Methods:

Cell Culture and Transfection

HeLa and the human breast cancer MDA-MB-231 cells were from ATCC. Human HT1080 fibrosarcoma cells, wild-type and MT1-MMP$^{-/-}$ mouse dermal fibroblasts were previously described(Sabeh et al., 2004). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 unit/ml penicillin, 100 μg/ml streptomycin, and 1 mM sodium pyruvate (all GIBCO BRL)in a humidified 95% air, 5% CO_2 incubator at 37°C.

DNA plasmids were transfected using Lipofectamine 2000 reagent according to the supplier's protocol (Invitrogen).

Inhibitors

Actin filaments, microtubules or membrane lipid rafts were disrupted by 1 hr incubation with cytochalasin D (0.2 μM; from Sigma)(Wang et al., 2005), Nocodazole (1 μM; from Sigma)(Wang et al., 2005), or Methyl-β-cyclodextrin (5 mM, from Sigma)(del Pozo et al., 2004), respectively. GM6001 (20 μM, Calbiochem), TIMP-1 (2.5 μg/ml, Calbiochem), and TIMP-2 (2.5 μg/ml, Calbiochem) were pre-incubated with cells for 20 min at 37°C before the cells were stimulated with 50 ng/ml EGF application.

Immunoblotting (IB)

HeLa cells transfected with different constructs were starved in 0.5% FBS for 36-48 hr before stimulated with EGF stimulation (50 ng/ml) or kept as control. The cells were then lysed in a buffer containing 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton-X-100,

0.1% SDS, 5 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, and 10 μg/ml Leupeptin. The lysate was centrifuged, and the supernatant was subjected to SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was then blocked with 5% bovine serum albumin, followed by incubation with the primary antibody. A rabbit polyclonal anti-MT1-MMP antibody (Lehti et al., 2005; Lehti et al., 2000), mouse monoclonal anti-GFP antibody (BD Biosciences) and anti-Actin antibody (Santa Cruz Biotech) were used for immunoblotting (IB). The bound primary antibodies were detected by using goat IgG-horseradish peroxidase conjugate (Santa Cruz Biotechnology) and the ECL detection system (Amersham Pharmacia Biotech).

Immunostaining

All immunostaining experiments were conducted at room temperature if not otherwise specified. In brief, Cells were fixed in 4% paraformaldehyde, and kept without treatment as control or permeabilized with 0.1% TX-100. The cells were then incubated with a primary Rabbit anti-GFP antibody (ab290; from Abcam), which recognizes ECFP/EYFP/YPet, followed by an anti-Rabbit secondary antibody conjugated with Rhodamine. The images were collected by using an epi-fluorescence microscopy system (Zeiss Axiovert 200).

Supplemental Figure 1. ECFP/YPet emission ratio images before and after EGF stimulation of HeLa cells transfected with MT1-MMP biosensor and different plasmids [MT1: MT1-MMP; Vector: Empty vector; MT1 (E/A): catalytically-inactive mutant of MT1-MMP with E240A mutation within its catalytic domain; MT1ΔTM: MT1-MMP

with its transmembrane domain truncated; MT1ΔCT: active MT1-MMP with its cytoplasmic tail deleted; MMP-2; and MMP-9].

Supplemental Figure 2. The same cell groups as described in Fig. 4A were lysed and subjected to immunoblotting. The upper gel panel represents the blotting with anti-GFP antibody, illustrating the protein levels of the MT1-MMP biosensor (upper arrow, intact biosensor; lower arrow, cleaved biosensor). The lower gel panel depicts actin levels used as a loading control.

Supplemental Figure 3. (**A**) Representative ECFP/YPet emission ratio images of HeLa cells transfected with MT1-MMP biosensor and different groups as indicated in the presence of 10% FBS. (**B**) Bar graphs represent the ECFP/YPet emission ratio (mean ± S.D.) averaged over the entire cell body as described in (**A**). The * sign represents a statistically significant difference between various samples and the vector control.

Supplemental Figure 4. HT-1080, wild-type, and MT1-MMP-/- dermal fibroblasts were lysed and MT1-MMP levels analyzed by Western blotting. The upper gel panel shows the blots performed with a rabbit polyclonal anti-MT1-MMP antibody that detects human and mouse MT1-MMP as previously described (Lehti et al., 2005; Lehti et al., 2000). The lower gel panel shows actin levels used as a loading control.

Supplemental Figure 5. Representative time course of normalized ECFP/YPet emission ratio averaged over the wild-type and MT1-MMP-/- fibroblast cells, as indicated. The cells were transfected with the MT1-MMP biosensor and maintained in 10% FBS before subjected to 20 μM GM6001.

Supplemental Figure 6. HeLa cells co-transfected with MT1-MMP biosensor and MT1-MMP expression vectors were plated on fibronectin before EGF stimulation. ECFP/YPet

emission ratio images representing the MT1-MMP activity were collected by focusing on the cell rear. White arrows point to the cell rear with low ECFP/YPet emission ratio.

Supplemental Figure 7. (A) The fluorescence intensity images of ECFP after EGF stimulation for 60 min in non-patterned cells cultured on fibronectin as shown in Fig. 5A. (**B**) 2D histograms of ECFP fluorescence intensity vs the ratiometric signals of ECFP/YPet in HeLa cells grown on Fn before and after EGF stimulation for 60 min are shown with each dot representing values from a single pixel of the images. These Gaussian-like distributions of 2D histograms suggests that the ratiometric FRET signals are independent of its associated fluorescence intensity. (**C**) The pixel frequency distributions of ECFP/YPet emission ratio in HeLa cells grown on Fn before (Blue) or after (Red) EGF stimulation for 60 min. Only pixels inside the fluorescent cell are counted. The frequency was normalized by the total number of intracellular pixels before and after EGF stimulation. An overall shift to higher ratio values can be observed following 60 min EGF stimulation.

Supplemental Figure 8. (A) A representative DIC image of HeLa cells cultured on micro-patterned parallel Fn stripes (10 μm in width). **(B)** Fluorescence intensity images of ECFP after EGF stimulation for 60 min in cells cultured on patterned fibronectin as shown in Fig. 5C.

Supplemental Figure 9. EGF-induced MT1-MMP activity is regulated by the integrity of cytoskeleton. Bar graphs represent the percentile change of ECFP/YPet emission ratio (mean ± S.D.) of the MT1-MMP biosensor in response to EGF in the cells treated with control medium, Cyto D, or nocodazole. The emission ratios were averaged over the

entire cell body. The * and # signs represent a statistically significant difference between the indicated groups.

Supplemental Table 1. The parameters of dichroic mirrors, excitation and emission filters for FRET as well as different fluorescence proteins.

Movie 1: EGF-induced FRET response in HeLa cells expressing both the biosensor and MT1-MMP.

Movie 2: EGF induced a spatially directional FRET response in HeLa cells grown on Fn expressing the biosensor and MT1-MMP.

Movie 3: EGF induced an accumulation and co-localization of EGFR-GFP and MT1-MMP-mCherry at the leading edge in migrating HeLa cells seeded on Fn (Left: EGFR-GFP; Right: MT1-MMP-mCherry).

Movie 4: EGF induced a high FRET response at the cell leading edge along micro-patterned Fn stripes (10 μm in width) in HeLa cells expressing the biosensor and MT1-MMP.

Movie 5: EGF induced an accumulation and co-localization of EGFR-GFP and MT1-MMP-mCherry at the cell leading edge along micro-patterned Fn stripes (10 μm in width) in HeLa cells (Left: DIC Images; Middle: EGFR-GFP; Right: MT1-MMP-mCherry).

References:

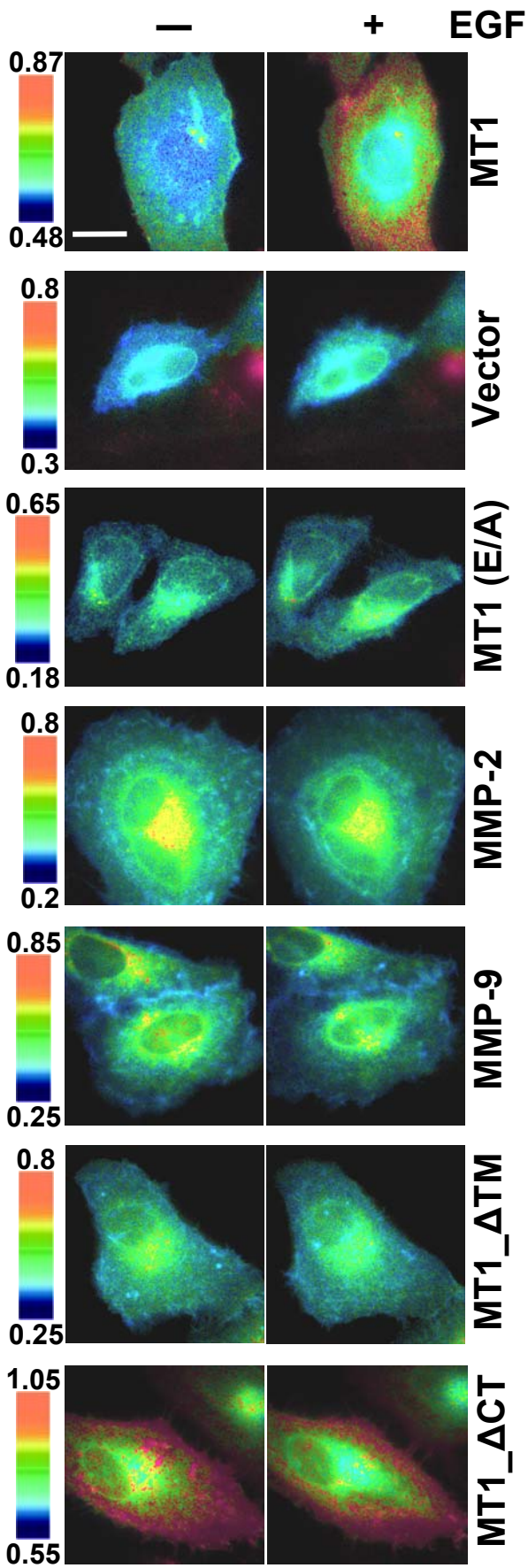
del Pozo, M.A., Alderson, N.B., Kiosses, W.B., Chiang, H.H., Anderson, R.G. and Schwartz, M.A. (2004) Integrins regulate Rac targeting by internalization of membrane domains. *Science*, **303**, 839-842.

Lehti, K., Allen, E., Birkedal-Hansen, H., Holmbeck, K., Miyake, Y., Chun, T.H. and Weiss, S.J. (2005) An MT1-MMP-PDGF receptor-beta axis regulates mural cell investment of the microvasculature. *Genes Dev*, **19**, 979-991.

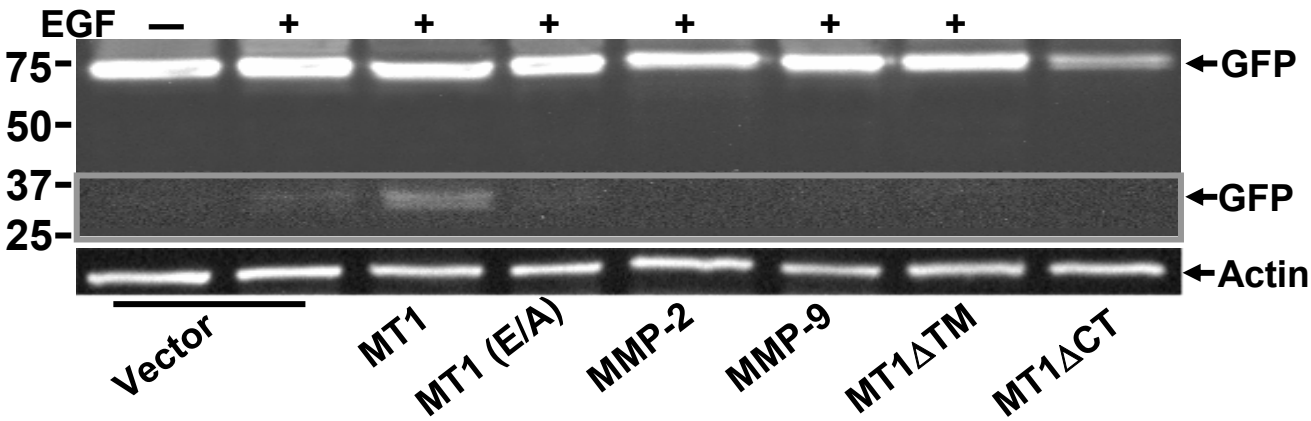
Lehti, K., Valtanen, H., Wickstrom, S.A., Lohi, J. and Keski-Oja, J. (2000) Regulation of membrane-type-1 matrix metalloproteinase activity by its cytoplasmic domain. *J Biol Chem*, **275**, 15006-15013.

Sabeh, F., Ota, I., Holmbeck, K., Birkedal-Hansen, H., Soloway, P., Balbin, M., Lopez-Otin, C., Shapiro, S., Inada, M., Krane, S., Allen, E., Chung, D. and Weiss, S.J. (2004) Tumor cell traffic through the extracellular matrix is controlled by the membrane-anchored collagenase MT1-MMP. *J Cell Biol*, **167**, 769-781.

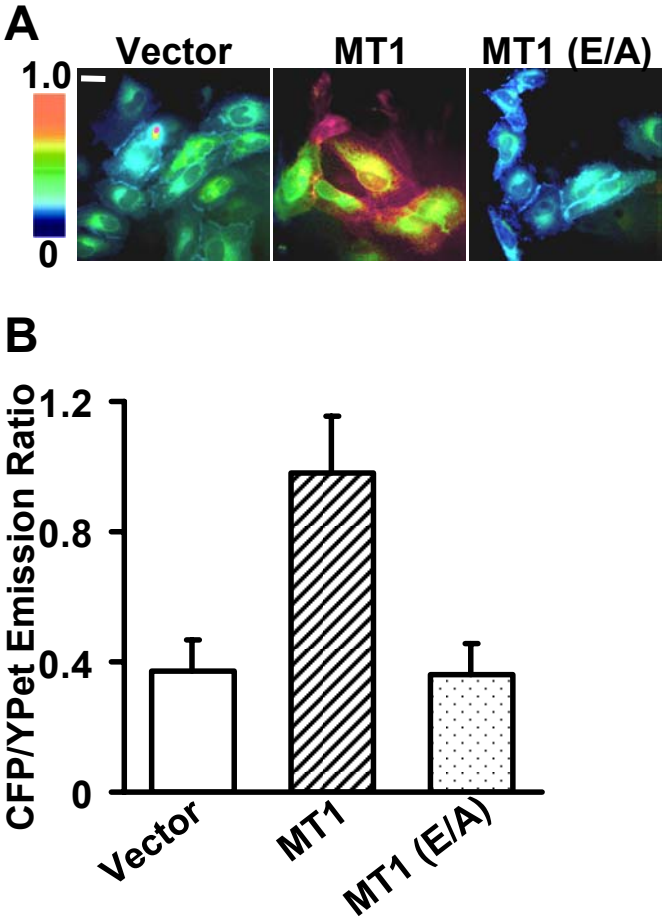
Wang, Y., Botvinick, E.L., Zhao, Y., Berns, M.W., Usami, S., Tsien, R.Y. and Chien, S. (2005) Visualizing the mechanical activation of Src. *Nature*, **434**, 1040-1045.



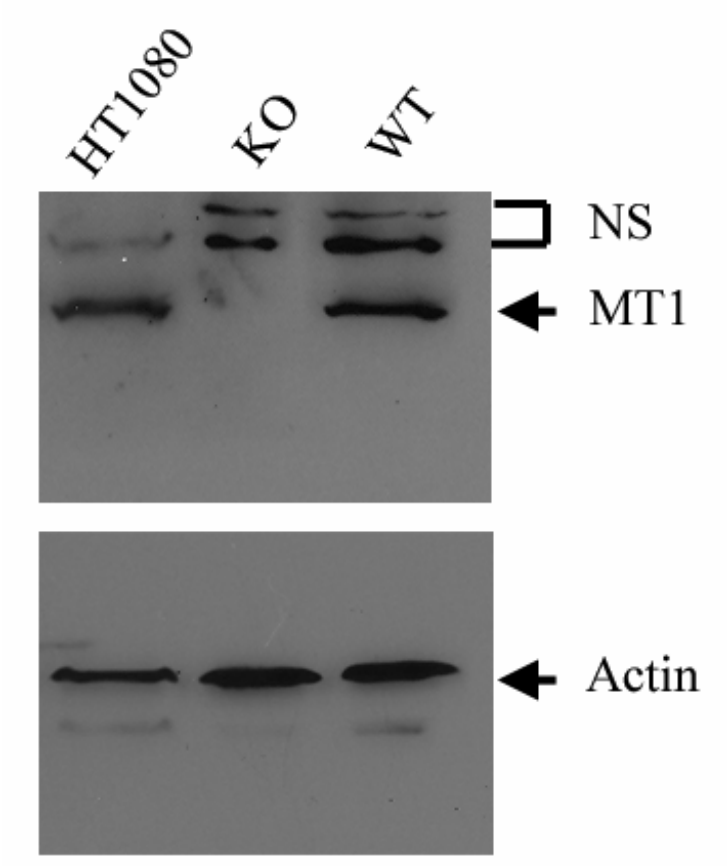
Supplemental Figure 1



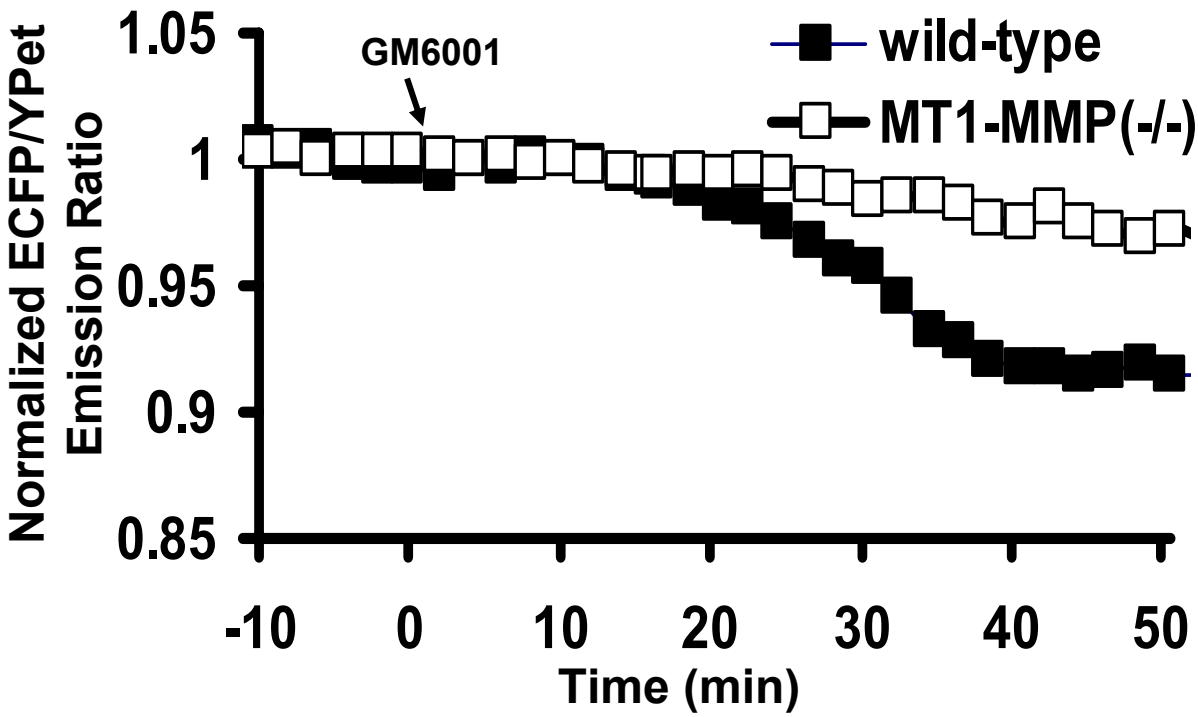
Supplemental Figure 2



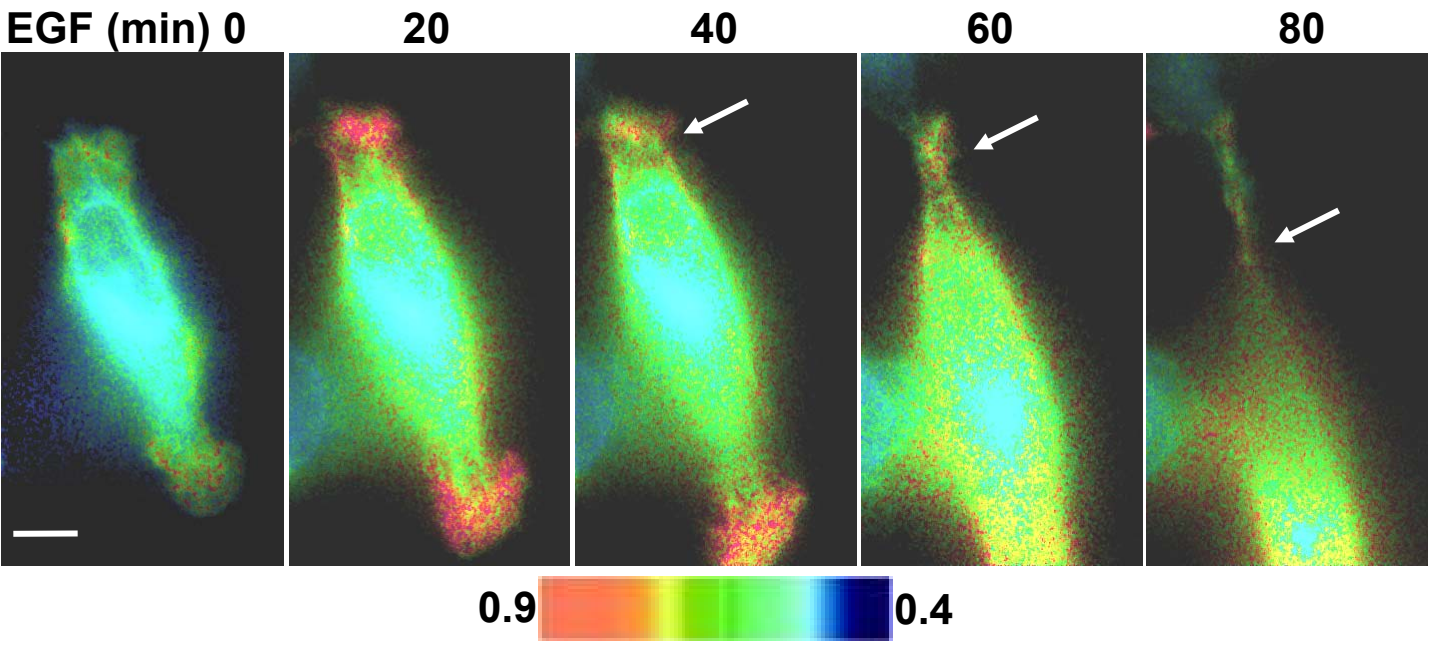
Supplemental Figure 3



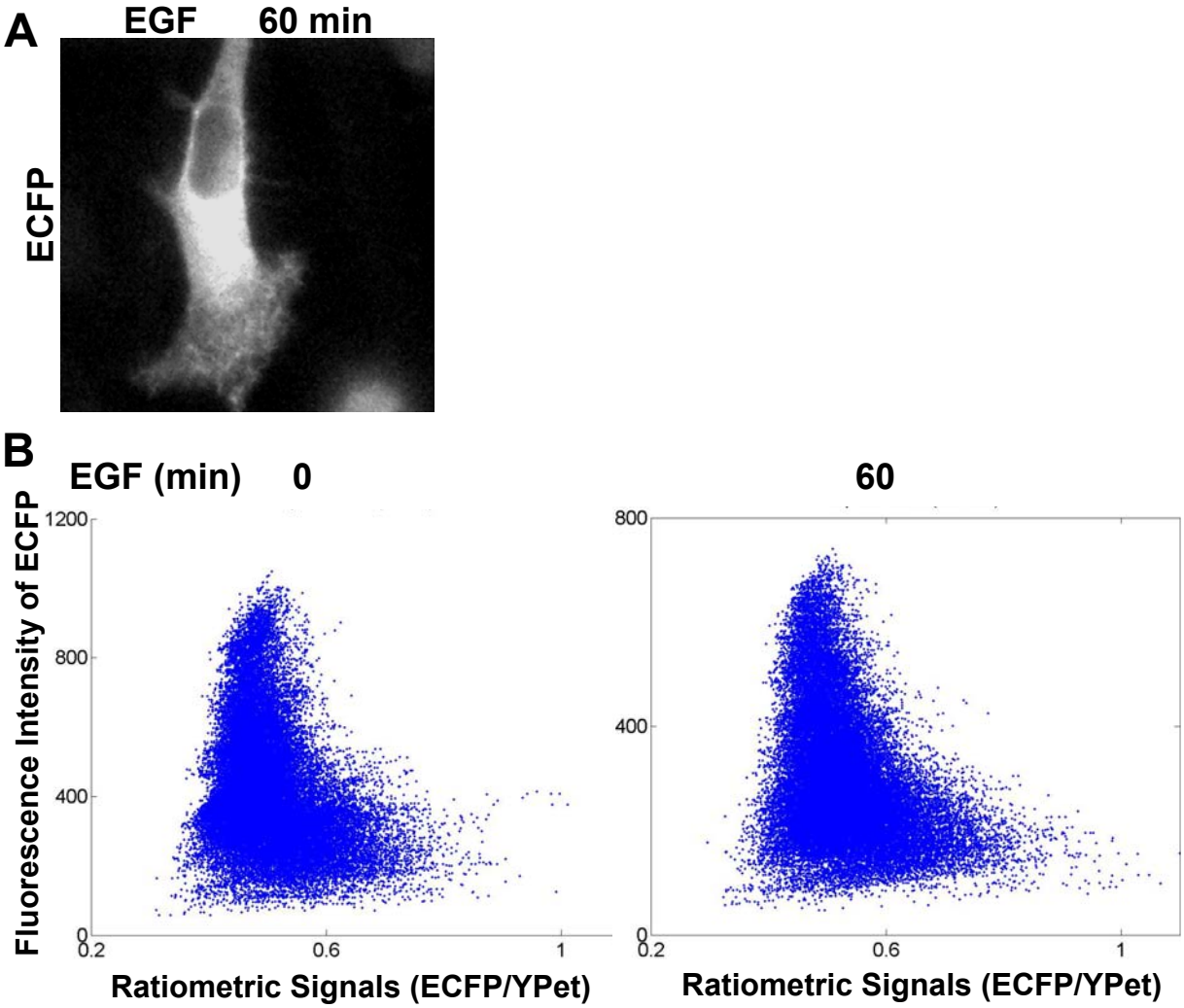
Supplemental Figure 4



Supplemental Figure 5



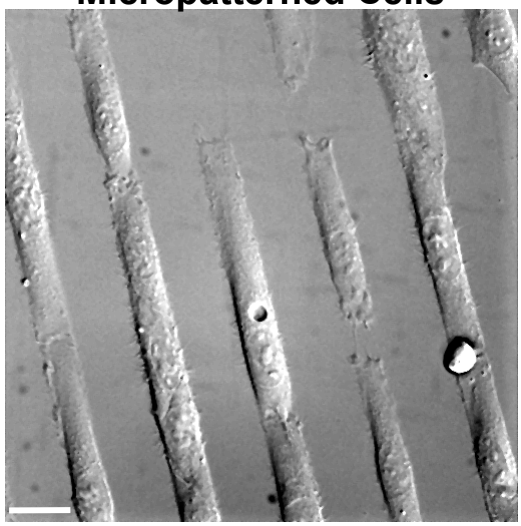
Supplemental Figure 6



Supplemental Figure 7

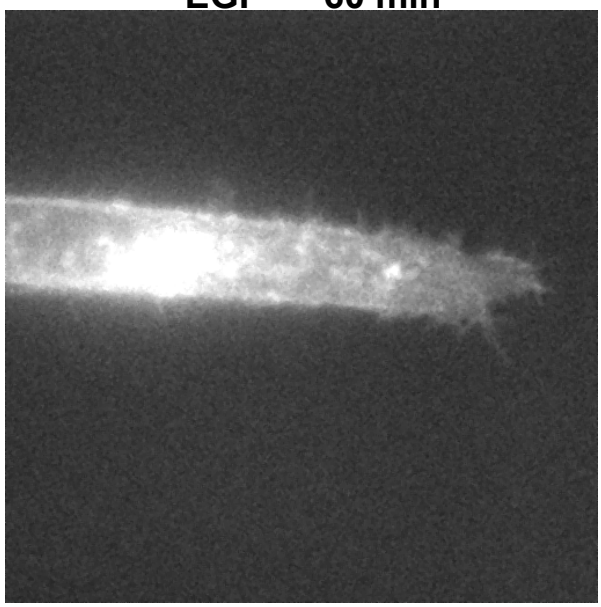
A

Micropatterned Cells

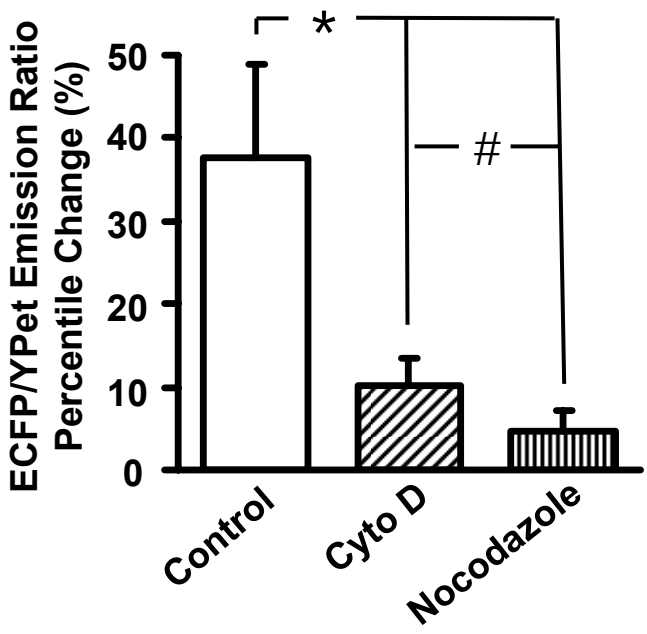


B

EGF 60 min



Supplemental Figure 8



Supplemental Figure 9

Supplementary Table 1. The settings of filters for Fluorescence imaging

	Excitation Filter (nm)	Dichroic Mirror (long pass; nm)	Emission Filter (nm)
CFP	420/20	450	475/40
YFP (FRET)			535/25
GFP	495/10	515	535/25
mCherry	560/40	595	653/95