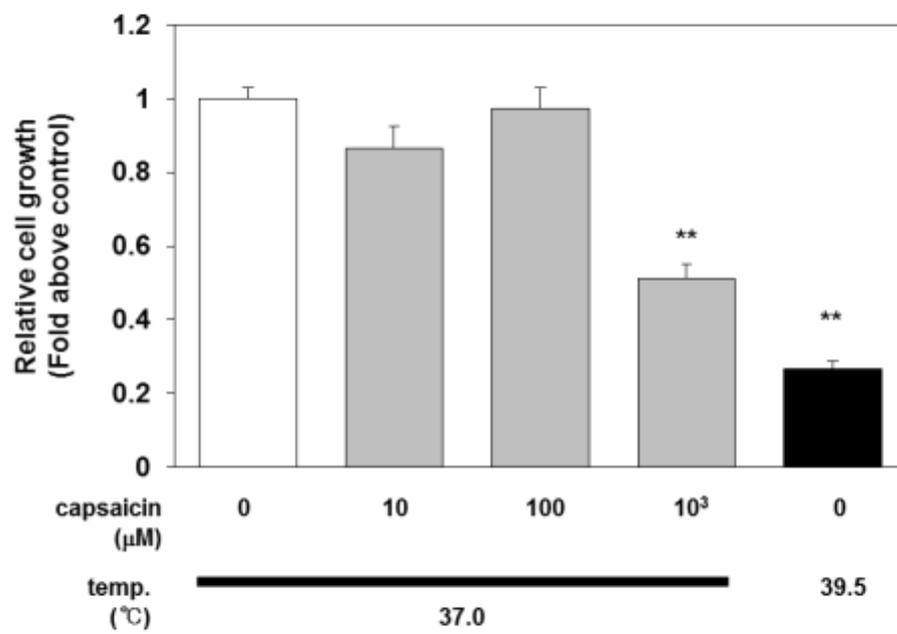


Figure 3

2 days

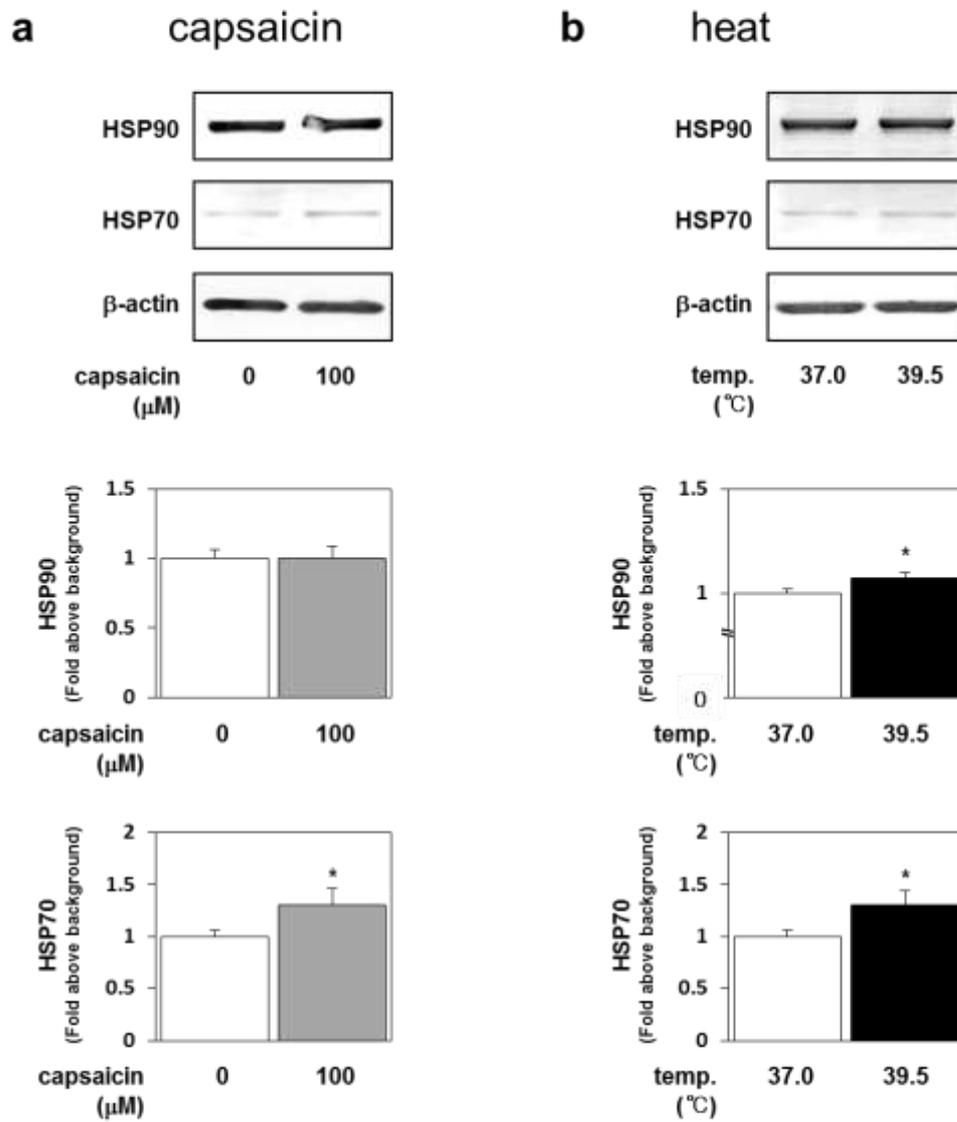


Figure 4

2 days

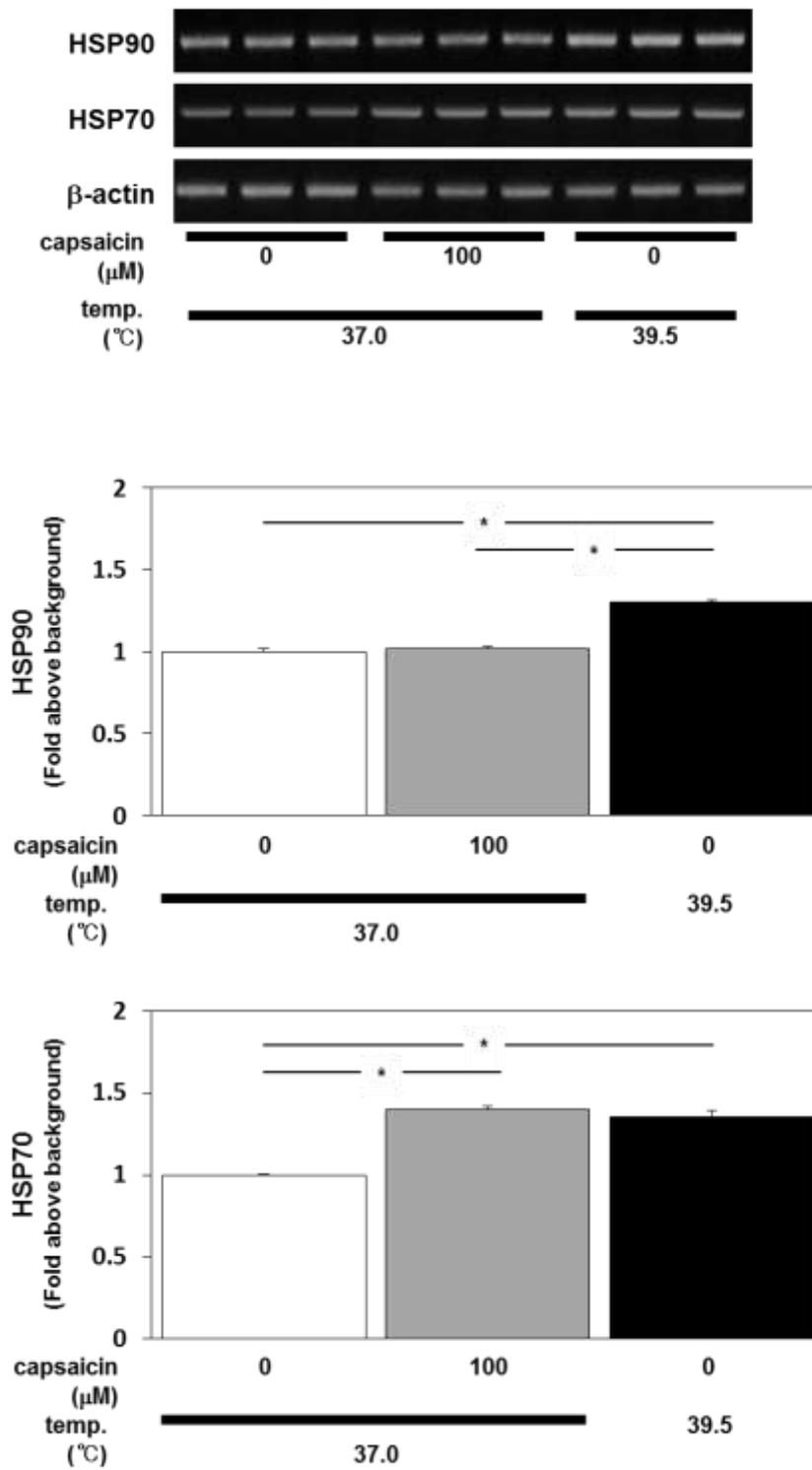


Figure 5

2 days + Heat Shock (HS)

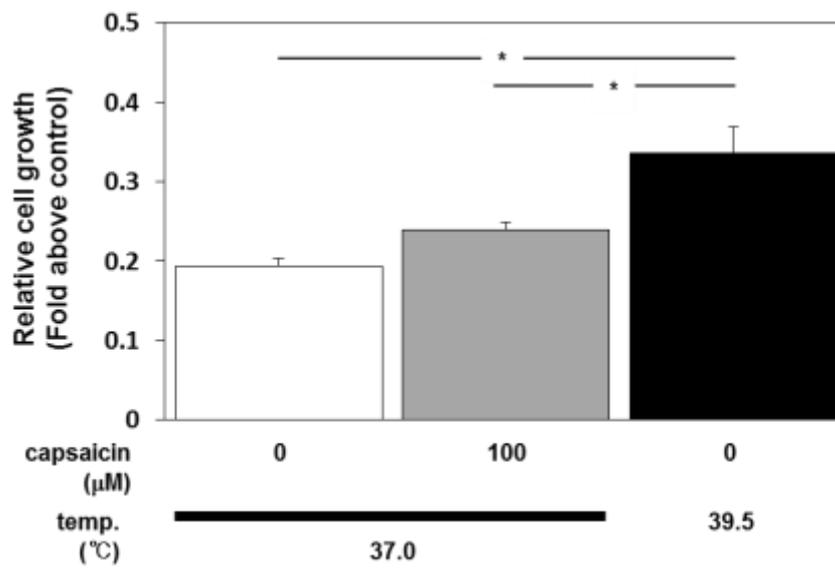
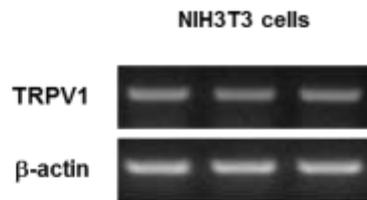


Figure 6

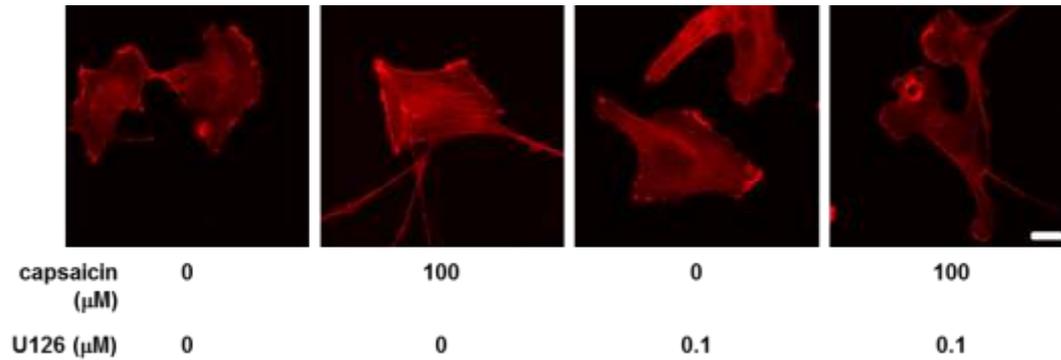


Supplemental Figures Caption

Fig. S1. Expression of TRPV1 in NIH3T3 cells without any stimulation
TRPV1 expression was detected in cells by RT-PCR using mRNA prepared from NIH3T3 mouse fibroblast cells.

To evaluate the expression pattern of transient receptor potential vanilloid 1 (TRPV1) mRNA in the cells, reverse transcription-mediated polymerase chain reaction (RT-PCR) was performed as follows. Briefly, RNA was extracted from the cells and reverse transcribed using the reverse transcriptase ReverTra Ace (TOYOBO, Tokyo, Japan). PCR-based subtype-specific gene amplification for TRPV1 and β -actin was performed with LA Taq (TAKARA, Tokyo, Japan) using the following sets of primers: 5'-GACAGCTACTACAAGGGCCA-3' and 5'-TTCTGCAGCAGGAACTTCAC-3' for TRPV1 and 5'-ATGGTGGGTATGGGTCAGAAG-3' and 5'-CTGGGGTGTGAAGGTCTCAA-3' for β -actin. The PCR conditions were 35 cycles of amplification (30 s at 94 °C; 30 s at 55 °C; 30 s at 72 °C) or 25 cycles of amplification (30 s at 94 °C; 30 s at 60 °C; 30 s at 72 °C) for TRPV1 or β -actin, respectively.

Supplemental Figure 1



Supplemental Figure Caption

Fig. S2. ERK inhibitor prevents capsaicin-induced stress fiber formation in NIH3T3 cells

F-actin was visualized with TRITC-labeled phalloidin under an inverted EVOS fluorescence microscope .

The cells were pretreated with or without U126 (0.1 μM, Cell Signal Technology Inc.) for 10 min and then stimulated with capsaicin (100 μM) for 15 min.

To evaluate the actin cytoskeletons, the cells were fixed in 3.7% (v/v) formaldehyde in Dulbecco's phosphate buffered saline and processed as described previously (Sugimoto et al, 2016). F-actin was visualized with TRITC-labeled phalloidin under an inverted EVOS fluorescence microscope (Life Technologies Japan, Tokyo, Japan).

Supplemental Figure 2