Francavilla et al., http://www.jcb.org/cgi/content/full/jcb.200903030/DC1

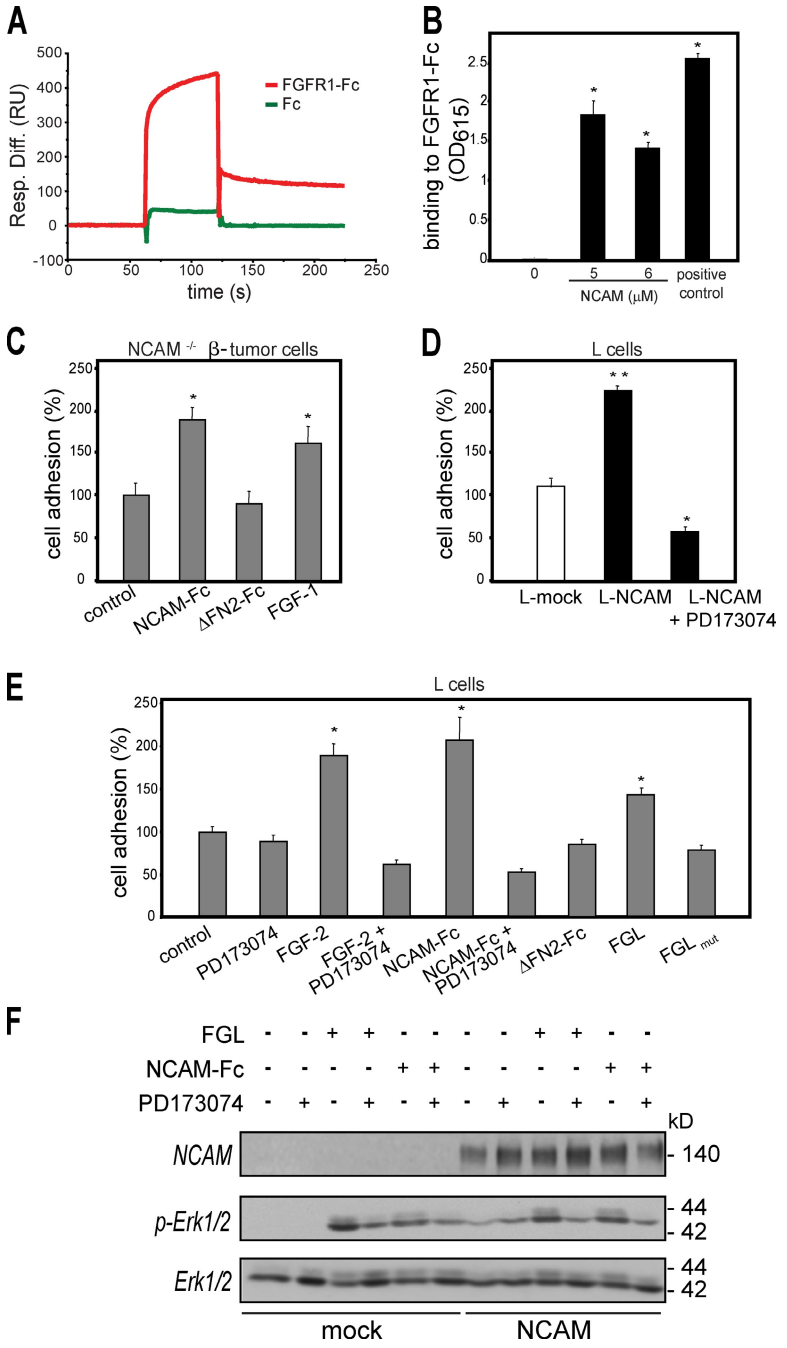


Figure S1. **NCAM binds to immobilized FGFR1 and promotes FGFR-mediated cell adhesion.** (A) The binding of NCAM to FGFR1-Fc was studied by using a Biacore 2000 instrument. FGFR1-Fc (10 µg/ml in 10 mM sodium acetate buffer, pH 4.3) was immobilized on a CM5 sensor chip via amine coupling kit (Biacore) according to the manufacturer's instructions. As a control, 10 µg/ml human Fc (EMD) in 5 mM maleate buffer, pH 6.0, were immobilized on a separate flow cell. Two blank surfaces were also prepared for reference curve subtraction. The binding experiments were performed at 25°C in HBS running buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, and 0.005% Tween 20). A 5 µM solution of HA-Myc-His–tagged NCAM ectodomain in PBS, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 3 mM NaN_3 was passed over the flow cells coupled to FGFR1-Fc or Fc with a flow rate of 30 µl/min. (B) Solid phase–binding assay. The Fc-tagged ectodomain of FGFR1-α (isoform IIIc; R&D Systems) was immobilized on 96-well plates (0.3 pmol/well) precoated with 0.67 pmol/well of goat anti–human Fc (Sigma-Aldrich). The human Fc fragment alone was also immobilized as a negative control. 0, 5, or 6 µM HA-Myc-His–tagged NCAM ectodomain in PBS was added in the presence of 1 mM $CaCl_2$ and 1 mM $MgCl_2$. The positive control consisted of a Myc-tagged scFv raised against

the ectodomain of FGFR1 (Francavilla et al., 2007) used at 30 nM. The binding of NCAM or anti-FGFR1 scFv to FGFR1 was revealed using anti-Myc monoclonal antibody (9E10) and europium-conjugated goat anti–mouse antibody (PerkinElmer). Europium was revealed using enhancement solution (DELFIA; PerkinElmer), and the fluorescence was read at 615 nm in a plate reader (Victor; PerkinElmer). Background signal was determined by omitting the anti-Myc antibody, whereas specific binding was measured by subtracting the values obtained in Fc-coated wells. Data are expressed as mean ± SD of two experiments performed in triplicate. *, $P < 0.05$. Both surface plasmon resonance and solid phase–binding assays confirmed the binding of NCAM ectodomain to immobilized FGFR1-Fc. (C) $NCAM^{-/-}$ β-tumor cells were stimulated for 10 min with NCAM-Fc, ΔFN2-Fc, or FGF-1. The latter was used because these cells are more responsive to FGF-1 than FGF-2 (Cavallaro et al., 2001). After the treatment, cells were subjected to adhesion assays on collagen IV–coated wells as described previously (Cavallaro et al., 2001). (D) L cells transfected with either an empty vector (L-mock) or with NCAM (L-NCAM; Francavilla et al., 2007) were subjected to adhesion assays on collagen IV either in the presence or in the absence of PD173074. (E) L cells were stimulated for 10 min with FGF-2, NCAM-Fc, ΔFN2-Fc, FGL, or FGL_{mut}. When needed, cells were preincubated with PD173074 or DMSO alone for 2 h before the stimulus. (C–E) Adherent cells were counted and are indicated as a percentage of the control (untreated cells ± SEM). Results refer to three experiments performed in triplicate. *, $P < 0.005$. (F) HeLa cells were transfected with an empty vector (mock) or NCAM. After serum starvation, cells were stimulated with FGL or NCAM-Fc for 10 min either in the absence or presence of PD173074 before cell lysis and immunoblotting for NCAM (top), phospho-Erk1/2 (middle), and total Erk1/2 (bottom).

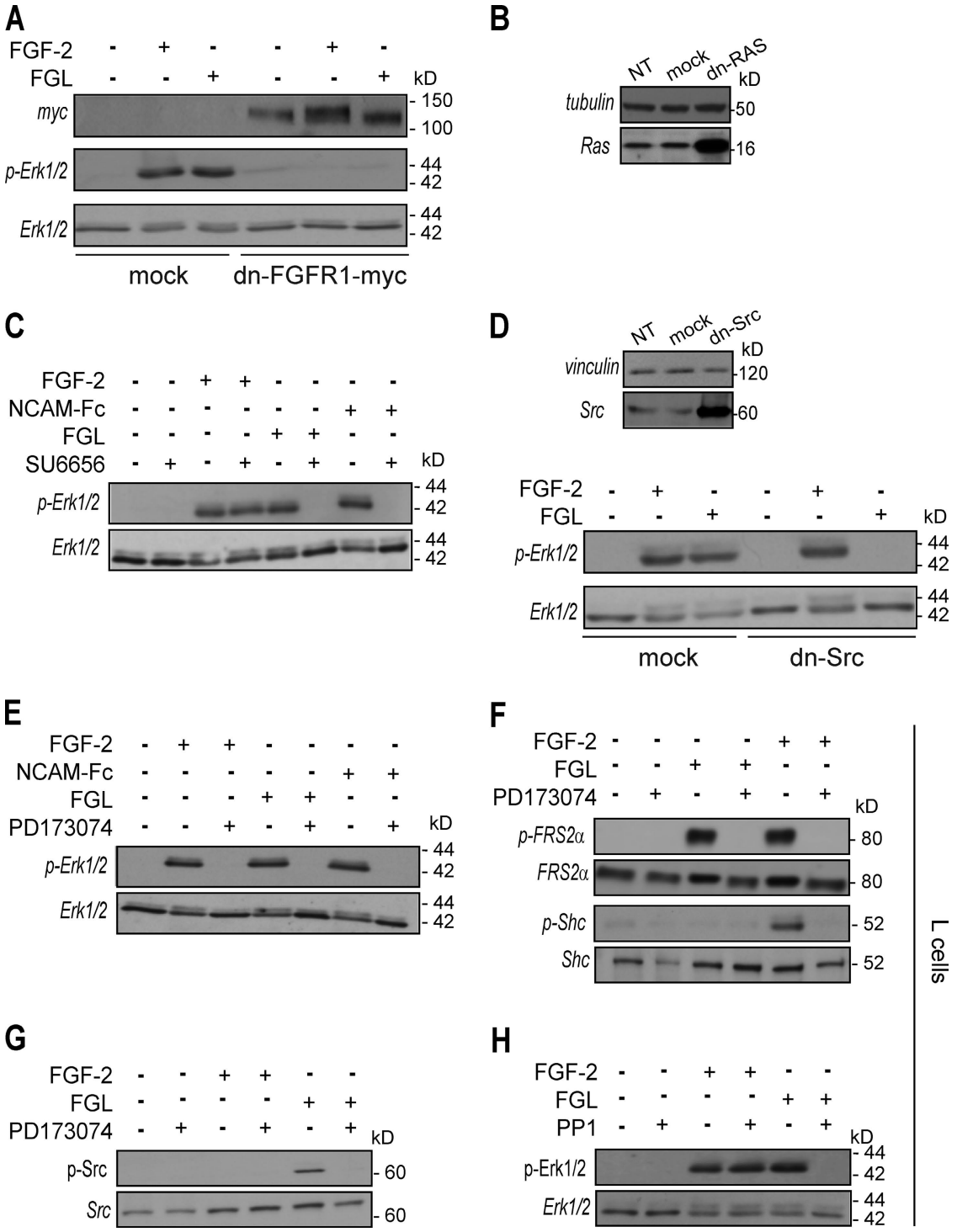


Figure S2. **NCAM and FGF activate distinct FGFR-mediated signaling pathways.** (A) HeLa cells were transfected with an empty vector (mock) or with Myc-tagged dn-FGFR1 (dn-FGFR1–Myc) and stimulated for 10 min with either FGF-2 or FGL. Cells were lysed and immunoblotted for Myc to confirm the ectopic expression of dn-FGFR1–Myc (top) and for phospho (middle)- or total Erk1/2 (bottom). (B) HeLa cells transfected with dn-Ras or with an empty vector (Fig. 1 B) were lysed and immunoblotted for Ras or tubulin. NT, nontransfected. (C) HeLa cells were stimulated for 10 min with FGF-2, FGL, or NCAM-Fc with or without a pretreatment with 20 μM SU6656. Cells were lysed and immunoblotted for phospho (top)- or total Erk1/2 (bottom). (D) HeLa cells were left untransfected (NT) or transfected either with an empty vector (mock) or dn-Src. Cell extracts were probed for Src to confirm the ectopic expression of dn-Src and vinculin to verify equal loading (top). Cells were stimulated for 10 min with either FGF-2 or FGL followed by immunoblotting for phospho- or total Erk1/2 (bottom). (E–H) L cells were stimulated for 10 min with FGF-2, FGL, or NCAM-Fc with or without a pretreatment with PD173074 for 2 h (E–G) or with PP1 for 30 min (H). Cells were lysed and immunoblotted for phospho–FRS-2α, phospho-Shc, phospho-Src, and phospho-Erk1/2. The same lysates were immunoblotted for total FRS-2α, Shc, Src, and Erk1/2 as indicated.

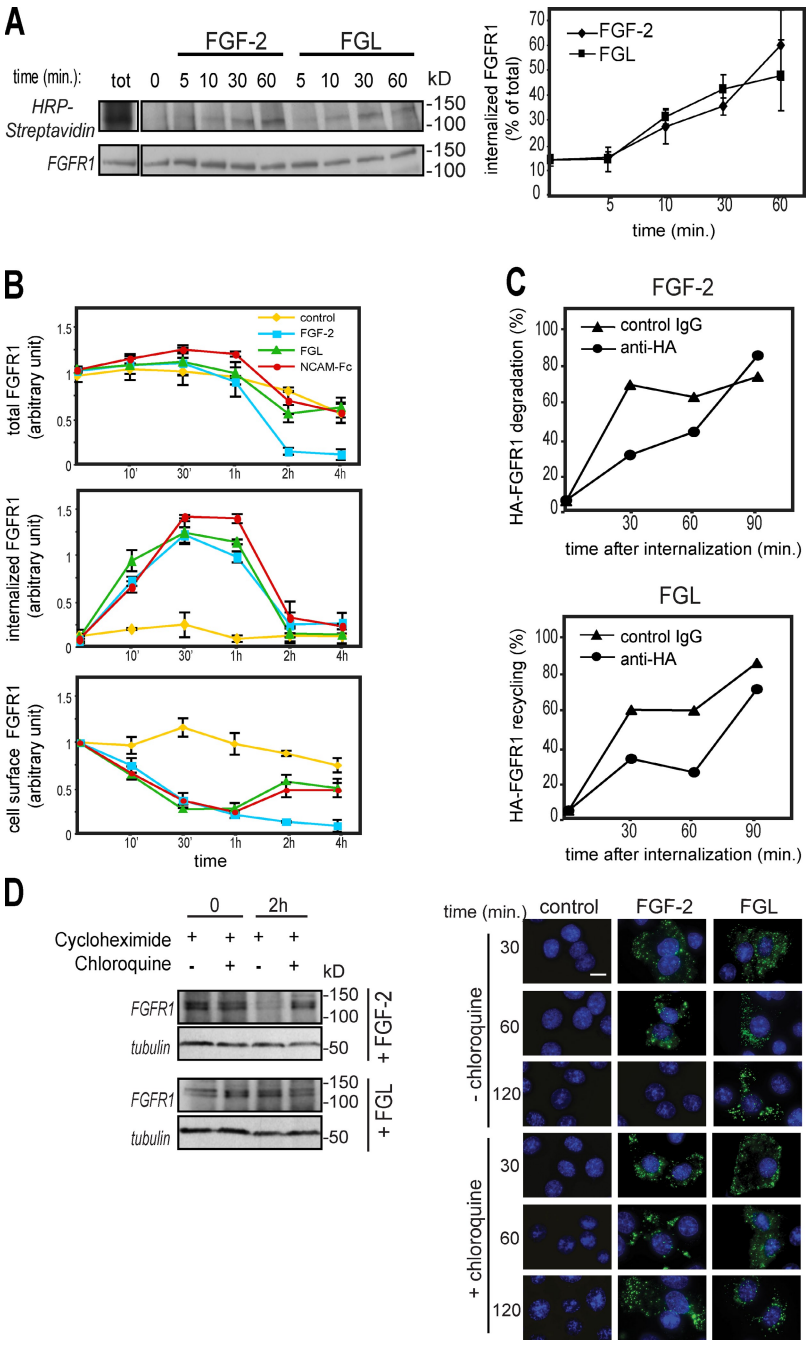


Figure S3. **Internalization, degradation, and recycling of FGFR1.** (A) The internalization of FGFR1 in cells stimulated with FGF-2 or FGL was measured after cell surface biotinylation, as described in Materials and methods. The results of a representative experiment (left) and the mean ± SD from three independent experiments, where the rate of FGFR1 internalization is expressed as a percentage of total FGFR1 (right), are shown. (B) The presence (top), internalization (middle), and recycling (bottom) of HA-FGFR1 in HeLa cells stimulated for the indicated time lengths were quantified as described in Materials and methods. Values represent the means ± SD from three independent experiments. (C) HA-FGFR1–transfected HeLa cells were subjected to surface biotinylation and preincubated with the anti-HA antibody or with a control, isotype-matched antibody before stimulation with FGF-2 (top) or FGL (bottom). FGFR1 degradation (top) and recycling (bottom) were determined as described in Materials and methods and Fig. 3 C. (D, left) HeLa cells were treated with cycloheximide for 2 h in the presence or absence of 0.5 mM chloroquine and stimulated with FGF-2 or FGL for 2 h. Cell lysates were immunoblotted for FGFR1 and tubulin. FGF-2–induced FGFR1 degradation was blocked by chloroquine, whereas no degradation and no effect by chloroquine were observed in FGL-stimulated cells. (right) HA-FGFR1–transfected HeLa cells were treated with anti-HA antibody as described in Fig. 3 A and stimulated with FGF-2 or FGL in the presence or absence of 0.5 mM chloroquine for 30, 60, or 120 min. Cells were acid washed, fixed, permeabilized, and stained with Alexa Fluor 488–conjugated secondary antibody to visualize the internalized HA-FGFR1 (green). After 120 min of FGF-2 stimulation, HA-FGFR1 was no longer detectable in cells not pretreated with chloroquine, whereas it accumulated in chloroquine-treated cells, supporting the notion of lysosomal degradation of FGFR1 upon FGF-2 stimulation. Bar, 10 µm.

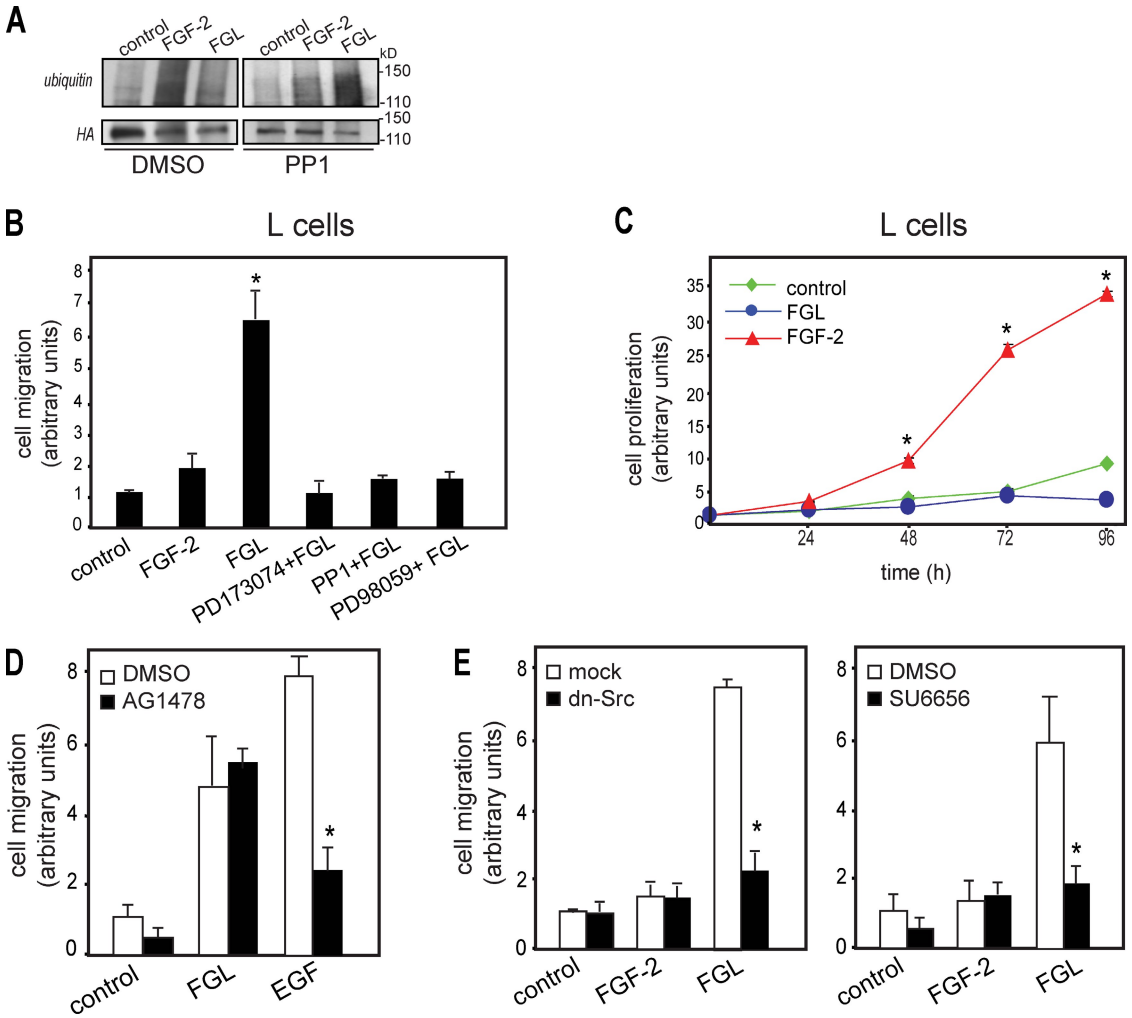


Figure S4. **FGF promotes cell proliferation, whereas NCAM induces cell migration via FGFR1, Src, and Erk1/2.** (A) HeLa cells transfected with HA-FGFR1 were pretreated with either DMSO or PP1 before stimulation with FGF-2 or FGL. Cell extracts were immunoprecipitated with anti-HA antibody and immunoblotted for ubiquitin (top) or HA (bottom). Src inhibition resulted in FGL inducing ubiquitination of FGFR1 to a comparable level as FGF-2. (B) L cells were stimulated with FGF-2 or FGL alone or in the presence of PD173074, PP1, or PD98059 and subjected to migration assays in modified Boyden chambers as described in Materials and methods. (C) L cells were treated with FGF-2 or FGL for 0–96 h and subjected to cell proliferation assay as described in Materials and methods. (B and C) *, $P < 0.005$ relative to untreated cells. (D) HeLa cells were pretreated with DMSO or AG1478 before migration assays. *, $P < 0.005$ relative to DMSO-treated cells stimulated with EGF. (E, left) HeLa cells were transiently transfected with an empty vector (mock) or dn-Src. (right) Alternatively, cells were pretreated with DMSO or SU6656. Cells were stimulated with FGF-2 or FGL and subjected to migration assay in modified Boyden chambers for 16 h as described in Materials and methods. *, $P < 0.005$ relative to mock-transfected or DMSO-treated cells stimulated with FGL. Src activity was required for FGL-induced cell migration. Data represent the mean ± SEM from three independent experiments.

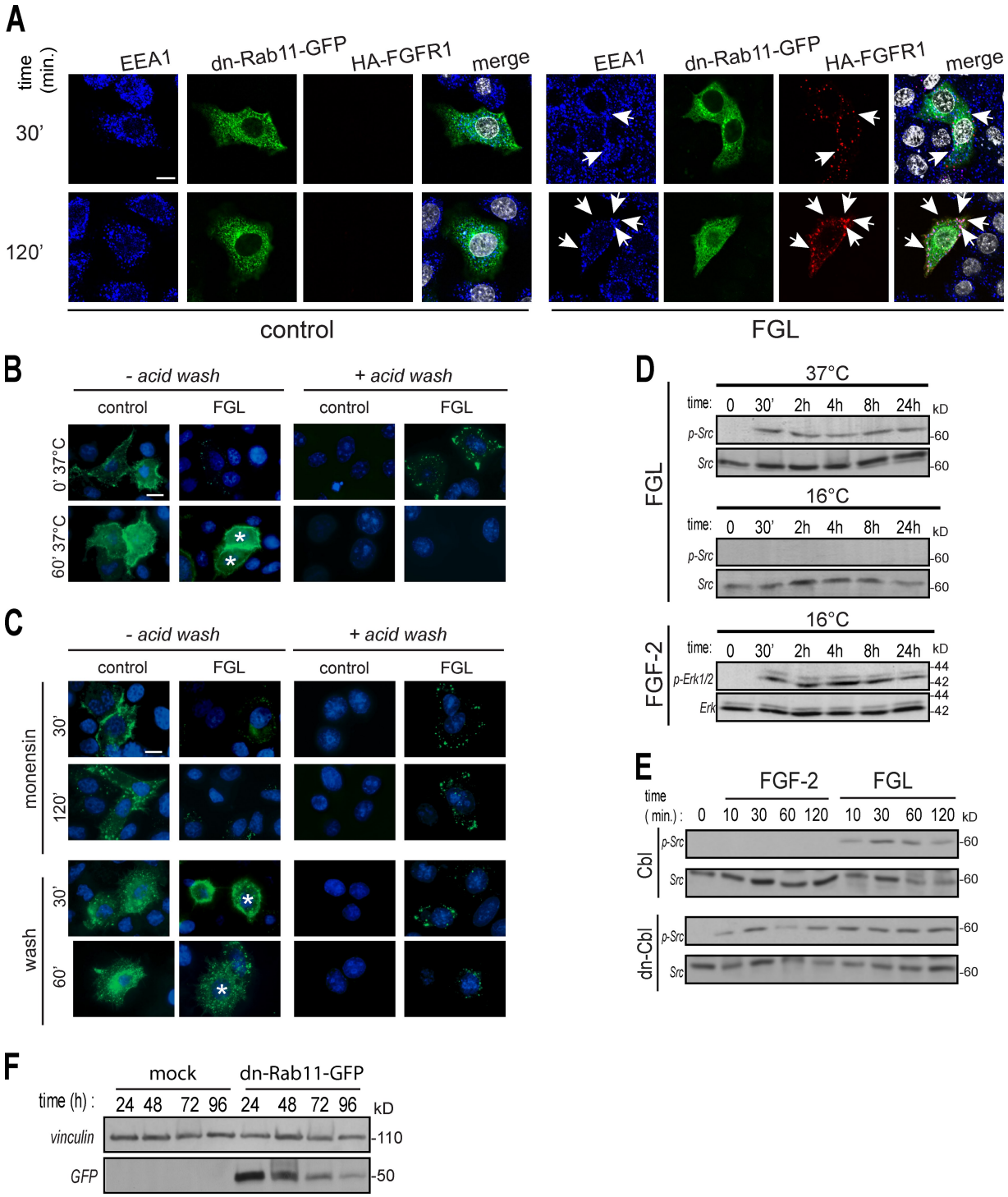
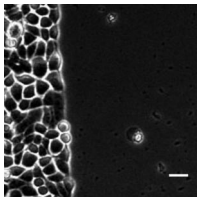
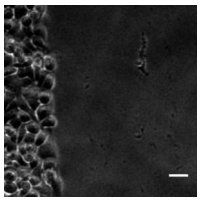


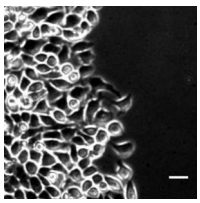
Figure S5. **FGFR1 recycling is required for NCAM-induced cell migration.** (A) HeLa cells were cotransfected with HA-FGFR1 (red) and dn-Rab11–GFP (green). Cells were stimulated with FGL for 30 or 120 min followed by acidic wash, permeabilization, fixation, and staining for EEA1 (blue). In dn-Rab11–expressing cells, HA-FGFR1 is retained in EEA1-positive early endosomes even at late time points, as revealed by the merge of red and blue staining (arrows). (B) HA-FGFR1–transfected HeLa cells were incubated with anti-HA as described, stimulated with FGL, and incubated at 16°C for 4 h. Half of the samples were either fixed immediately (−acid wash) or acid washed, fixed, and permeabilized (+acid wash), before staining with Alexa Fluor 488–conjugated secondary antibody. The other half was incubated at 37°C for 1 h to allow FGFR1 recycling and either fixed or acid washed, fixed, and permeabilized. FGFR1 recycling was observed only upon reincubation of cells at 37°C (asterisks). (C) HA-FGFR1–transfected HeLa cells were treated with anti-HA as described and stimulated with FGL for 30 or 120 min in the presence of 100 µM monensin. The samples were either fixed (−acid wash) or acid washed, fixed, and permeabilized (+acid wash) before staining with Alexa Fluor 488–conjugated secondary antibody (monensin). Part of the samples treated for 120 min with FGL was extensively washed with medium and put back at 37°C for an additional 30 or 60 min to allow FGFR1 recycling (wash). Cells were either fixed or acid washed, fixed, and permeabilized before staining with Alexa Fluor 488–conjugated secondary antibody. FGFR1 recycling was observed only upon removal of monensin (asterisks). Thus, both temperature shift and monensin treatment blocked HA-FGFR1 recycling. (D) HeLa cells were incubated either at 37 or at 16°C and stimulated with FGL or FGF-2 for the indicated time intervals. (top and middle) Cell lysates were immunoblotted for phospho- or total Src. (bottom) Cells incubated at 16°C were also stimulated with FGF-2 for the indicated time intervals followed by cell lysis and immunob-



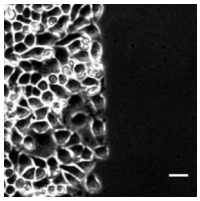
Video 1. **Migration of control HeLa cells.** HeLa cells were subjected to monolayer-wounding assays. Phase-contrast time-lapse video microscopy was performed at the wound edge. Frames were taken every 15 min for a total of 24 h. The display rate is 12 frames per second. Bar, 30 μm.



Video 2. **Migration of FGF-2–stimulated HeLa cells.** HeLa cells were subjected to monolayer-wounding assays and stimulated with FGF-2. Phase-contrast time-lapse video microscopy was performed at the wound edge. Frames were taken every 15 min for a total of 24 h. The display rate is 12 frames per second. Bar, 30 μm.



Video 3. **Migration of FGL-stimulated HeLa cells.** HeLa cells were subjected to monolayer-wounding assays and stimulated with FGL. Phase-contrast time-lapse video microscopy was performed at the wound edge. Frames were taken every 15 min for a total of 24 h. The display rate is 12 frames per second. Bar, 30 μm.



Video 4. **Migration of NCAM-Fc–stimulated HeLa cells.** HeLa cells were subjected to monolayer-wounding assays and stimulated with NCAM-Fc. Phase-contrast time-lapse video microscopy was performed at the wound edge. Frames were taken every 15 min for a total of 24 h. The display rate is 12 frames per second. Bar, 30 μm.

lotting for phospho- or total Erk1/2. NCAM-induced sustained activation of Src was lost at 16°C. In contrast, FGF-2 retained its ability to induce Erk1/2 activation, confirming that the temperature shift had no effect on FGFR intrinsic activity. (E) HeLa cells stably transfected with either Cbl or dn-Cbl were stimulated with FGF-2 or FGL for the indicated time intervals. Cell lysates were immunoblotted for phospho- or total Src. In dn-Cbl–expressing cells, FGF-2 induced the sustained activation of Src. (F) HeLa cells were transfected with an empty vector or GFP-tagged dn-Rab11, and the expression of the transgene at the indicated time points was monitored by immunoblotting of cell lysates for GFP using vinculin as a loading control. The expression of dn-Rab11 was maintained throughout the 96-h period. Bars, 10 μm.

References

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