Mechanosensitive Signaling in Myofibroblasts as a Target for Anti-Fibrotic Therapy

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METHODS

Antibodies, reagents and plasmids

Anti-α-SMA antibody and anti-collagen I antibody were purchased from Sigma (St. Louis, MO). Anti-MKL1 antibody was purchased from Bethyl Laboratories (Montgomery, TX). Anti-Bcl-2, anti-Bcl-xL, anti-Mlc-1, anti-caspase 3, anti-caspase 9, anti-cytochrome *c*, anti-phospho-Ezrin (Thr567)/Radixin (Thr564)/Moesin (Thr558) (ERM), anti-ERM, anti-phospho MLC_{20} and anti-MLC_{20} antibodies were from Cell Signaling (Danvers, MA). Anti-SRF and anti-GAPDH antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho MYPT-1 antibody was from Millipore (Billerica, MA). Anti-VDAC and anti-lamin A/C antibodies were from Abcam (Cambridge, MA). TGF-β1 was from R&D (Minneapolis, MN). Latrunculin B and jasplakinolide were from BD Biosciences (San Jose, CA). CCG-1423 was from Cayman Chemical (Ann Arbor, MI).

Human Bcl-2 promoter was described previously (17913397). A 1096-bp promoter fragment containing two MKL1-SRK binding sequences (CArG boxes) was amplified by genomic PCR using a pair of primers: 5'-CAGGAGGAGGAGAAAGGGTG-3' and 5'-TTCTGGTGTTTCCCCCTTGG-3'. The fragment was cloned into pGL3-basic luciferase reporter vector (Promega, Madison, WI). PCR-based mutagenesis was performed to inactivate CArG-box 1 (C-565/A, G-559/A, and G-558/A), CArG-box 2 (C-1190/A, C-1189/A, G-1182/A, and G-1181/A) and CArG-box 1 and box 2. Plasmids expressing wild-type dominant negative MKL1 cDNA (dnMKL1) and constitutively active MKL1 cDNA (caMKL1) were provided by Dr. Kenji Sobue (Osaka University, Japan)(1). MKL1 cDNA fragments were subcloned into pEGFPC1 vector (Clontech, Mountain View, CA) to facilitate identification of exogenous MKL1 expression.

Lung (myo)fibroblast isolation, culture, transfection, sorting and treatment

Lungs were minced in sterile phosphate-buffered saline and tissue pieces were placed in 100-mm tissue culture dishes containing Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS), 1% penicillin/streptomycin/glutamine, nonessential amino acids, and sodium pyruvate (supplemented DMEM). Medium was replenished every 3 d. After 14 d, cells growing out of the explants were trypsinized and plated in supplemented DMEM. Control lung fibroblasts were propagated and maintained on 0.5 kPa soft polyacrylamide gels to avoid matrix stiffness-induced myofibroblast differentiation (e.g. plastic and glass surfaces). Lung (myo)fibroblasts were used between passages 6 and 10.

Plasmid transfection was performed using a Nucleofector device (Amaxa, Inc., Cologne Germany) following the manufacturer's instructions. All transfections were performed with the combination of cell line Nucleofector Kit R and program O-017. Cells expressing EGFP and EGFP-tagged caMKL1 and dnMKL1 were sorted using a FACSTARPlus Cell Sorter (Becton-Dickinson, Mountain View, CA) under sterile conditions.

Lung (myo)fibroblasts were grown in DMEM containing 10% FBS until 80% confluence. Cells were rinsed with DMEM + 0.1% FBS twice and were incubated in DMEM + 0.1% FBS containing 0 - 50 μM fasudil in the presence or absence of 1 μM CCG-1423, 1 μM latrunculin B, 200 nM jasplakinolide and 2 ng/ml TGF-β1 for 24 hr.

Cell extracts and subcellular fractionation

Nuclear proteins and cytoplasmic proteins were isolated using NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific) as described in our previous studies(2). For

mitochondria isolation, 2×10^7 cells were harvested and washed with PBS. Mitochondrial and cytosolic fractions were isolated with the use of the Mitochondria Isolation Kit for cultured cells (Thermo Scientific). The mitochondrial pellet was lysed in 1x Laemmli buffer, and the cytosolic supernatant was mixed with 10x Laemmli buffer to a final concentration of 1x. Both fractions were quantified by the micro-bicinchoninic acid method (Thermo Scientific) before analysis by immunoblotting. Protein content and purity of the fractions were checked by probing the blots with cytosolic protein GAPDH and mitochondria outer membrane protein VDAC.

Preparation of soft and stiff polyarylamide hydrogels and mechanical testing

PA gels with tunable stiffness were fabricated using a published protocol (3). Gel surfaces were coated with 0.1 mg/ml rat tail collagen I (BD Biosciences). PA gel mechanical properties were measured using an MFP-3D-BIO Atomic Force Microscope (AFM) (Asylum Research; Santa Barbara, CA) in contact mode. Samples were probed with a 4.74 μm diameter beaded-tip (Bruker; Camarillo, CA), and cantilever spring constants were measured prior to sample analysis using the thermal fluctuation method (4), with nominal values of 20-30 mN/m. Young's modulus was obtained from force-indentation profiles using a Hertzian model and sample Poisson's ratio of 0.5. A minimum of 20 independent measurements were obtained and analyzed for both soft and stiff PA gels.

F/G-actin content

Relative proportions of F-actin and G-actin were determined using a kit from Cytoskeleton (Denver, CO). Blot images were scanned. Densitometry was performed using ImageJ.

Collagen gel contraction assay

Cell pellets were mixed with 8 volumes of rat tail type I collagen suspension, 1 volume of 10x concentrated DMEM and 1 volume of reconstitution buffer (2% sodium bicarbonate and 4.77% HEPES dissolved in 0.05N NaOH) at a concentration of $2x10^5$/ml. Cell-populated collagen solution was immediately poured into 24-well-plate (0.5ml/well) and incubated at 37°C for 20-30mins to permit complete gelation. Gels were gently released from the plate by spatula and overlaid with culture media. Gel images were taken at 48 hr.

Quantitative real-time PCR

Total RNA was isolated using TRIzol reagent (Invitrogen). 1 μg total RNA was reversely transcribed into cDNA with a cDNA Synthesis Kit (Thermo Scientific). Quantitative PCR reactions were carried out in a Bio-Rad iCycler. Specific primers used for real-time PCR are: α-SMA 5'-GACCGAATGCAGAAGGAGAT-3' and 5'-CCACCGATCCAGACAGAGTA-3'; Bcl 5'-CTGCACCTGACGCCCTTCACC-3' and 5'-CACATGACCCCACCGAACTCAAAGA-3'; Bcl-xL 5'-GATCCCCATGGCAGCAGTAAAGCAAG-3' and 5'-CCCCATCCCGGAAGAGTTCATTCACT-3'; Mcl-1 5'-AGCTGCATCGAACCATTAGC-3' and 5'-TCCTGATGCCACCTTCTAGG-3'; 18S rRNA (internal reference control) 5'-GTAACCCGTTGAACCCCATT-3' and 5'-CCATCCAATCGGTAGTAGCG-3'. Relative quantification was calculated using the comparative C_T method (5).

Immunoblot analysis

Cell lysates containing 10 - 40 μg total proteins were loaded onto SDS-polyacrylamide gels under reducing conditions. After electrophoresis, proteins were electrophoretically transferred from the gels to nitrocellulose at 100 V for 1.5 hr at 4°C. Membranes were blocked in casein solution (1% casein, 25 mM Na_2HPO_4, pH 7.1) for 1 hr at room temperature. Primary antibodies were diluted in TBS-T and casein solution (1:1) at a working concentration recommended by manufactures. Membranes were incubated with primary antibodies at room temperature for 1 hr. After extensive washing, membranes were incubated with peroxidase-conjugated secondary antibodies (0.1 μg/ml) diluted in TBS-T for 1 hr at room temperature. Immunodetection was carried out by chemiluminescence.

RhoA activation and Rho kinase activity assay

RhoA activation was determined by affinity precipitation of GTP-bound RhoA (active RhoA) using glutathione S-transferase-rhotekin (GST-RBD) fusion proteins. Briefly, cells were lysed with RhoA-RBD buffer, and then incubated with GST-RBD-bound Sepharose 4B for selective pull-down of active RhoA. Detection of active RhoA was performed by Immunoblot.

Rho kinase activity was assessed using ROCK Activity Assay kit (Cell Biolabs, San Diego, CA). Plates were pre-coated with substrates corresponding to the C terminus of MYPT1, which contains a threonine residue that was phosphorylated by ROCK. The specific detector antibody was horseradish peroxidase (HRP)-conjugated anti-phospho-MYPT1 (AF 20). The amount of phosphorylated substrates was measured by binding with HRP-conjugated AF20 which catalyzed the conversion of TMB to a yellow solution. Results were quantified by a plate reader at 450 nm.

Promoter activity assay

4 μg of Bcl-2 promoter constructs were co-transfected with 50 ng of Renilla luciferase-expressing control vector (pRL-CMV) (Promega) into 1x 10^6 lung myofibroblasts. Transfected cells were cultured for 48 hrs. Cells were lysed with 1x passive lysis buffer (Promega). Luciferase activities were determined using a Dual-Luciferase reporter assay system (Promega). The relative light units were measured by an Orion Microplate Luminometer (Berthold, Germany). The firefly luciferase activity corresponding to a specific Bcl-2 promoter construct was normalized to the renilla luciferase activity of the same sample. Results are expressed as fold changes compared with the mean firefly/renilla ratio of the cells cultured on soft gels taken as a unit.

Immunohistochemistry and histology

5 μm lung tissue sections were immersed in 10 mmol/L sodium citrate buffer (pH 6.0) and heated at 100°C for 10 minutes to unmask antigen. Sections were blocked with 5% normal goat serum and stained with anti-α-SMA antibody at 2.5 μg/ml. Endogenous peroxidase activity was quenched with 3% H_2O_2. Staining was developed with biotinylated secondary antibodies, streptavidin-peroxidase and 3-amino-9-ethylcarbazole chromogen (Vector Laboratories, Burlingame, CA). Masson's trichrome stain for collagen and H&E stain were performed using a kit from Poly Scientific (Bay Shore, NY) according to the manufacturer's recommendations. Images were obtained with a Nikon Eclipse TE 300 microscope equipped with Spot Insight CCD camera and MetaMorph software version 6.2 r4 (Universal Imaging Corp., Downington, PA).

Hydroxyproline content assay

Mouse right lungs were homogenized in 2.0 ml distilled water and incubated with 125 ul of 50% trichloroacetic acid on ice for 20 min. Samples were centrifuged and the pellets were mixed with 1 ml 12 N hydrochloric acid and baked at 110°C for 14-18 h. Dry samples were dissolved in 2 ml deionized water. 200 µl samples (or standards) were added to 500 µl 1.4% chloramine T in 0.5 M sodium acetate/10% isopropanol (Fisher Scientific, Pittsburgh, PA) and incubated for 20 min at room temperature. 500 µl Ehrlich's solution (1.0 M p-dimethylaminobenzaldehyde in 70% isopropanol/30% perchloric acid) (Fisher Scientific, Pittsburgh, PA) was added, mixed, and incubated at 65°C for 15 min. Optical density of each sample and standard was measured at 550 nm and the concentration of lung hydroxyproline was calculated from a hydroxyproline standard curve.

Caspase activity assay

Caspase activity assays were performed based on spectophotometric detection of the chromophore p-nitroaniline (pNA) after cleavage from the labeled substrate LEHD-pNA (for caspase 9) and DEVD-pNA (for caspase 3) (Millipore).

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FIGURE LEGENDS

Figure 1S: **Fasudil does not induce significant smooth muscle cell apoptosis in bleomycin-treated fibrotic mouse lungs**. Frozen lung tissue sections from mice treated with bleomycin (Bleo) and fasudil were stained for TUNEL (green), α-SMA (red) and nuclei (blue). Images showed a bronchiole and an adjacent pulmonary artery. Scale bar: 20 μM.

Figure 2S: **Fasudil does not activate death receptor-mediated extrinsic apoptotic pathway**. IPF lung myofibroblasts were treated with 25 μM fasudil or an equal volume of PBS for 24 hr. Cleavage of caspase 8 was determined by immunoblot analysis. GAPDH was used as loading control. Caspase 8 activity was measured with a colorimetric assay. Bar graphs are the means ± SD of three separate experiments.

Figure 3S: **Fasudil does not promote normal lung fibroblasts to undergo mitochondria-dependent apoptosis**. Control lung myofibroblasts were treated with 25 μM fasudil or an equal volume of PBS for 24 hr. Cleavage of caspase 9 and caspase 3 was determined by immunoblot analysis. GAPDH was used as loading control. Caspase activities were measured with a colorimetric assay. Bar graphs are the means ± SD of three separate experiments.

Figure 4S: **Lung myofibroblasts express total actin equivalent to control lung fibroblasts**. Levels of total actin were determined by immunoblot analysis. GAPDH was used as loading control. Relative density of total actin was normalized to GAPDH. Bar graphs represent mean ± SD; n = 3 per group

Figure 5S: **Fasudil inhibits lung fibroblast-to-myofibroblast differentiation by inhibition of MKL1 nuclear translocation**. **A**, To evaluate forced MKL1 nuclear translocation on fasudil-modulated lung fibroblast-to-myofibroblast differentiation, lung fibroblasts were cultured on soft and stiff PA gels and treated by 25 μM fasudil, 4 ng/ml TGF-β1, 200 nM jasplakinolide (Jas) or overexpression of caMKL1. Myofibroblast differentiation was assessed by α-SMA expression. **B**, To evaluate disruption of MKL1 nuclear signaling on fasudil-modulated lung fibroblast-to-myofibroblast differentiation, lung fibroblasts cultured on soft and stiff PA gels were treated with 4 ng/ml TGF-β1, 1 μM CCG-1423, 1 μM latrunculin B (LatB) or overexpression of dnMKL1. Myofibroblast differentiation was assessed as described above.

Figure 6S: **Fasudil does not alter cofilin phosphorylation and subcellular localization**. **A**, IPF lung myofibroblasts were treated with 25 μM fasudil for 24 hr. Cell lysates were collected at the indicated time points. The levels of phosphorylated cofilin (Ser3), total cofilin and GAPDH were determined by immunoblot. **B**, Subcellular localization of cofilin in the mitochrondrial (Mito) and cytoplasmic (Cyto) fractions were determined by immunoblot. VDAC and GAPDH were used as loading controls for mitochrondrial and cytoplasmic proteins, respectively.

Figure 1S

Bleo + Fasudil

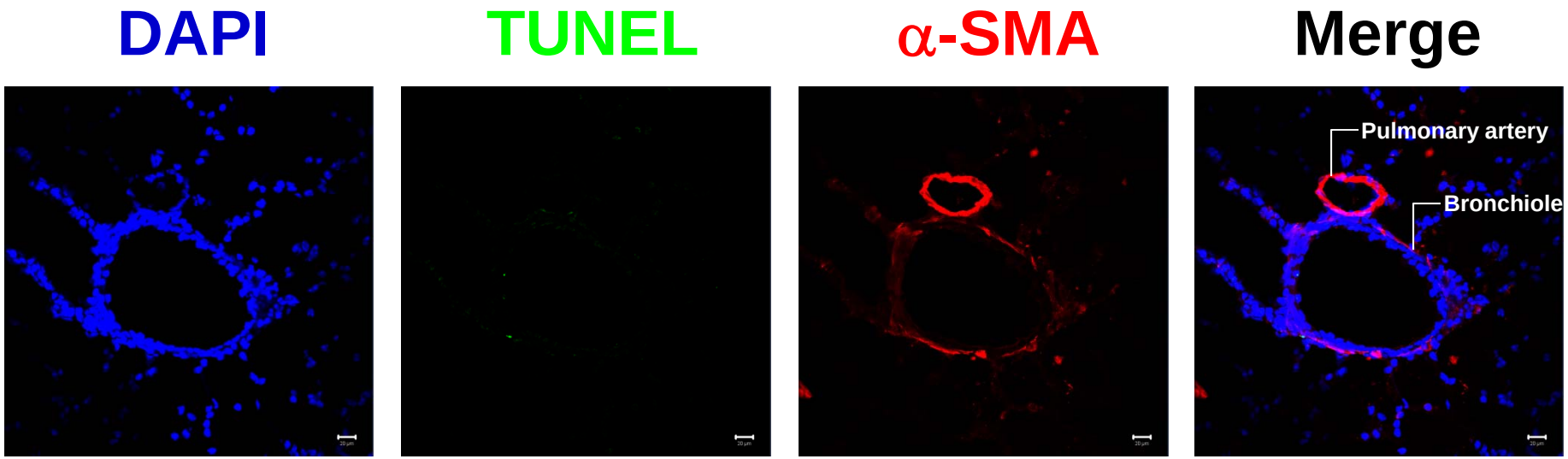


Figure 2S

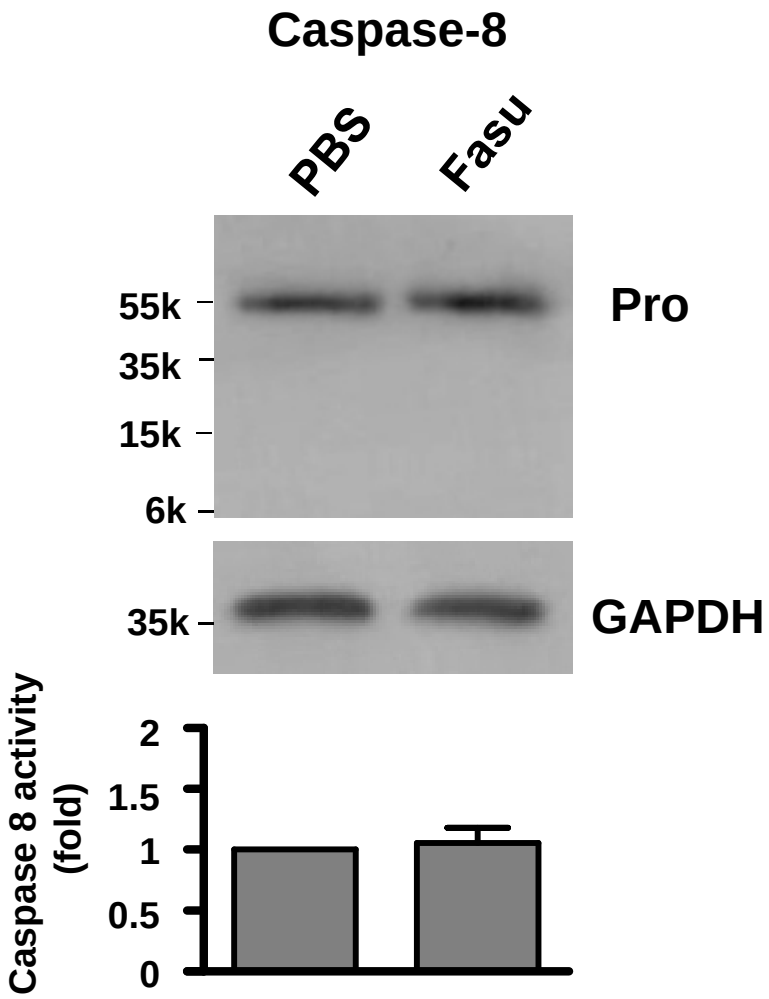
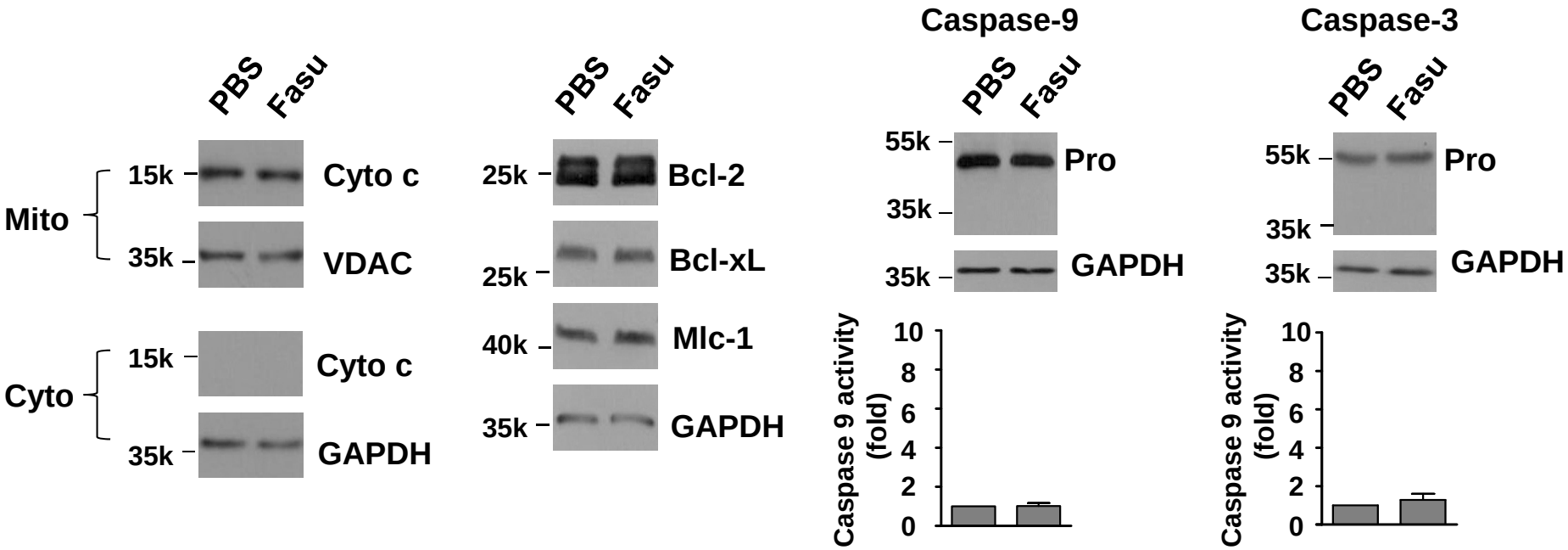

Caspase-8
PBS
Fasu
55k
35k
15k
6k
Pro
35k
GAPDH
Caspase 8 activity (fold)
2
1.5
1
0.5
0

Figure 3S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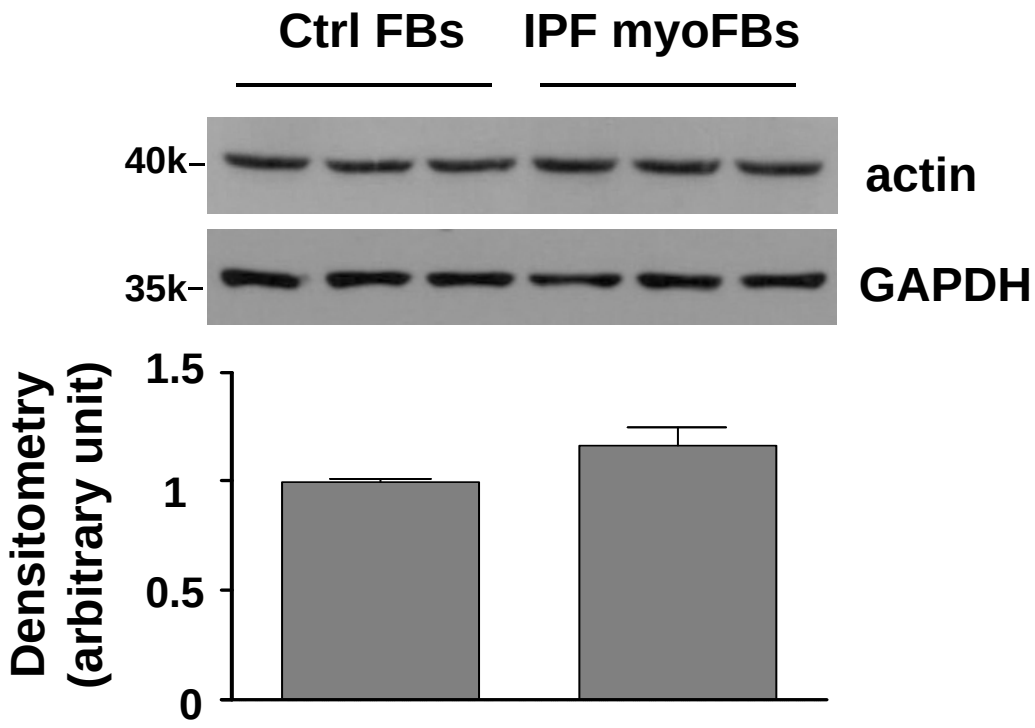
Fasu
Mito
15k
Cyto c
35k
VDAC
Cyto
15k
Cyto c
35k
GAPDH
PBS
Fasu
25k
Bcl-2
25k
Bcl-xL
40k
Mlc-1
35k
GAPDH
Caspase-9
PBS
Fasu
55k
Pro
35k
GAPDH
Caspase 9 activity (fold)
10
8
6
4
2
0
Caspase-3
PBS
Fasu
55k
Pro
35k
GAPDH
Caspase 9 activity (fold)
10
8
6
4
2
0

Figure 4S


Ctrl FBs
IPF myoFBs
40k–
actin
35k–
GAPDH
Densitometry (arbitrary unit)
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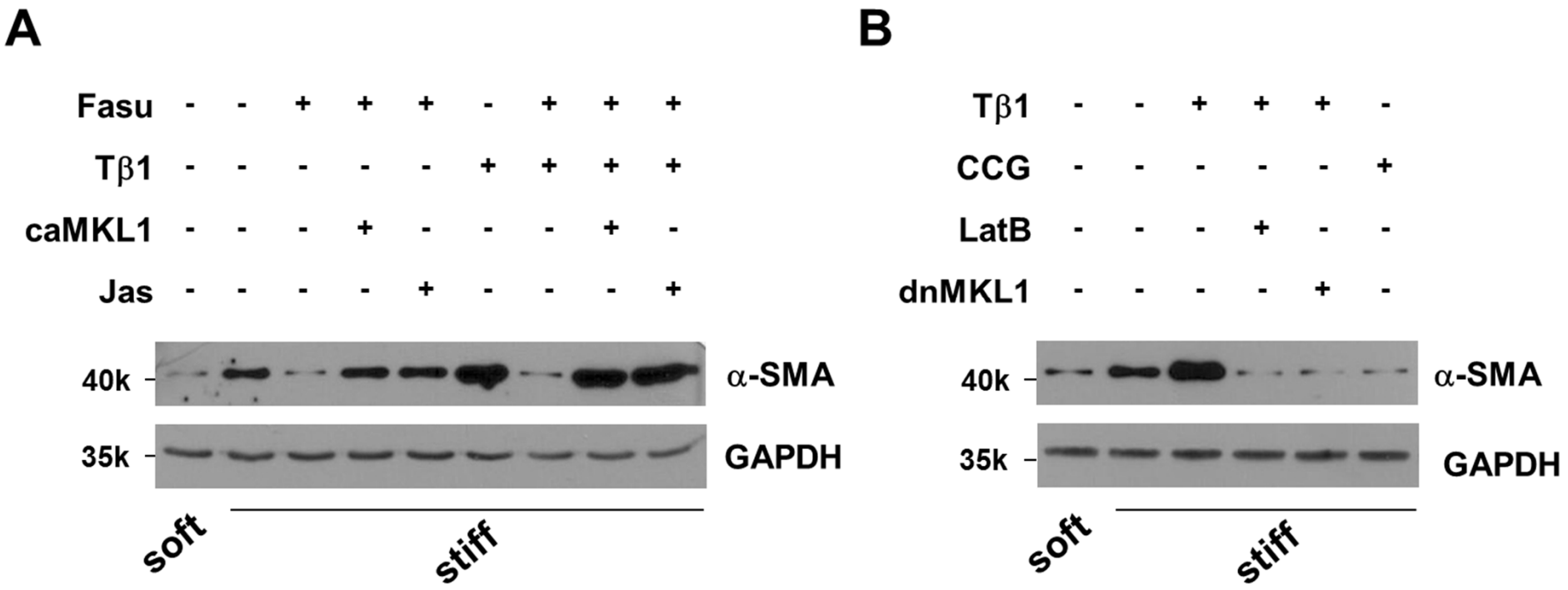
1
0.5
0

Figure 5S


A
Fasu - - + + + - + + +
Tβ1 - - - - - + + + +
caMKL1 - - - + - - - + -
Jas - - - - + - - - +
40k
α-SMA
35k
GAPDH
soft
stiff
B
Tβ1 - - + + + -
CCG - - - - - +
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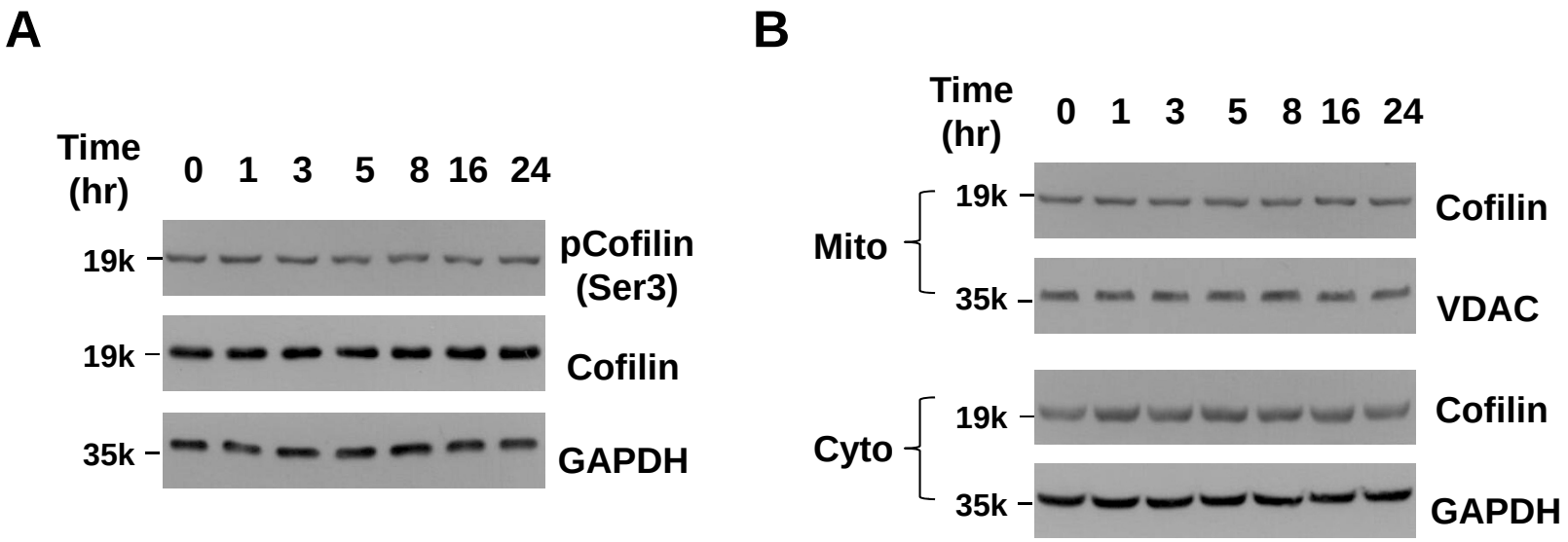
dnMKL1 - - - - + -
40k
α-SMA
35k
GAPDH
soft
stiff

Figure 6S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Time (hr)
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19k
pCofilin (Ser3)
19k
Cofilin
35k
GAPDH
B
Time (hr)
0 1 3 5 8 16 24
Mito
19k
Cofilin
35k
VDAC
Cyto
19k
Cofilin
35k
GAPDH