

Supplementary Information

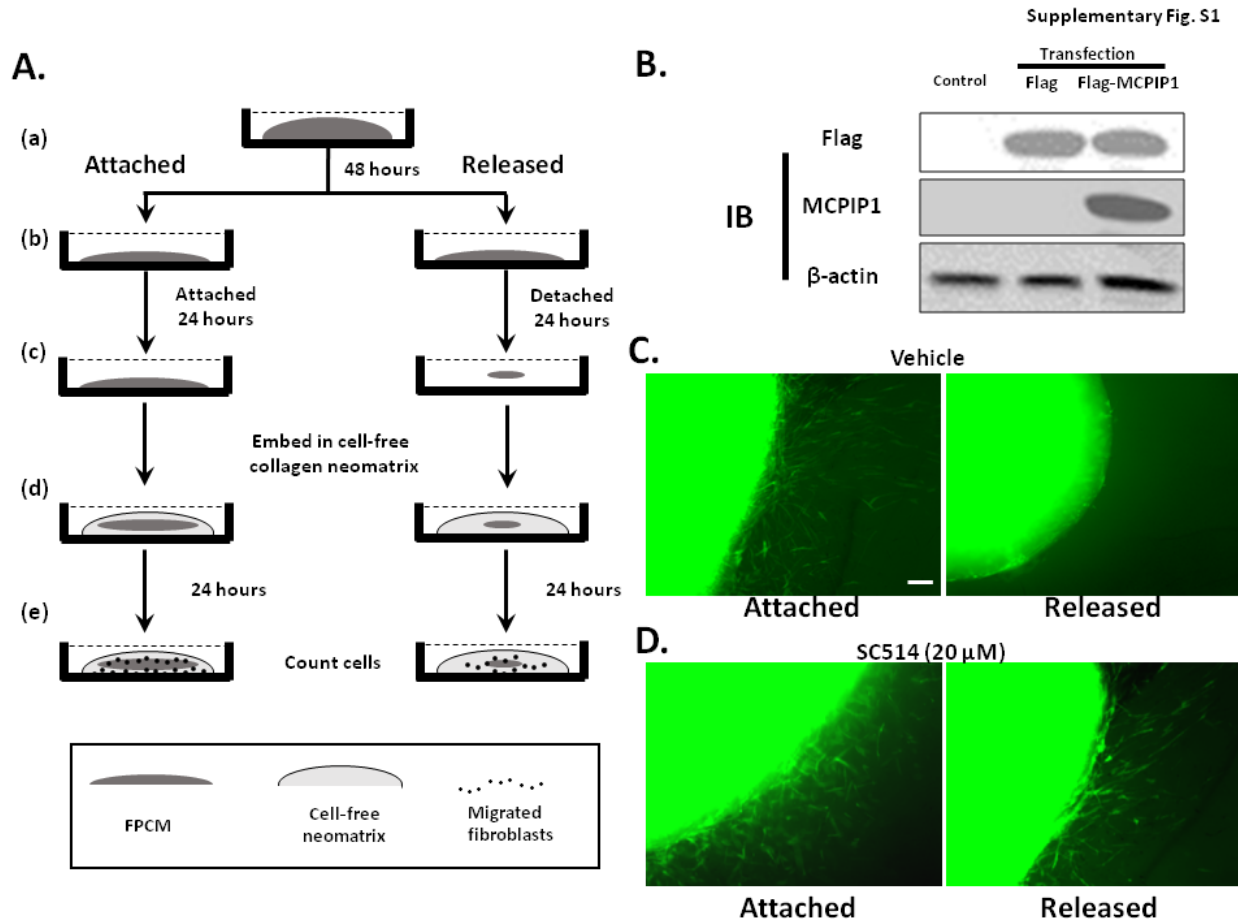
Manuscript title: "MCPIP1 regulates fibroblast migration in 3D collagen matrices downstream of MAP kinases and NF- κ B"

Authors: Chao, Dai, Peña, Doyle, Guenther, Carlson

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[Supplementary Fig. S1]

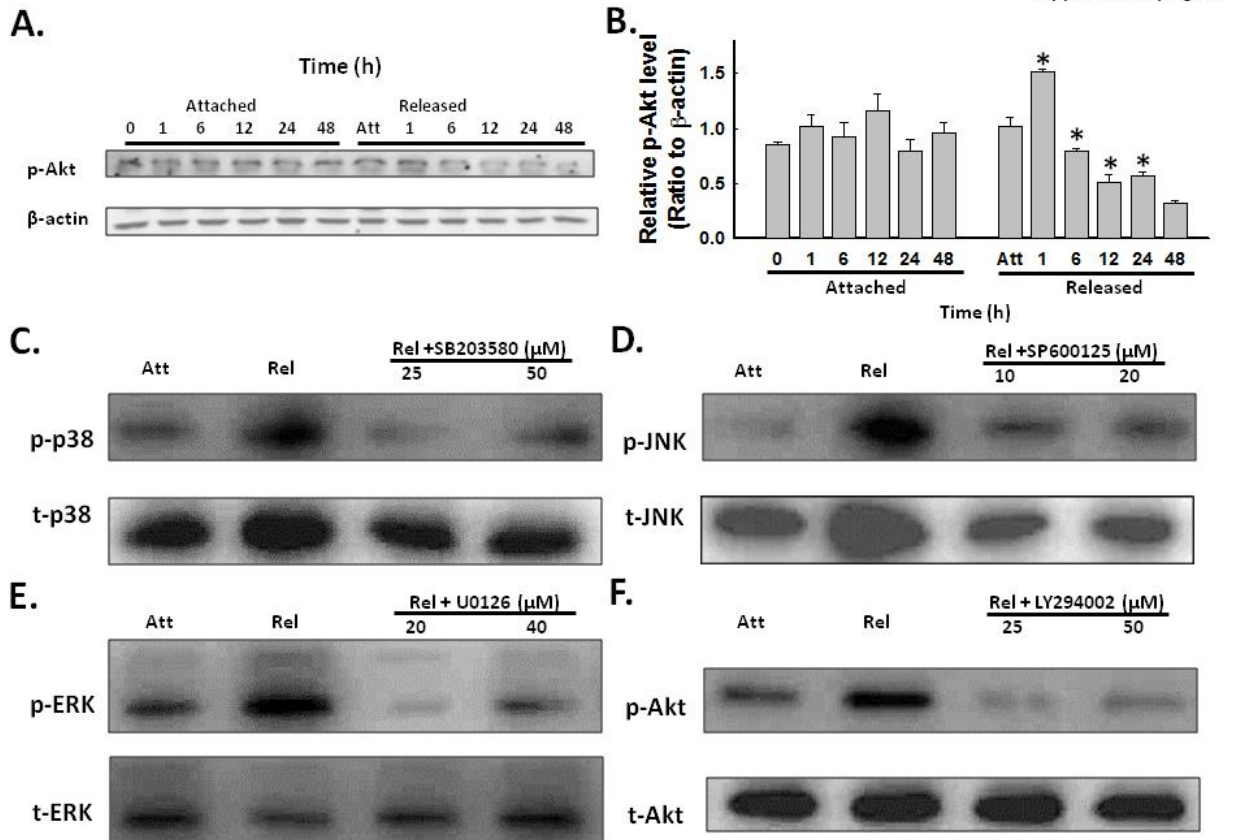


Supplementary Fig. S1. Nested collagen matrix model.

(A) Nested collagen matrix model. A modified version of the nested collagen matrix model(Grinnell *et al.*, 2006) was utilized to evaluate the motility of GFP-labeled fibroblasts(Chao *et al.*, 2014) in the 3D collagen matrix. For the nested attached matrix, an FPCM set up in standard fashion (a) was incubated in the attached state for 72 h (b-c), then removed from the culture well and embedded (nested) into fresh acellular collagen matrix solution (d); see Methods for details. The nested matrix was incubated for 24 h in serum-supplemented medium, and then cell migration was quantified (e) as described under Methods. The procedure for the nested released matrix was similar, except that the initial attached incubation was 48 h (a-b); the matrix then was detached, followed by 24 h incubation in the released state (b-c) prior to nesting (d). All acellular neomatrices remained attached to the culture dish for the duration of each experiment.

(B) Confirmation of MCPIP1-Flag vector transfection. In order to confirm transfection of HFFs with the MCPIP1-Flag vector, immunoblotting for both Flag and Flag-tagged MCPIP1 in the attached FPCM 72 hours of transfection was performed. Expression of Flag was high in HFFs transfected with either Flag vector or MCPIP1-Flag vector, while detection of MCPIP1 only occurred after transfection with the MCPIP1-Flag vector. (C-D) Effect of SC-514 pretreatment on fibroblast migration in the nested attached vs. released nested matrix. Migration of GFP-expressing fibroblasts out of nested matrices was decreased 24 hr after matrix release with vehicle pretreatment, but pretreatment with SC-514 restored fibroblast migration in the release matrix to a level similar to that seen in the attached matrix (see Fig. 6l of the main manuscript for quantification of migration). Representative images show fibroblast migration at the interface between the nested matrix and the restrained cell-free matrix 24 h after nesting. Scale bar = 80 μm .

[Supplementary Fig. S2]

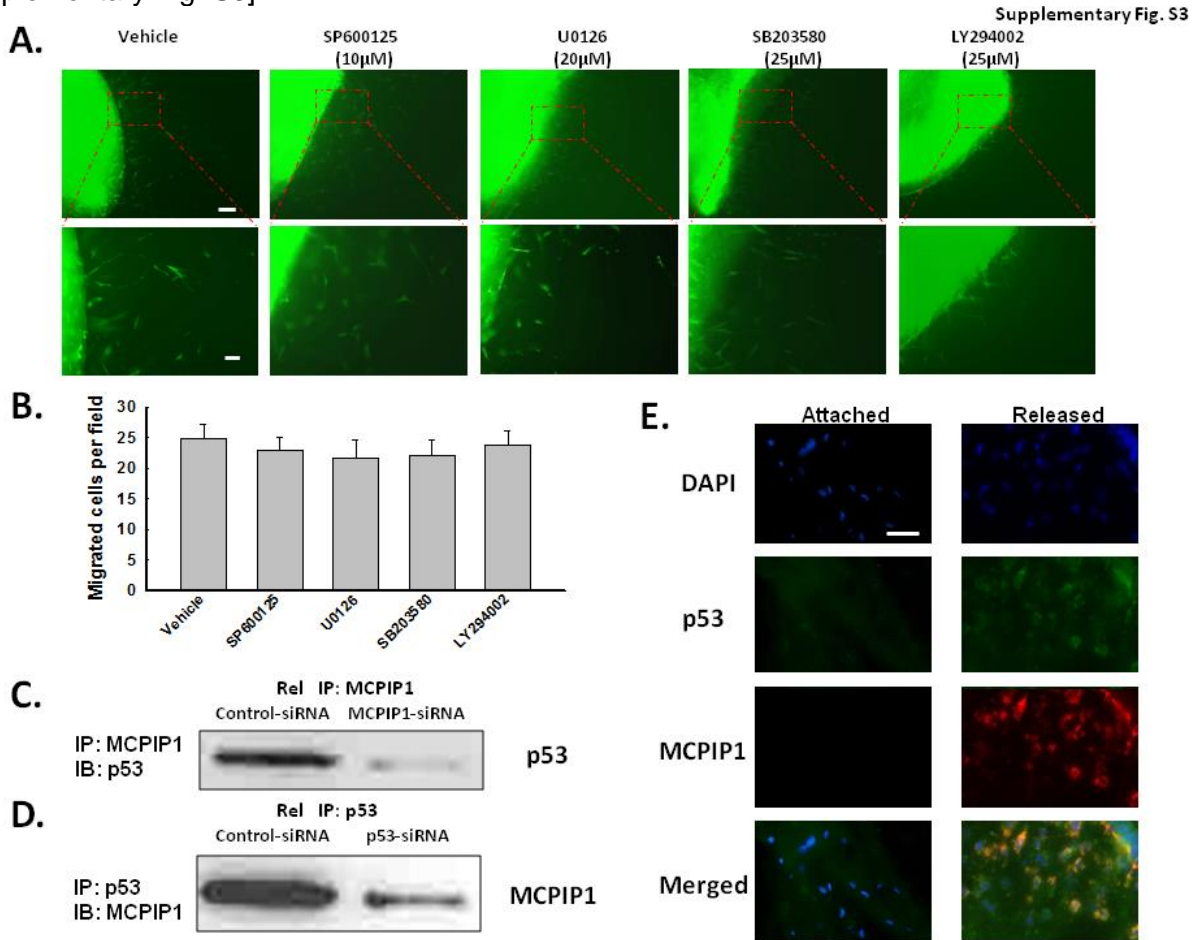


Supplementary Fig. S2. Effect of pharmacologic inhibitor of MAP kinases and PI3K blocker on phosphorylation in 3D model.

(A-B) Effect of FPCM release on Akt phosphorylation (48 h time course). Previous experimentation demonstrated that Akt dephosphorylation occurs about 24 hours after release of the FPCM.(Carlson and Longaker, 2004; Tian *et al.*, 2002; Xia *et al.*, 2004) In our current studies (Fig. 4), we saw transient phosphorylation of Akt occurring 1-6 h after matrix release. In order to determine whether we were obtaining results that conflicted with published data, we assessed phosphorylation of Akt at later time points (up to 48 h) after release (i.e., we repeated a previously-published experiment). As shown in these panels, the phosphorylation of Akt did not show significant change during the 48 h observation period in attached FPCM. In the released FPCM, a rapid and transient phosphorylation of Akt was detected, beginning at 1 h (consistent with Fig. 4). At the 12 h time point, however, Akt phosphorylation in the released matrix decreased relative to the attached matrix, which was consistent with previously-published data.(Carlson and Longaker, 2004; Tian *et al.*, 2002; Xia *et al.*, 2004)

(C-F) Effect of kinase inhibitors on phosphorylation of the target kinases. In order to demonstrate that the dose of each kinase inhibitor that was utilized was adequate to suppress kinase activity, attached FPCMs were pretreated with two doses of each kinase inhibitor (SB203580 in panel C, SP600125 in panel D, U0126 in panel E, and LY294002 in panel F), and then immunoblotting for the respective phospho-proteins of target kinases was performed 30 min after matrix release. With inhibitor pretreatment, reduction of kinase phosphorylation after matrix release was complete or near-complete for p38, ERK and Akt, and ~80% complete for JNK.

[Supplementary Fig. S3]



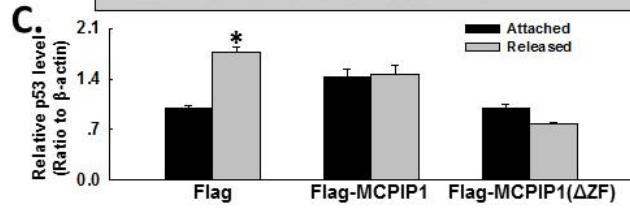
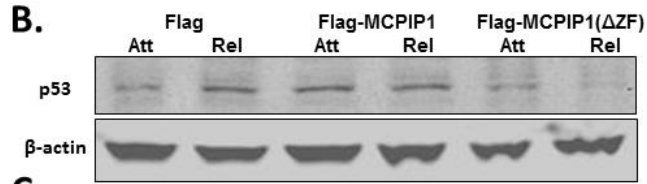
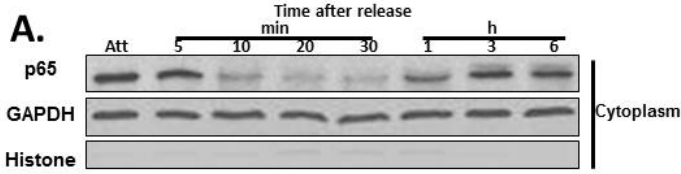
Supplementary Fig. S3. Effect of FPCM release on Akt phosphorylation (48 h time course).

(A-B) Effect of pharmacological inhibition of MAP kinases or Akt on fibroblast migration out of the attached FPCM. The experiment shown in this panel was intended as a control experiment for the data shown in Fig. 5E-F, in which only data from the nested released matrix were shown. In Supplementary Fig. S3A, an experimental protocol identical to that in Fig. 5E-F was utilized in nested attached matrices. Pretreatment with SP600125, U0126, SB203850, or LY294002 did not effect fibroblast migration out of the nested attached matrix. Upper and lower scale bars are 200 and 80 μm , respectively.

(C-D) Effect of MCPIP1 or p53 knockdown on the co-localization of MCPIP1 and p53 in the released FPCM. The experiment shown in this panel was intended as a control experiment for the data shown in Fig. 7B-C. Matrices underwent RNAi for MCPIP1 or p53 as described in the methods, and then lysates from 1-day released matrices underwent immunoprecipitation for MCPIP1 or p53, followed by immunoblotting for p53 or MCPIP1, respectively, as described in the Methods. Treatment with siRNA against either MCPIP1 or p53 markedly reduced/eliminated the ability of the immunoprecipitation procedure to pull down the p53 or MCPIP1, respectively, which suggested that the co-localization of MCPIP1 and p53 observed in Fig. 7B-C was specific.

(E) Immunocytochemistry of MCPIP1 and p53 in the attached vs. 1 day-released FPCM. Blue = DAPI; green = MCPIP1; red = p53; scale bar = 20 μm . This panel represents a duplicate experiment of that shown in Fig. 7A, performed with a different strain of human foreskin fibroblasts.

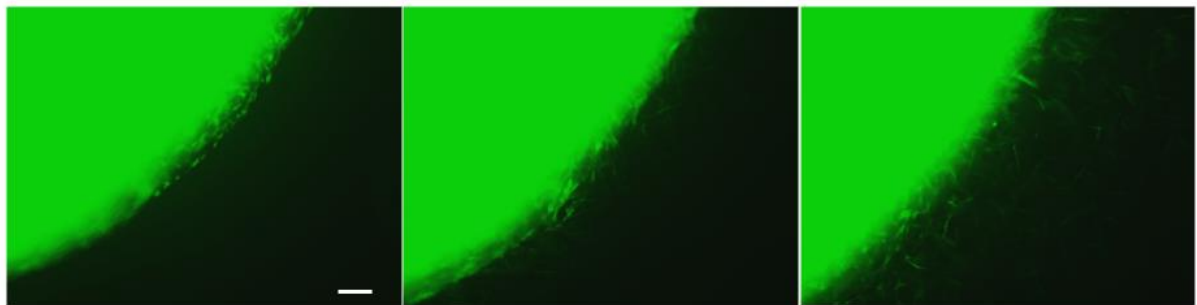
[Supplementary Fig. S4]



G.

0h

12h



D.

Supplementary Fig. S4

Vehicle

TPCK (25μM)

Att

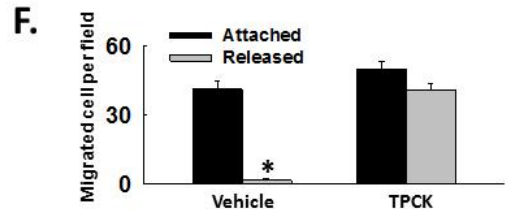
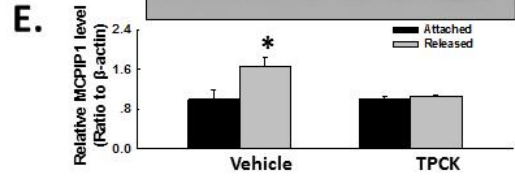
Rel

Att

Rel

MCPIP1

β-actin



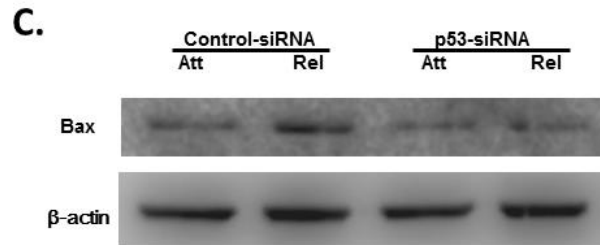
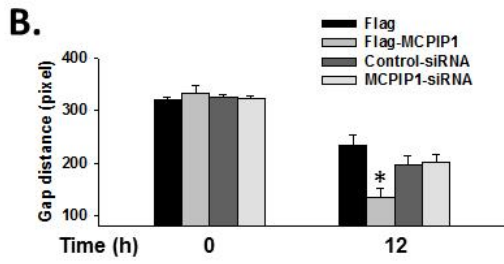
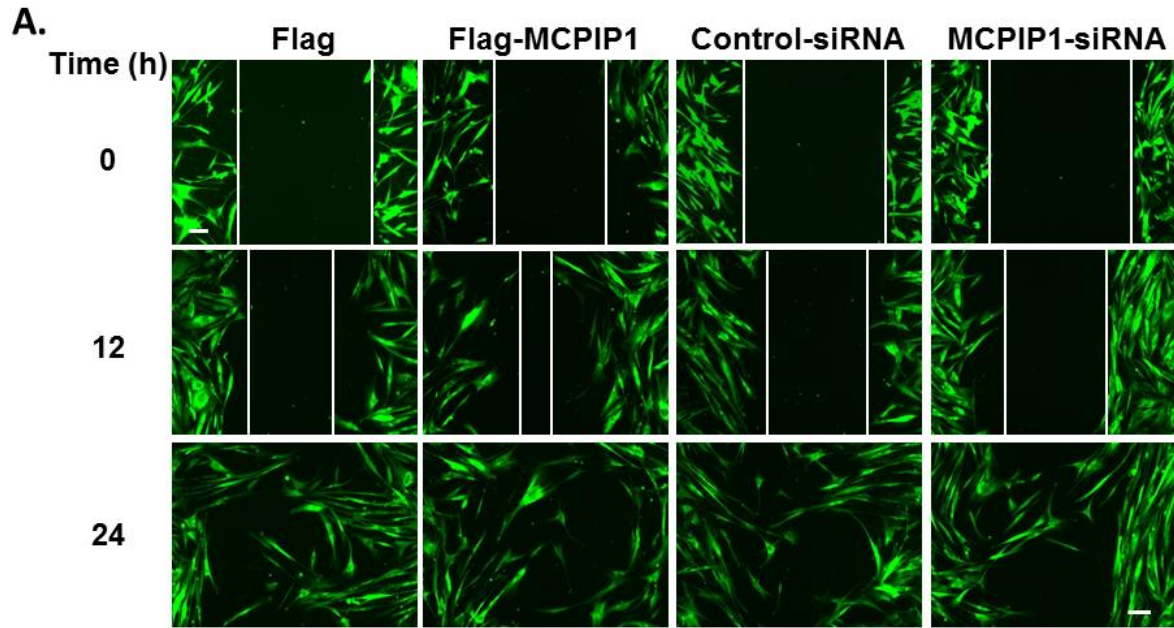
24h

Supplementary Fig. S4. Effect of pharmacological inhibition of MAP kinases or Akt on fibroblast migration out of the attached FPCM.

(A) NF- κ B p65 expression after FPCM release (immunoblots of cytoplasmic fraction); n = 4 experiments for densitometry; complementary data for Fig. 4A. (B-C) To determine whether the enhancement of deubiquitination by MCPIP1 was important for MCPIP1-induced upregulation of p53 in the stress-released FPCM, MCPIP1(Δ ZF) (an MCPIP1 construct with a mutation that eliminates the enhancement of deubiquitination (Liang *et al.*, 2010)) was transfected into fibroblasts, and p53 immunoblotting was performed in lysates of attached vs. 1 d stress-released matrices. The lack of p53 induction after stress-release in matrices made from fibroblasts that expressed MCPIP1(Δ ZF) suggested that enhancement of deubiquitination by MCPIP1 was important for induction of p53 after matrix stress-release. (D-F) Effect of TPCK pretreatment on MCPIP1 induction and fibroblast migration. The data in these panels were intended as companion data to that shown in Fig. 6G-H of the main manuscript, which showed analogous experiments using the selective inhibitor of NF- κ B activation, SC-514. TPCK is a less selective inhibitor of NF- κ B activation, in that TPCK has general activity against serine proteases; see text associated with Fig. 6 in the main manuscript. Pretreatment of matrices with TPCK resulted in abrogation of MCPIP1 induction associated with FPCM stress release (24 h time point; immunoblot of whole cell lysates). Densitometry of MCPIP1 from four separate experiments of the type shown in panel C is shown in panel D; *p < 0.05 vs. attached (unpaired t-test). Panel E shows that pretreatment of matrices with TPCK resulted in disinhibition of migration of GFP-expressing fibroblasts out of the nested released FPCM (analogous data to Fig. 6I); results from three separate experiments. Plot bars represent mean \pm S.E.M.; *p < 0.05 vs. attached.

(G) Migration of fibroblasts out of the nested FPCM. These images were intended as companion data to Figure 1A, and were included here to provide some larger images showing migration of the GFP-expressing fibroblasts in the nested FPCM model. Attached collagen matrices embedded with GFP-expressing human foreskin fibroblasts (Chao *et al.*, 2014) were nested into acellular collagen matrices in the presence of serum, as described in the Methods. Fluorescent microscopic images were captured at 0, 12, and 24 h after nesting. Scale bar = 80 μ m.

[Supplementary Fig. S5]



Supplementary Fig. S5. Effect of MCPIP1 on cell migration in a scratch assay.

(A-B) Migration of GFP-expressing fibroblasts in a 2D (monolayer) scratch assay was assessed. Ectopic expression of MCPIP1-Flag increased cell migration across the scratch gap, while MCPIP1 RNAi have no effect. Scale bar = 80 μm . **(C)** Effect of FPCM stress-release on Bax expression. FPCM stress- release induced expression of Bax, an effect which was abolished p53 RNAi.

Supplementary Materials and Methods

Reagents

Fetal bovine serum (FBS), normal goat serum, Dulbecco's Modified Eagle Medium (DMEM; #1200-046), and 10X-MEM (11430-030) were obtained from Life Technologies™; Amphotericin B (BP2645) and GlutaMax™ Supplement (35050-061) were obtained from Gibco®; Pen Strep (15140-122) was obtained from Fisher Scientific; and PureCol® type I bovine collagen (3 mg/mL) was obtained from Advanced Biomatrix. Antibodies for p53 (SC6243, from rabbit), MCPIP1 (SC136750, from goat), and β -actin (SC8432, from mouse) were obtained from Santa Cruz Biotechnology®, Inc. DharmaFECT® transfection reagent (T-0001-03) for short interfering RNA (siRNA) and ON-TARGETplus TP53 siRNA (L-003329-00-0005) were obtained from Thermo Scientific. The plasmids for expression of Flag and Flag-MCPIP (Liang *et al.*, 2010) were a gift Dr. Mingui Fu through Dr. Shilpa Buch. The Flag-tagged MCPIP1 expression plasmid was generated by insertion of the human MCPIP1 coding fragment into the pCMV-MAT-Tag-Flag-1 vector (Sigma-Aldrich®, www.sigmaaldrich.com) at the HindIII and BamHI sites.

Plasmid and siRNA transfection

The Flag-tagged MCPIP1 expression plasmid was generated by inserting the human MCPIP1 coding fragment into the pCMV-MAT-Tag®-FLAG®-1 vector (Sigma-Aldrich®, catalog number C5989) at the *Hind*III and *Bam*HI sites. The resultant fusion protein consisted of MCPIP1 with the MAT-Tag-Flag sequence attached to the N-terminus of MCPIP1. The MAT-Tag-Flag sequence added 15 amino acids to the MCPIP1 protein. The MCPIP1(Δ ZF) plasmid was generated by mutating K311C312 into GG using Flag-MCPIP1 as a template. Plasmid and siRNA transfection were performed on the FPCM model as previously described (Carlson *et al.*, 2007), with some modifications. siRNAs specific for p53 and MCPIP1 (pool of 3-4 unique siRNAs for each target) were obtained from Thermo Scientific Dharmacon RNAi Technologies (Accell SMART Pool). Flag/ Flag-MCPIP plasmids were transfected using DharmaFECT® 1 (Thermo Scientific) according to the manufacturer's protocol. Briefly, for a single collagen matrix, separate 17.5 μ L aliquots of serum-free DMEM were combined with 3 μ L of transfection reagent, 3 μ L of siRNA stock (5 μ M in water), or 1.5 μ L of plasmid stock (200 ng/ μ L in water), and then incubated separately at room temperature (RT, c. 22°C) for 15 min. These solutions (containing transfection reagent, siRNA, or plasmid) then were mixed together and incubated at RT for another 15 min. During this time, HFFs were prepared per the FPCM protocol at a concentration

of 5.0×10^6 cells/mL in serum-free DMEM. Each transfection solution was mixed with 40 μ L of this cell suspension, and this mixture was incubated at RT for 15 min. The cell-transfection solution then was added to the FPCM set-up process at the cell addition step per routine. After the collagen polymerization step, transfected matrices were cultured in serum-free DMEM for 24 h, and then the medium was replaced with 5% FBS in DMEM (supplemented with 50 μ g/mL ascorbic acid). Matrices were incubated for an additional 48 h prior to initiating an experiment (i.e., matrix release). The transfection efficiency was determined 3 days after transfection with immunoblotting.

Nested matrix model and cell migration

The nested collagen matrix model was utilized as previously described (Chao *et al.*, 2014; Grinnell *et al.*, 2006; Miron-Mendoza *et al.*, 2010) with some modifications; refer to Supplementary Fig. S1A. For the nested attached matrix, a standard FPCM was incubated in the attached state for 72 h with 5% FBS in DMEM; the FPCM then was removed from the culture well, and placed onto a 60 μ L aliquot of fresh acellular collagen matrix solution (neomatrix solution) that was centered inside a 12 mm-diameter score on the bottom of a new culture well. A 140 μ L aliquot of neomatrix solution then was used to cover the newly-transferred FPCM. The neomatrix was allowed to polymerize for 1 h at 37°C and 5% CO₂, and then 2 mL of DMEM with 5% FBS was added to the well. The same procedure was followed for the nested released matrix, except that the initial incubation of the FPCM was 48 h in the attached state, followed by detachment, and then 24 h incubation in the released state (see Supplementary Fig. S1A).

Cell migration out of the nested FPCM and into the acellular neomatrix was quantified 24 h after nesting with fluorescent microscopy. Digital images (constant dimension of 1000 \times 800 μ m) were captured with a EVOS® FL Cell Imaging microscope (Life Technologies, Grand Island, NY) from 3-5 randomly-selected microscopic fields at the interface of the nested FPCM with the acellular neomatrix (unfixed samples). Fibroblast migration out of the nested FPCM was quantified by counting the number of cells that had migrated clear from the nested matrix into the cell-free matrix. The number of cells that had migrated out of the nested matrix per field was averaged from these digital micrographs. All migration experiments were repeated in cells from five different donors.

In vitro scratch assay

Cell migration in a 2D culture system was evaluated using an *in vitro* scratch assay (Rodriguez-Menocal *et al.*, 2012). Briefly, GFP-HFFs were seeded into 24-well plates (1×10^5 cell/well) and cultured in 10% FBS in DMEM for 24 h, which resulted in confluence of ~70-80%. Using a sterile 200 μ L pipette tip, a line was scratched in the monolayer across the center of the well, keeping the pipette tip perpendicular to the plate. A second straight line then was scratched perpendicular to the first line to create a cross-shaped gap that was devoid of cells and about 800 μ m wide. Each well was washed twice with 1 mL DMEM to remove any non-adherent cells, and then the well was filled with fresh 10% FBS in DMEM. Digital images of the cell gap were captured at set intervals, and the gap width was measured using ImageJ. Each experiment was repeated with three different cell strains.

Gel contraction assay

FPCM contraction was determined by the floating matrix contraction assay originally described by Bell *et al.* (Bell *et al.*, 1979), with minor modifications. Briefly, matrices were set up per the routine described above, allowed to polymerize, covered with 5% FBS in DMEM, and then were released from the culture well with a sterile spatula. Matrices were incubated at 37°C for a specified interval, and then fixed with 4% paraformaldehyde in PBS overnight at 4°C. Fixed matrices were imaged with a desktop flatbed scanner. Matrix area subsequently was measured from the scans using ImageJ (<http://rsb.info.nih.gov/ij/>), and was expressed as a ratio of final (i.e., released) matrix area to initial (attached) matrix area.

Immunoblotting

Immunoblotting was utilized as previously described (Carlson *et al.*, 2004; Carlson *et al.*, 2013), with minor modifications. FPCMs were collected from culture wells, washed with PBS, and the matrices were lysed using a Mammalian Cell Lysis kit (MCL1-1KT, Sigma-Aldrich®) following the manufacturer's instructions. Briefly, two FPCMs to be extracted were transferred to a 1.5 mL microfuge tube containing 120 μ L of ice-cold lysis buffer, and homogenized (50 strokes) using a polypropylene pestle (USA Scientific, Inc. Ocala, FL). Samples were clarified by centrifugation (20,000 *g*, 20 min, 4°C: Eppendorf 5417R centrifuge), and the supernatant (100 μ L) was transferred to a microfuge tube. The supernatant was mixed with 25 μ L of 5x loading buffer (no. 161-0767; Bio-Rad; Hercules, CA) and boiled for 5 min. Samples were subjected to SDS-PAGE using 12% polyacrylamide gels, and transferred to PVDF membranes (Millipore). Western blots were probed with primary antibodies overnight. Secondary antibodies were goat anti-mouse or -rabbit IgG conjugated to alkaline phosphatase (1:5,000). Signals were detected

by chemiluminescence (SuperSignal West Dura Chemiluminescent Substrate, Thermo Scientific). Each Western blot was repeated using cells from three different donors. A single representative immunoblot for all related blots is shown in a given Figure. Immunoblot densitometry was performed using ImageJ (<http://rsb.info.nih.gov/ij/>) on scanned blot images.

Immunocytochemistry

Immunocytochemistry was performed as previously described (Carlson *et al.*, 2007), with some modifications. FPCMs were prepared as described above and cultured in 5% FBS in DMEM for 2 days. Attached or released matrices were fixed with 4% paraformaldehyde in PBS at 4°C overnight. Fixed, intact matrices were permeabilized for 30 min at room temperature (RT) with 0.3% Triton X-100 in PBS. Permeabilized samples were blocked with 10% normal goat serum (NGS; Life Technologies) in 0.3% Triton X-100/PBS at RT for 2 h. Blocked samples were incubated with primary antibodies in 10% NGS/0.3% Triton X-100/PBS at 4°C overnight. The samples then were washed three times with PBS, and treated with donkey anti-rabbit (conjugated to Alexa-Fluor® 488) and donkey anti-mouse (conjugated to Alexa-Fluor® 576) secondary antibodies for 2 h at RT. After three PBS washes, samples were mounted with Prolong® Gold antifade reagent with DAPI (P36931; Life Technologies). Slides were examined with an EVOS FL fluorescence microscope.

Immunoprecipitation

The procedure for immunoprecipitation was performed as described by Peng *et al.* (Peng *et al.*, 2008) Briefly, attached or released FPCMs were washed with PBS twice, followed by lysis in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris, pH8.0, 150mM NaCl, 0.1% SDS, 1.0% NP-40, and 0.5% sodium deoxycholate) containing proteinase and phosphatase inhibitors. For each sample, 200 µg protein was used for immunoprecipitation. The sample protein was incubated with 2 µg diluted p53 or MCPIP1 antibody overnight at 4°C, followed by incubation with 20 µL of protein A-sepharose (sc-2003, Santa Cruz) for 3 h at 4°C. The mixture then was centrifuged at 6000 *g* for 30 s, the cell pellets were rinsed twice with RIPA buffer, and boiled in 2x immunoblot loading buffer for 4 min. After another spin at 6000 *g* for 30s, the supernatants were subjected to immunoblotting as described above for detection of MCPIP1 or p53.

Chromatin Immunoprecipitation (ChIP) Assay

The ChIP assay was performed according to the manufacturer's instructions (EZ-ChIP™, Cat. No. 17-371; EMD Millipore). All materials were provided by the kit except as specified. Briefly, 108 µL of 18.5% fresh formaldehyde was added directly into each well containing an FPCM (2 mL medium/well), yielding a final concentration of 1% formaldehyde. Fixation proceeded for 10 min at room temperature, followed by quenching with 10X glycine. The FPCMs then were washed with 2 mL of ice-cold PBS containing the 1x protease inhibitor cocktail. The FPCMs were homogenized (50 strokes) using a polypropylene pestle in kit lysis buffer to harvest nuclei. DNA was sheared by sonication (5 sets of 10-s pulses on wet ice with 30% maximum power) using sonicator (Cole Parmer High Intensity Ultrasonic Processor/Sonicator, 50-watt, 2 mm tip). Sheared DNA solution (50 µL) then was mixed with 20 µL of protein A magnetic beads, and then 5 µg of the corresponding antibodies (specific to NF-κB (#8242, Cell Signaling Technology®), Histone (#3636, Cell Signaling Technology®), or IgG (ab2410, Abcam) diluted in 450 µL of dilution buffer was added. The DNA-bead-antibody mixture was incubated overnight at 4°C on a rotator. The magnetic beads (bound to the antibody/chromatin complex) then were washed with 0.5 mL each of a series of cold wash buffers in the order of low salt buffer, high salt buffer, LiCl buffer, and finally Tris-EDTA buffer. The cross-linked protein/DNA complexes were reversed to free DNA by incubation at 62°C for 2 h, and then purified using DNA purification spin columns, following the manufacturer's instructions. Finally, the purified DNA was amplified via PCR (Chao *et al.*, 2013) to identify the promoter region containing the NF-κB binding site GGGGTTCCCC. The sequence of the primers used to identify the MCPIP1 promoter bound by NF-κB was: anti-sense 5'-ACAGGCAGATAGATGGTGGG-3'; anti-sense 5'-TGTCTTCCGGCTCATGCTA-3'.

Computational prediction pipeline for NF-κB binding sites and primer design

In order to predict MCPIP1 promoter bound by transcription factor NF-κB (NF-κB miRNA target/binding sites), three publicly-available algorithms were used: TargetScan (<http://www.targetscan.org/>), miRanda (<http://www.microrna.org/>) and PicTar (<http://pictar.bio.nyu.edu/>). Network visualization was carried out using Cytoscape (<http://cytoscapeweb.cytoscape.org/>). Primers are designed encompassing the predicted binding site using Primer3 (<http://frodo.wi.mit.edu/>) or Primer Express (Applied Biosystems) for real-time PCR primer pairs. The designed primers were typically 20–25 oligomers with a T_m range of 58–63°C (maximum T_m difference 2°C), and amplified a product of 50–150 bp. The melting temperature was calculated based on the Nearest-Neighbor thermodynamic parameters. The GC content was set between 40 and 55%; the concentration of monovalent cation was set

at 50 mM; the concentration of annealing oligos was set at 50 nM; and the maximum 3' complementarity was set at 3.00.

Statistics

Data are expressed as mean \pm s.e.m. Unpaired numerical data were compared using the unpaired t-test (two groups) or ANOVA (more than two groups), with statistical significance set at $p < 0.05$.

Fibroblast populated collagen matrix (FPCM) protocol

1. Warm DMEM (Life Technologies, Cat. No. 1200-046) and 0.05% trypsin/EDTA (Life Technologies, Cat. No. 25300-054) to 37°C.
2. Sterilely inscribe a 24-well culture dish (BD no. 353047) with a 12 mm diameter circle in each well, using a geometry compass with two sharp points; keep the 24-well plate warm in the 37°C incubator until step 18.
3. Obtain 1-2 (not more than two) T75 flasks of subconfluent human foreskin fibroblasts (HFFs; passage < 10) from 37°C/5% CO₂ incubator.
4. Trypsinize cells: wash T75 flask with 5 mL of PBS @ room temperature (RT), aspirate, then incubate with 2 mL of trypsin/EDTA for ~5 min at 37°C.
5. During trypsin incubation, make solution DMEM containing 10% FBS; will need 10 mL for each T75 flask
6. Upon completion of trypsin incubation, add 8 mL of 10% FBS/DMEM to each T75 flask, swirl gently.
7. Transfer cell suspension from T75 flask into a 15 mL plastic conical tube (if starting with one T75 flask) or into a 50 mL plastic conical tube (if starting with two T75 flasks); wash each T75 flask once with 2 mL of 10% FBS/DMEM and add these washings to the conical tube.
8. Centrifuge cell solution for 4 min at 600 *g*.
9. Aspirate/discard supernatant, and gently resuspend cell pellet in 10 mL of DMEM (if starting with one T75 flask) or 20 mL of DMEM (if starting with two T75 flasks).
10. Using the resuspended cell pellet solution from step 9, load both ends of a hemacytometer and obtain a 4-square count from both ends.
11. Calculate the cell concentration (cell per mL) as below; *FSC* = four square count.

$$cell\ per\ ml = \frac{(FSC_1 + FSC_2)}{8} \cdot 10,000$$

12. While counting cells, centrifuge the rest of the cell suspension from step 9 for 4 min at 600 *g*; aspirate/discard the supernatant, and use the cell pellet for next step.
13. Using the cell per mL from step 11, determine the mL of DMEM (*x* in the equation below) to resuspend the cell pellet from step 12; this will yield a HFF stock of 5 x 10⁶ cell per mL.

$$x = \frac{(cell\ per\ ml)}{500,000} \quad [if\ starting\ with\ one\ T75\ flask]$$

$$x = \frac{(2) \cdot (\text{cell per ml})}{500,000} \quad \text{[if starting with two T75 flasks]}$$

14. Place the following reagents on ice: HFF stock (from step 13); DMEM; 10x MEM (Life Technologies, no. 11430-030); 0.1 N NaOH; and PureCol type I bovine collagen (Advanced Biomatrix; 3 mg/mL).
15. Using Supplementary Table 1, find the volumes of reagents (DMEM, 10x MEM, 0.1 N NaOH, PureCol, and HFF stock) that will make two more matrices than required by the planned experiment.
16. Using a 50 mL plastic conical tube, gently combine aliquots per step 15 in the following order:
 - DMEM
 - 10x MEM
 - 0.1N NaOH
 - PureCol
 - HFF stock

This will be the FPCM solution; keep this on ice until step 17, in order to prevent premature polymerization of the collagen.

17. Warm the FPCM solution of step 16 in a 37°C water bath for 4 min, with gentle swirling by hand. Do not let the tube sit idle during this 4 min period, but do not shake or otherwise over-agitate the tube; doing the latter will decrease cell viability.
18. Immediately after step 17, pipet 200 µL of collagen gel solution as a bead into the center of each inscribed circle of each well that will be utilized in the 24-well plate. The pipetted bead should expand to fill the 12 mm circle, but should retain an oblong bead shape. If the FPCM solution breaks outside of the circle, then that well will not be usable. Do step 18 quickly, until all wells have been pipetted, all the while swirling the FPCM solution frequently to keep cells from sinking to the bottom of the tube.
19. Without breaking the pipetted beads of the nascent collagen matrices, carefully place the 24-well plate into the 37°C/5% CO₂ incubator, and allow 1 hr for the collagen to polymerize.
20. Make vitamin C (ascorbic acid) solution while gels are polymerizing: 50 mg ascorbate acid (Sigma, Cat. No. A-4403) in 1 mL H₂O, and passed through a 0.2 µM filter.
21. Also during the 1 hr collagen polymerization phase, make fresh growth medium: 5% FBS in DMEM, and add 1 µL of vitamin C stock (50 mg/mL) per mL of FBS/DMEM (final vit C concentration = 50 µg/mL).
22. After the collagen polymerization step, add 2-3 mL of fresh growth medium to each well.
23. Incubate at 37°C/5% CO₂ for ~48 h prior to beginning an experiment.

References (Supplementary Information only)

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