Supporting Information

Klarlund 10.1073/pnas.1210992109

SI Methods

Antibodies and Other Reagents. Antibodies to c-fos were from Santa Cruz Biotechnology, antibodies to nonmuscle myosin II heavy chain A were from Covance, and LA1 antibody was from R&D Systems. Alexa Fluor–conjugated secondary antibodies and phalloidin were from Invitrogen. Cell-permeable exoenzyme C3 transferase from *Clostridium botulinum* was from Cytoskeleton. UO126, PP2, and SKI were from EMD Biosciences, tyrphostin AG 1478 was from Enzo Life Sciences, and PI-103 was from Cayman Chemical. The green fluorescent protein–actin vector was from Clontech. Tissue culture plastics were from BD Falcon or Greiner Bio-One. Agarase was from Fermentas. EGF was from Invitrogen, cell culture reagents were from Mediatech, and other reagents and supplies were from Thermo-Fisher Scientific.

Tissue Culture and Immunofluorescence. HCLE cells (1) were propagated in human keratinocyte serum-free medium (Invitrogen) supplemented with 0.3 mM $CaCl_2$, 25 μg/mL bovine pituitary extract, and 0.2 ng/mL EGF. When confluent, they were differentiated by incubation for 2 d in F12: Dulbecco's modified Eagle's medium (1:1) with 10% (vol/vol) newborn calf serum. The agarose strips or droplets were removed and cells transferred to MEM with 10% (vol/vol) newborn calf serum. Incubation in the latter medium was found to enhance the appearance of purse strings. To block division, cells were treated with mitomycin C (0.5 mM) for 2 h in differentiation medium, washed, and used for experiments. For immunofluorescence analysis, cells were fixed with 3.7% (wt/wt) formaldehyde at the end of experiments and processed as described previously (2). Images were acquired on a Nikon TE2000-E microscope with a CoolSNAP camera (Photometrics).

Treatment with LA1 Antibody and Mechanical Wounding. For antibody treatment, cells were cultured in 1.5-cm tissue culture wells with 1 × 11 mm agarose strips in 300 μL medium, treated as described above in the presence of 10 μg/mL LA1 antibody or nonimmune IgG. Small holes were induced by stabbing confluent cell layers with a 200-μL pipette tip sharpened by cutting with a razor blade. Because the holes were of very irregular shapes, suitable regularly shaped small round holes were first identified at the beginning of the assay (20–30 holes per dish), their locations were recorded with a laboratory marker, and the number of holes closed after 10 h was counted. To test for cell death, propidium iodide (1 μg/mL) was added to the tissue culture medium immediately after wounding, and the cells were washed after 3 min.

Live-Cell Imaging. Cells were seeded in six-well dishes containing agarose droplets or strips. Just before imaging, they were transferred to an incubation chamber (Oko Industries) to maintain constant temperature, CO_2 concentration, and humidity. Images were captured on a Nikon TI microscope and acquired with a Cascade camera (Photometrics).

1. Gipson IK, et al. (2003) Mucin gene expression in immortalized human corneal-limbal and conjunctival epithelial cell lines. *Invest Ophthalmol Vis Sci* 44:2496–2506.
2. Block ER, Matela AR, SundarRaj N, Iszkula ER, Klarlund JK (2004) Wounding induces motility in sheets of corneal epithelial cells through loss of spatial constraints: Role of heparin-binding epidermal growth factor-like growth factor signaling. *J Biol Chem* 279:24307–24312.

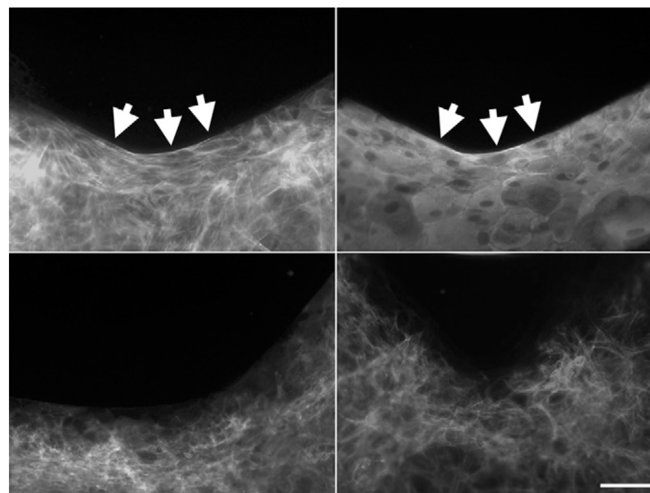


Fig. S1. Characterization of actin cables formed at concave edges in cells grown on thin plastic strips. Cells were stained with labeled phalloidin and antibodies to myosin IIA (*Upper Left* and *Right*, respectively). Cells were treated with 20 μM blebbistatin (2.5 h) or 5 μg/mL cell-permeable exoenzyme C3 transferase (6 h) and stained with labeled phalloidin (*Lower Left* and *Right*, respectively). Purse strings are indicated with arrows. (Scale bar: 100 μm.)

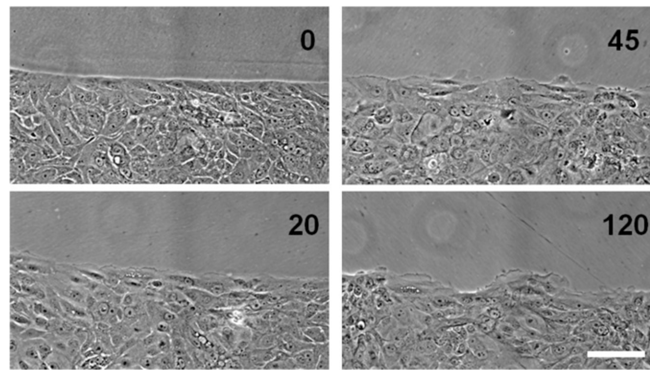


Fig. S2. Formation of lamellipodia after digestion of strips of agarose with straight edges. The times after initiation of digestion are indicated (min). Phase-contrast micrographs. (Scale bar: 100 μm.)

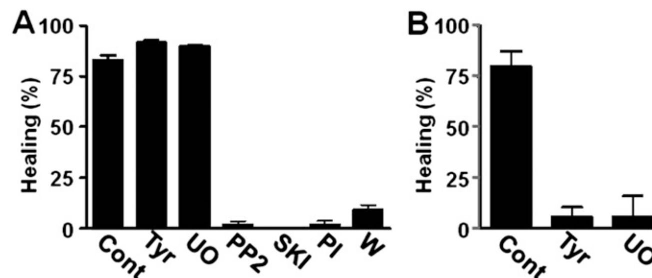


Fig. S3. Insensitivity of healing of small mechanically induced holes to EGFR inhibition. (*A*) The cells were incubated with the inhibitors used in Fig. 2*B* at the same concentrations. Values are means of the percentages of holes closed (triplicates ± SD). (*B*) To verify the efficacy of the tyrphostin AG 1478 and UO128 treatments, straight wounds were induced by scraping with a pipette tip, and the percentage of healing was calculated after photographing the wounds as described (1) (quadruplicates ± SD).

1. Tolino MA, Block ER, Klarlund JK (2011) Brief treatment with heparin-binding EGF-like growth factor, but not with EGF, is sufficient to accelerate epithelial wound healing. *Biochim Biophys Acta* 1810:875–878.

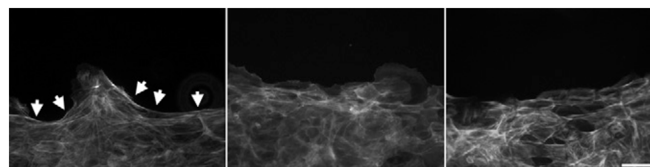


Fig. S4. Characterization of actin cables formed at concave edges in moving cell sheets. The cells were stained with labeled phalloidin after no treatment (*Left*), after treatment with 20 μM blebbistatin (2.5 h) (*Center*), or 5 μg/mL cell-permeable exoenzyme C3 transferase (6 h) (*Right*). Purse strings are indicated with arrows. (Scale bar: 100 μm.)

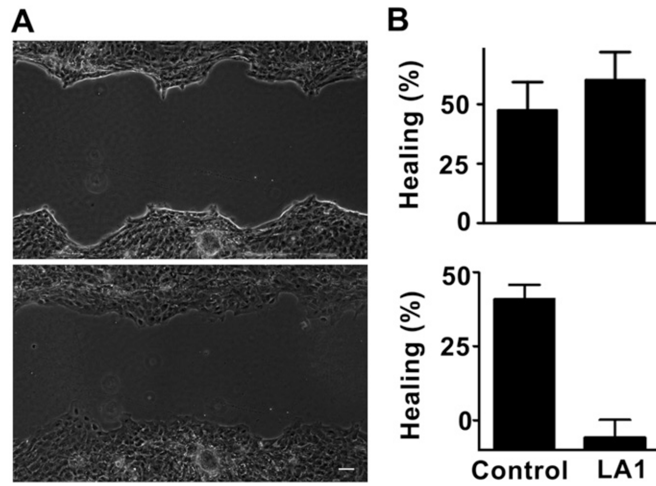


Fig. S5. Effects of LA1 antibody. (*A*) A strip of agarose was removed from a culture of HCLE cells, and healing was allowed to proceed for 12 h (*Upper*). LA1 antibody was added (10 μg/mL), and the cells incubated for a further 16 h (*Lower*). (Scale bar: 100 μm.) (*B*) Healing of small round wounds (*Upper*) or wounds with linear edges (*Lower*) with LA1 added, as indicated (four to six determinations ± SD).

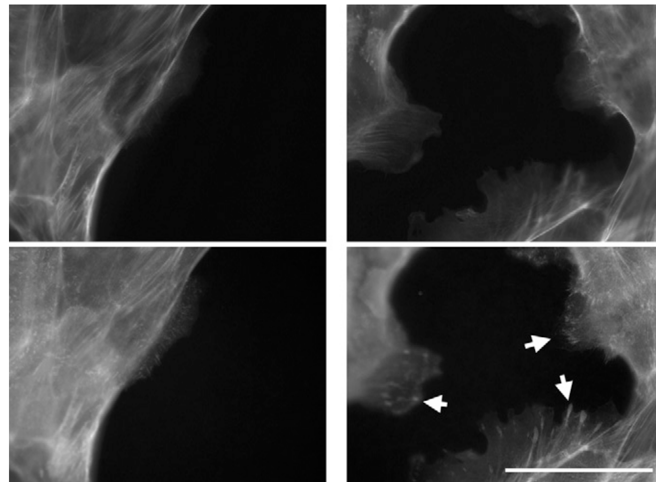


Fig. S6. Lamellipodia induced by EGF. Agarose droplets were digested with agarase without (left images, showing ruffles) or with 10 nM EGF (right images). Cells were stained with labeled phalloidin (upper images) and with antibodies to paxillin (lower images). Focal adhesions are indicated with arrows. (Scale bar: 100 μm.)

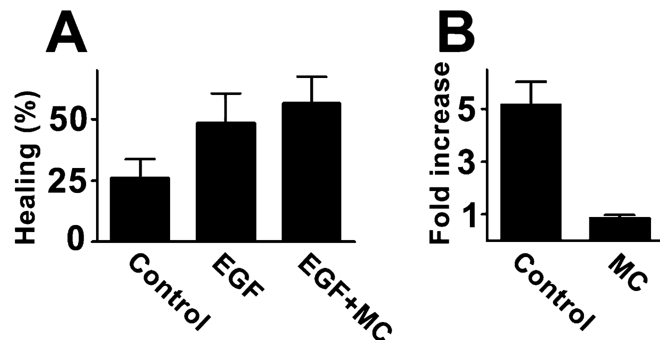


Fig. S7. Purse-string healing in mitocymin C–treated cells. (*A*) Cells were treated with mitomycin C (MC) and 10 nM EGF, where indicated, and allowed to heal for 4 h. (*B*) To verify the efficacy of the MC treatment, HCLE cells were counted the day after seeding and treated with the drug where indicated, and the fold increases in cell number were calculated after 2 d of further incubation. Quadruplicates ± SD.

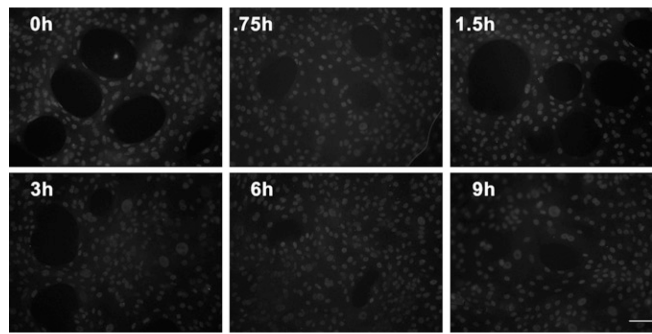


Fig. S8. c-fos transcription during purse-string healing. The cells were fixed at the indicated times after removal of the agarose droplets. (Scale bar: 100 μm.)

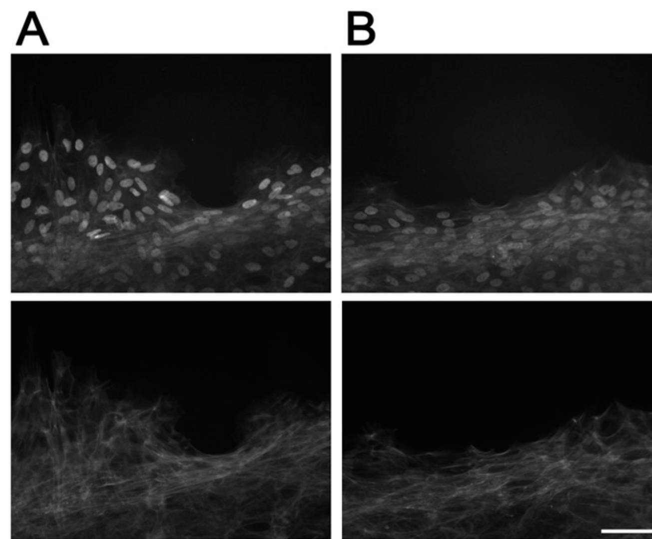
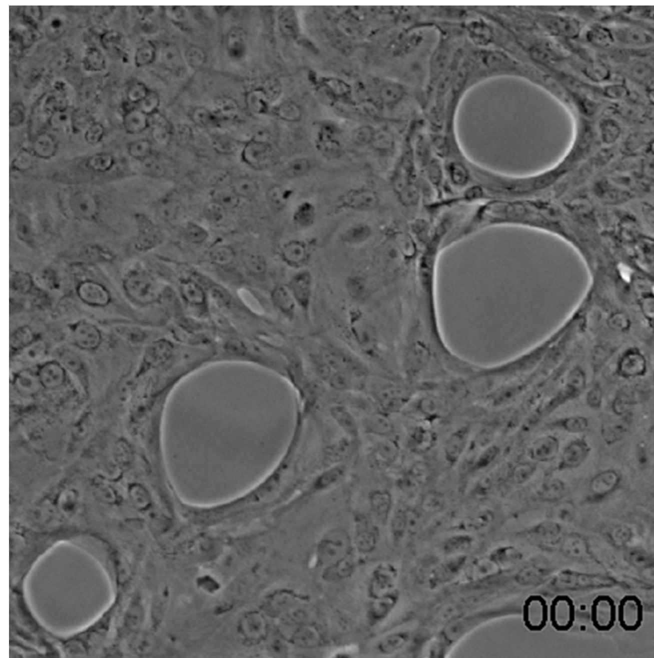
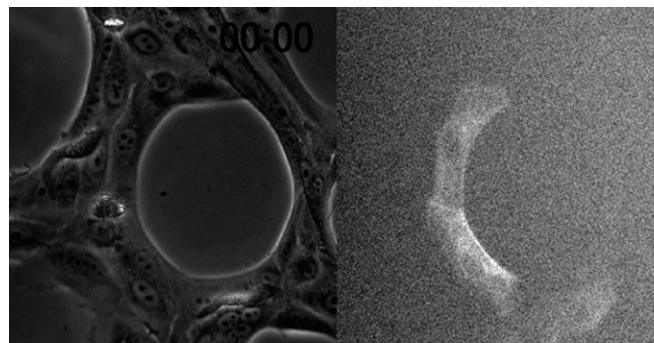


Fig. S9. Inhibition of c-fos transcription by the blocking antibody LA1. Twelve hours after removing the agarose to initiate movement, the cells were incubated with 10 μg/mL nonimmune IgG (*A*) or LA1 antibody (*B*) for 6 h before staining with antibodies to c-fos (upper images) and labeled phalloidin (lower images). After 6 h of incubation with LA1, the purse strings have largely disappeared. (Scale bar: 100 μm.)



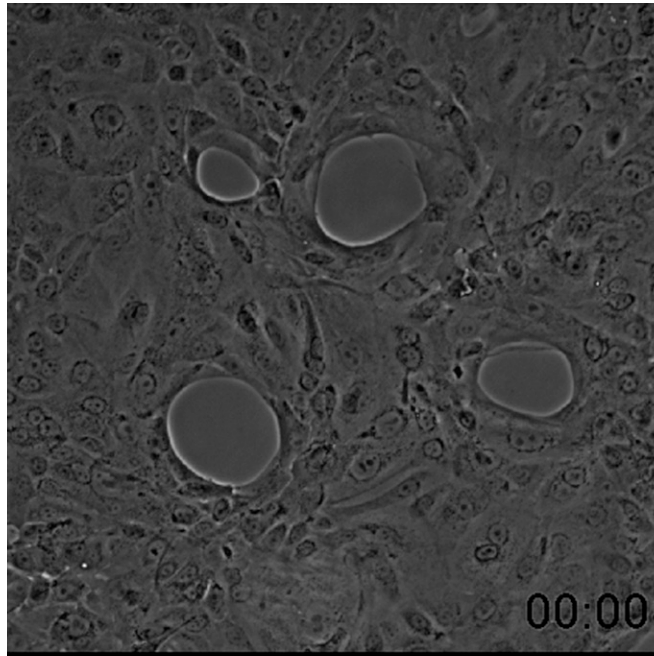
Movie S1. Healing after digestion of agarose droplets. The movies were acquired with a 10× phase-contrast objective at one frame per 10 min in this and the subsequent movies, except where indicated. The numbers indicate times after initiation of the movies (h:min).

Movie S1



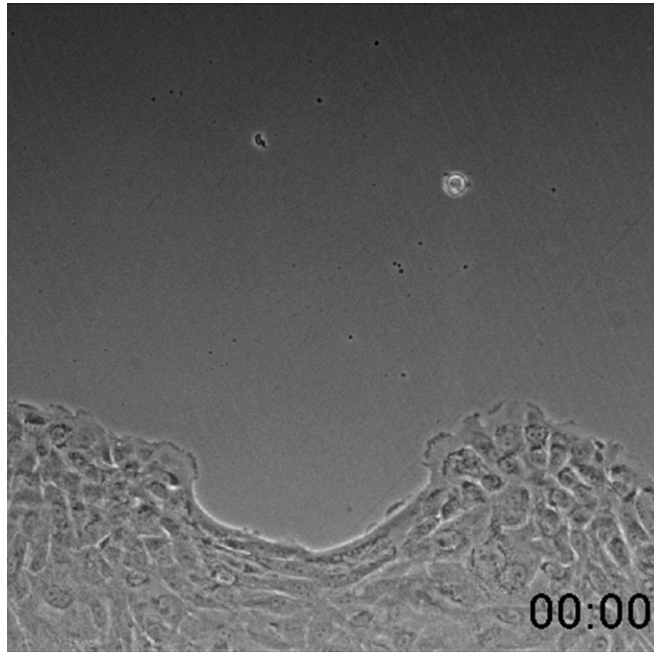
Movie S2. Localization of actin during healing. Cells were transfected with a vector coding for an enhanced green fluorescent protein/actin fusion protein. Micrographs in phase contrast (*Left*) or florescence (*Right*) (20×).

Movie S2



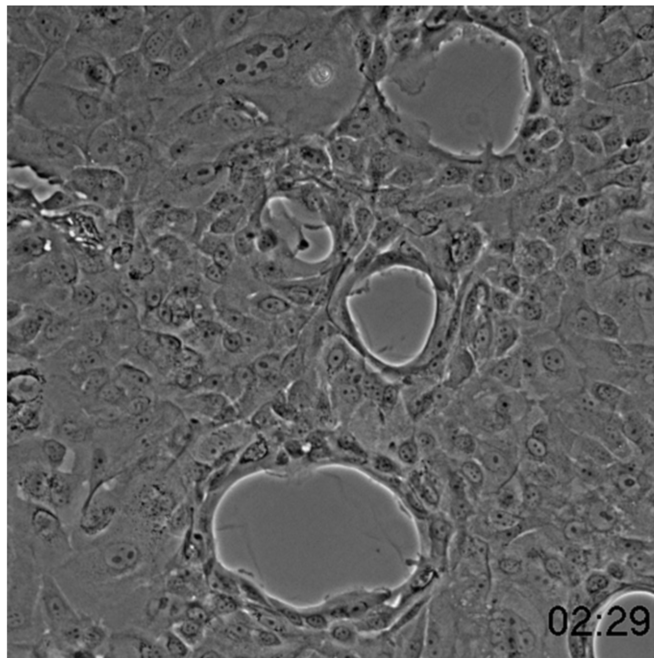
Movie S3. Healing after digestion of agarose droplets in the presence of 0.25 μM tyrphostin AG 1478. The drug was added at the start of digestion.

Movie S3



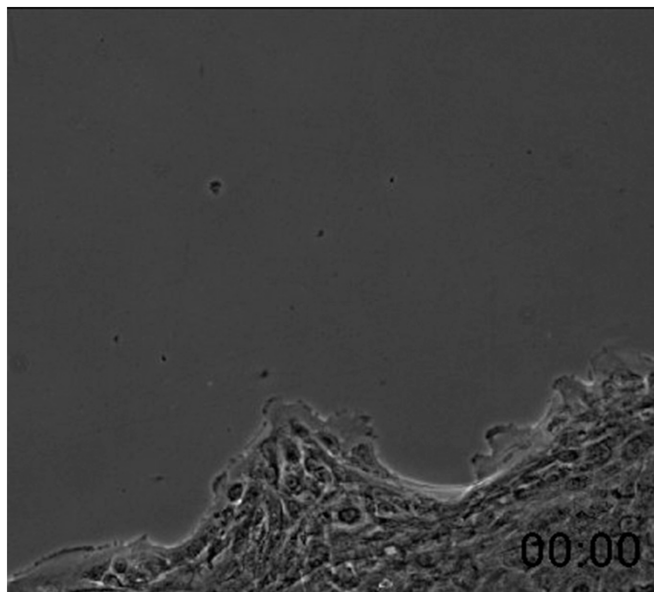
Movie S4. Effect of inhibition of the EGFR on the leading edge of moving HCLE cells. Cells were cultured around an agarose strip and acquisition of images started 16 h after removal of the strip. Tyrphostin AG 1478 was added 2.5 h after the start of the movie to a final concentration of 0.25 μM, and acquisition of images continued. Note that overall movement of the sheet is blocked but that the concave regions fill out.

Movie S4



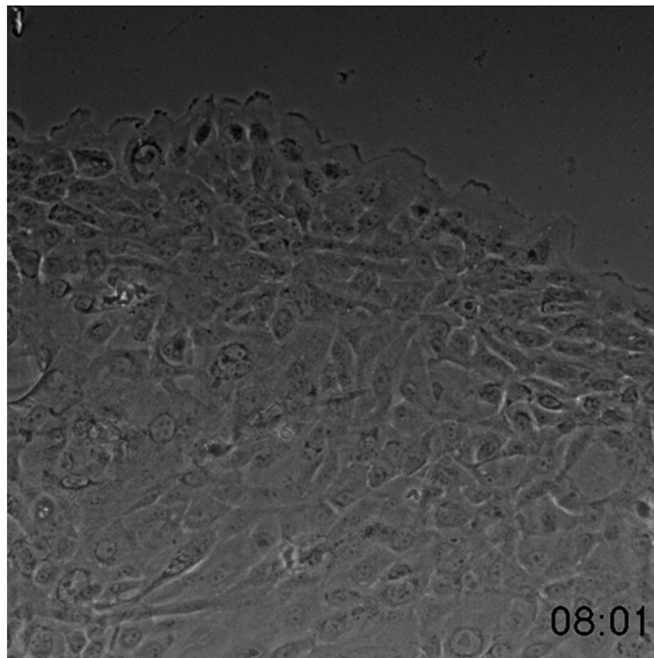
Movie S5. Effect of added EGF on healing induced by digestion of agarose droplets. EGF (10 nM) was added at the start of the digestion. Note the formation of numerous lamellipodia and general increased motility of the cells.

Movie S5



Movie S6. Dynamics of cells at a moving leading edge of HCLE cells. The movie was started 16 h after removal of the agarose strip.

Movie S6



Movie S7. Effect of EGF on the leading edge of moving HCLE cells. Acquisition of images started 16 h after removal of the strip, and 10 nM EGF was added 2.5 h later. Compare with Movie S6.

Movie S7