SUPPLEMENTARY INFORMATION

NUCLEAR TRAFFICKING OF THE SRF CO-ACTIVATOR MAL IS
DOWNREGULATED AT TENSIONAL HOMEOSTASIS
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Supplementary information Content

**Methods:** full details of the methods for paper figures and supplementary figures

**Supplementary figures with legend:**

- **Fig S1:** MAL nuclear accumulation is prevented or dramatically delayed in anchored gels.

- **Fig S2:** Controls showing A) the levels of cofilin downregulation in gels following siRNA treatment and B) the nuclear/cytoplasmic ratio of MAL in cells transfected with various LIMK and cofilin constructs.
Methods

Cells and reagents
NIH3T3 cells were routinely maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% foetal bovine serum (FBS, Sigma-Aldrich). NIH3T3 cells stably expressing tetracycline-inducible MAL-GFP or control vector have been previously described (Vartiainen et al., 2007). MALxxx-GFP denotes the previously described C-terminally GFP-tagged MAL R81A/R125A/R169A derivative (Vartiainen et al., 2007). The GFP-pyruvate kinase construct was made by subcloning a partial sequence of chicken PK (Kalderon et al., 1984) into pEGFP-C1 (Clontech).

For serum or drug stimulation, cells were starved overnight in 0.3% FBS and where indicated were stimulated with 15% FBS or 0.5μM jasplakinolide or 2.5μM cytochalasin D (4μM for saturation experiment) or 0.5μM mycalolide B for the indicated periods of time. For nuclear export experiments, starved cells were either treated with leptomycin B (50nM) alone in the presence of 0.3 or 15% FBS or preincubated for 5 min with latrunculin B (0.5μM) prior to treatment with leptomycin B or preincubated with latrunculin B for 15 min prior to washout and subsequent addition of leptomycin B with 0.3 or 15% FBS. All drugs were from Calbiochem with the exception of cytochalasin D (Sigma-Aldrich). NIH3T3 cells were transfected using Lipofectamin 2000 (Invitrogen) according to manufacturers instructions.

The shRNA constructs targeting cofilin1 and ADF have been characterised previously in NIH3T3 cells (Garvalov et al., 2007). Human cofilin WT, S3A and S3E in pmRFP-N1 as well as the LIMK1 in pCMV-Tag1 and LIMKCA (T508EE) in pCDNA3.1 were a gift from James Bamburg (Colorado State University).

Collagen gel preparation
NIH3T3 cells or MAL-GFP cells were cast at a final concentration of 2.5x10^5 cells/ml into a 1.5mg/ml collagen matrix of rat tail collagen type I (First Link, UK) in 35mm Mattek™ dishes, as previously described (Dahlmann-Noor et al., 2007). Following polymerisation, the gels were either manually detached from the edges of the well (free-floating gels; no mechanical tension) or left attached to the well (anchored gels; high
mechanical tension). Gels were maintained in DMEM with 10% FBS for 18-24h prior to being subjected to serum-starvation. High concentration collagen type I (BD Bioscience 9.03 mg/ml) was used to make gels of increasing collagen concentration.

**Mechanical stress assays**

MAL-GFP expressing cells were seeded on collagen-coated coverslips or within anchored collagen gels, grown in normal growth medium for 24h and starved in low serum (0.3% FBS) medium for a further 24h. The medium was replaced with L-15 medium (0.3% FBS) for live imaging using a 63x objective lens (Zeiss LD Plan Neofluor Korr NA 0.75) on an inverted Axiovert 100M (Zeiss) microscope. Images were captured every 60 sec with an ORCA-ER CCD digital camera (Hamamatsu Photonics UK) using Openlab acquisition software (Perkin Elmer). Using a microneedle controlled by a micromanipulator (Eppendorf), an individual cell (coverslip) or the matrix next to a cell (gel) was placed under stress and the tension was maintained throughout the timelapse. For gel shearing assays, cells were seeded in the gels for 1 hr or 24 hrs (with/without a further 24hrs in low serum) and stress was applied to the whole gel by pipetting it up and down twice with a plastic transfer pipette, followed by fixation and F-actin staining.

**Immunofluorescence**

For MAL-GFP localisation assays in fixed gels, gels were fixed in 4% formaldehyde in cytoskeleton buffer (CB) (10mM KCl, 137mM NaCl, 4 mM NaHCO$_3$, 0.4 mM KH$_2$PO$_4$, 1.1 mMNa$_2$HPO$_4$, 2mM MgCl$_2$, 5 mM PIPES, 2 mM EGTA, 5.5 mM glucose) for 30 min and permeabilised with 0.5% Triton X-100 for 30 min prior to blocking with 0.1M glycine in CB. Gels were then washed in TBS and incubated with rhodamine phalloidin (Molecular Probes, Invitrogen) in 1% BSA and 1% FBS in TBS for 30 min. Gels were washed in 1% BSA in TBS, with a final wash in TBS and mounted in a TBS/Glycerol (50:50) solution containing 6 g/l N-propyl gallate. Cells on coverslips were fixed in 4% formaldehyde in CB for 15 min and permeabilised with 0.5% Triton X-100 for 10 min prior to washing and subsequent incubation with rhodamine phalloidin before mounting. X-Y image stacks (step-size of 0.5 mm) were acquired using a 40x objective (Zeiss Plan Apochromat,NA 1.0) on an inverted Zeiss Axiovert 100S TV/BioRad Radiance 2000
confocal laser scanning system. Images were acquired under identical nonsaturating optical conditions using constant laser power and gain settings. Images were processed using either Volocity 4.0 (Improvision) software or Image J analysis packages. MAL nuclear/cytoplasmic ratio was calculated on single confocal slices taken within the central region of the cells by measuring the mean fluorescence grey level within a defined region of interest kept at constant size (5-10 μm in diameter) over the cytoplasm and over the nucleus. For quantitation of cofilin levels following siRNA transfection, the gels were fixed and stained for F-actin as above and further incubated overnight at 4°C with rabbit anti-cofilin antibodies (Cytoskeleton), followed by 2 hrs at room temperature with CF633 goat anti-rabbit antibodies (Biotium). After wash, the gels were mounted in a TBS/Glycerol with N-propyl gallate and viewed on the confocal. Images were acquired under identical non-saturating optical conditions using constant laser power and gain settings, and total cofilin levels were quantitated as mean grey levels over the whole cell area in Image J on single confocal slices taken within the central area of the cells.

**F-actin and G-actin analysis**

To measure G-actin levels in fixed cells, we used an established immunostaining method (Cramer et al., 2002), which was slightly modified to accommodate staining of cells within a collagen gel. Gels were fixed for 30 min in 4% formaldehyde containing 0.32M sucrose in CB, followed by permeabilisation in 0.5% triton X-100/CB for 30 min and a rinse in 0.1% tritonX-100/PBS. Samples were blocked for 30 min in 0.5% tritonX-100/2% BSA/PBS. To detect G- and F-actin respectively gels were incubated in Alexa Fluor 488-DnaseI and rhodamine-phalloidin (all from Molecular Probes, Invitrogen) in blocking solution for 40 min. Gels were rinsed in blocking solution twice followed by a final rinse in PBS. X-Y image stacks (step-size of 0.5 μm) were acquired using a 40x objective (Zeiss Plan Apochromat, NA 1.0) on an inverted Zeiss Axiovert 100S TV/BioRad Radiance 2000 confocal laser scanning system. Images were acquired under identical nonsaturating optical conditions using constant laser power and gain settings. Digital images were processed using Image J analysis software. Contours of cells were outlined and average fluorescence intensity was calculated from a sum of the stacks.
**Western blotting**

Following digestion of the gel in 0.05% Collagenase D, cells were centrifuged, counted, and resuspended in lysis buffer (containing protease inhibitor cocktail) at equal cell concentration. Lysates were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked in either 5% nonfat milk or BSA and immunoblotted with mouse monoclonal anti-actin (Chemicon; 1:2500), rabbit polyclonal anti-cofilin (Cytoskeleton; 1:1200), rabbit polyclonal anti-phospho-cofilin (Ser3) (Cell Signaling Technologies; 1:800), or rabbit anti-GAPDH (Sigma-Aldrich; 1:6000). Secondary antibodies polyclonal goat anti-rabbit/anti-mouse Ig-HRP were purchased from Dako (Dako, Denmark A/S). Proteins were visualised using chemiluminescence reagents (Pierce).

**Statistical analysis**

All graphs display mean and standard error. Statistical analysis was performed using the Students t test to establish significant differences and individual P values displayed (unless graphs have multiple tests).
REFERENCES


SUPPLEMENTARY FIGURES
Supplementary Figure S1 McGee et al
Supplementary Figure S2 McGee at al.
SUPPLEMENTARY FIGURE LEGENDS

Figure S1. MAL nuclear accumulation is prevented at tensional homeostasis

**A, B, MAL nuclear accumulation remains delayed and decreased in anchored gels even following treatment with high doses of cytochalasin D or mycalolide B.** MAL-GFP expressing NIH3T3 fibroblasts were seeded into free-floating and anchored gels and grown in normal growth medium for 24h, after which the medium was replaced with 0.3% FBS medium for 24h. The serum-starved cells were then incubated with medium containing 4 μM CytoD (A) or 0.5μM mycalolide B (B) for the indicated times and subsequently fixed in formaldehyde and stained with rhodamine-phalloidin. The nuclear/cytoplasmic ratio of MAL-GFP was determined on confocal images (8-27 cells per timepoint, n=2).

**C, Mechanical stress by gel shearing triggers MAL nuclear accumulation in free-floating gels.** MAL-GFP cells were seeded in free-floating (FF) collagen matrices and, after 1 hr, one set of matrices was rapidly sheared and fixed with formaldehyde. MAL-GFP localisation was monitored as the proportion of cells showing clear cytoplasmic, nuclear, or pancellular distribution. Inset shows representative MAL-GFP cytoplasmic localisation in a cell within a control gel (top) and nuclear localisation in a cell within a sheared gel (bottom). Graph shows the average of 2 experiments with 40-80 cells each per set. The proportion of cells showing nuclear staining is statistically different in the 2 sets (P=0.03).

**D, MALxxx-GFP, which cannot bind actin, is constitutively nuclear in both free-floating and anchored gels.** NIH3T3 fibroblasts were transiently transfected with MALxxx-GFP. Transfected cells were then seeded onto glass coverslips and into free-floating and anchored gels and grown in normal growth medium for 27h. The medium was then replaced with low serum (0.3% FBS) medium for 15h. The serum-starved cells were then fixed in formaldehyde. The graphs show the subcellular distribution (nuclear/pancellular/ cytoplasmic) of MALxxx-GFP. 60 cells (glass coverslips), 45 cells (free-floating gels) and 55 cells (anchored gels) were counted (n=1).
Figure S2. MAL nuclear import defect in anchored matrices is linked to increased cofilin expression. A, Cofilin levels in gels following siRNA transfection. MAL-GFP expressing fibroblasts were transfected with a control construct expressing mCherry with or without both cofilin and ADF shRNAi expressing constructs. After 24 hours, the cells were seeded in free-floating (FF) or attached (A) gels, and grown for 24 hours in medium with 10% serum followed by 24hrs in low serum. The cells were then left untreated (t0) or stimulated with 15% serum for 15 min (t15) and cofilin levels were monitored in mCherry expressing cells using confocal microscopy. Shown is the average fluorescence intensity for cofilin from one representative experiment, with 30-40 cells measured per condition. On average cofilin levels were increased 1.7 times in anchored compared to free-floating gels for control cells (n=2), while cofilin levels were down-regulated by half following siRNA treatment (average fluorescence level compared to corresponding control: 0.54 ± 0.11, n=4). B, Modulating cofilin phosphorylation levels does not rescue MAL trafficking in anchored gels. MAL-GFP expressing fibroblasts were transfected with a control plasmid expressing mCherry with or without LIMK or LIMK-CA expressing constructs. After 24 hours, the cells were seeded in gels, and grown for 24 hours in medium with 10% serum followed by 24hrs in low serum. The cells were then left untreated (t0) or stimulated with 15% serum for 15 min (t15) and MAL-GFP localisation was monitored on confocal images. Shown is the average of 2 experiments with 30-40 cells measured per condition in each experiment.