SUPPLEMENTAL MATERIAL

Xu et al., http://www.jem.org/cgi/content/full/jem.20100433/DC1

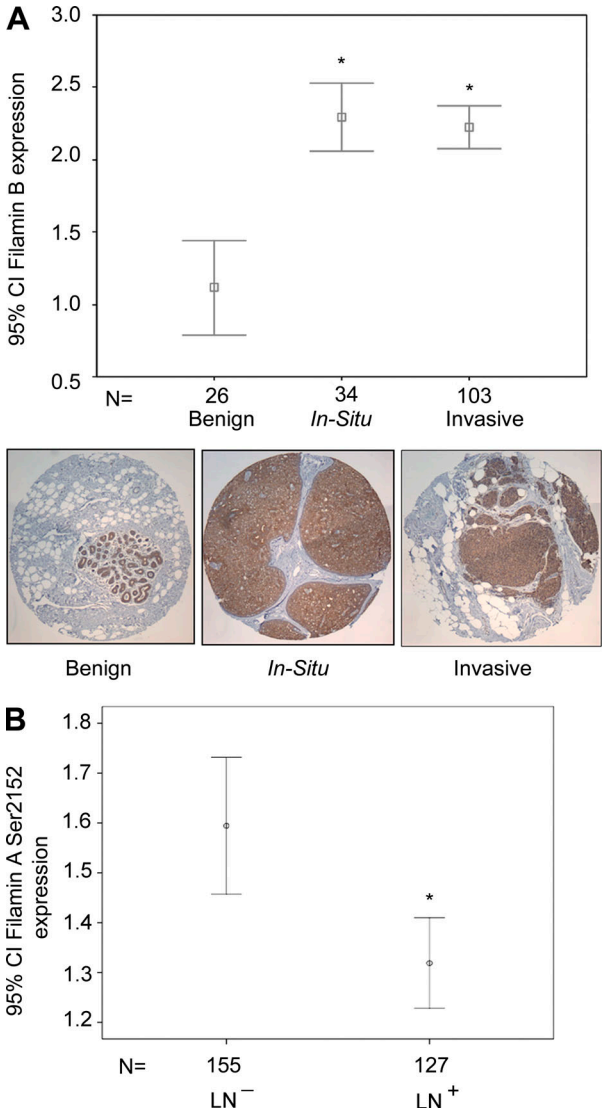


Figure S1. Expression of FLNb and phospho-FLNa in breast cancer patients with progressive disease. (A) FLNb expression levels were examined by blinded pathology analysis of a TMA evaluated using a categorical scoring method ranging from 0 to 3 (0, negative; 1, weak; 2, moderate; and 3, high). TMA cores were assigned a diagnosis (benign, in situ carcinoma, and invasive breast cancer). Histogram represents normalized mean of epithelial expression of FLNb levels (confidence intervals [CI] of 95% ± SEM) as determined by quantitative evaluation of immunohistochemical staining (*, $P < 0.005$). Representative immunohistochemistry images of tissue cores are shown. (B) An independent TMA was assembled from a total of 258 lymph node (LN)–positive and –negative breast cancer patients' samples. Histogram shown represents normalized mean of epithelial expression of phospho-FLNa-Ser2152 (confidence intervals of 95% ± SEM) as determined by quantitative evaluation of immunostaining. A significant decrease of phospho-FLNa-Ser2152 expression was observed in lymph node–positive ($n = 127$) versus lymph node–negative breast cancer ($n = 155$; mean epithelial expression of phospho-FLNa was 1.60 ± 0.13 and 1.32 ± 0.09 for lymph node–negative and –positive breast cancer, respectively; *, $P = 0.004$).

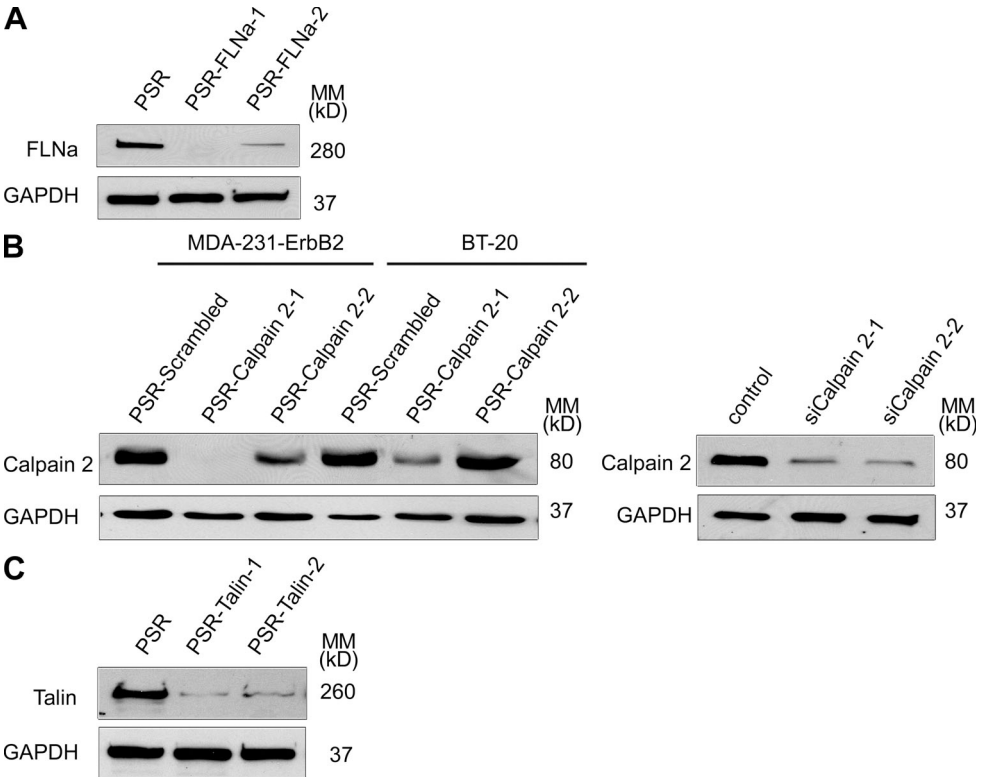


Figure S2. Western blot analysis shows efficient knockdown of FLNa, talin, and calpain 2 by targeting different sequences. (A) Western blots showing FLNa expression in BT-20 cell stably engineered to express FLNa-shRNA using PSR puro vector (PSR-FLNa). Control cells expressed PSR puro alone (PSR). (B, left) Western blots showing calpain 2 expression in MDA-231-ErbB2 and BT-20 cells stably engineered to express calpain 2 shRNA (PSR-Calpain 2) compared with control PSR cells. (right) Western blots showing transient knockdown of calpain 2 in BT-20 cells by transfection of two different siRNA oligonucleotides (targeting same sequences as shown in left). (C) Representative Western blots showing talin expression in BT-20 cells stably engineered to express talin shRNA (PSR-Talin) compared with control PSR cells. (A–C) GAPDH was used as an internal control. MM, molecular mass.

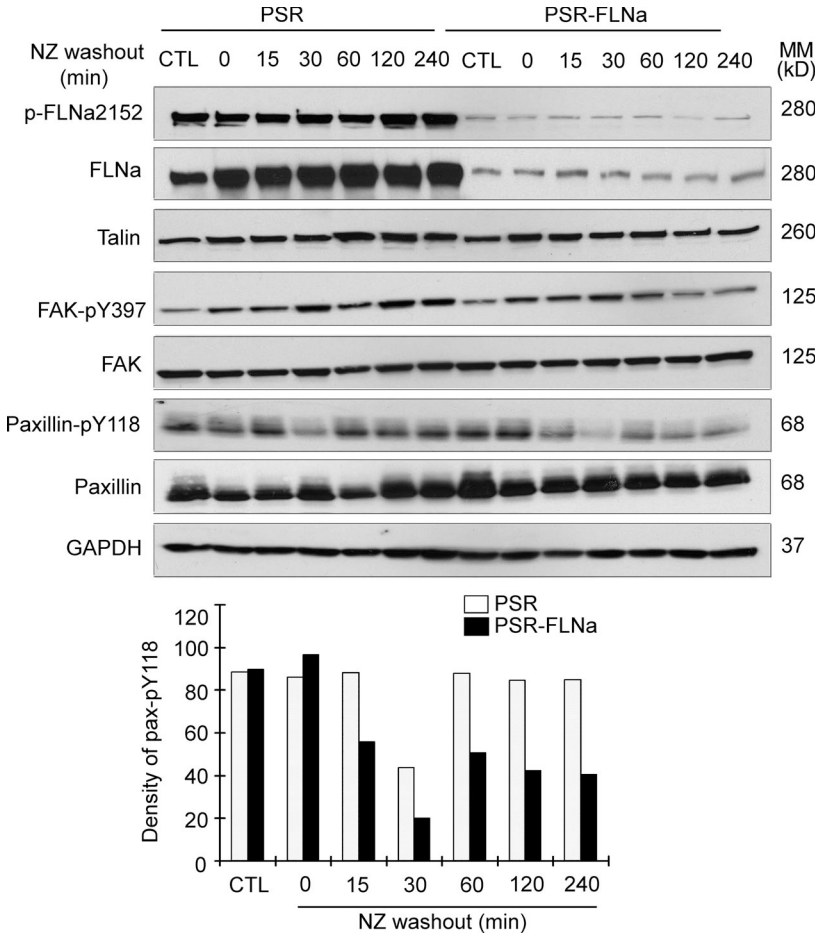


Figure S3. FLNa regulates FA disassembly. Serum-starved MDA-231-ErbB2 cells were incubated for 4 h with 10 µM nocodazole (NZ) treatment, and then the drug was washed out. At the indicated time after nocodazole washout, MDA-231-ErbB2 cell lysates were prepared and separated by SDS-PAGE and Western blotted with anti–FAK-pY397 and anti–paxillin-pY118 antibodies. Note that FLNa induced more reducing of these FA markers compared with control cells (start from 15 min). Antibodies that recognize total FAK, paxillin, and GAPDH were used as internal controls for loading. Bar graph represents the densitometry result as the percentage of density of PSR control at 0 min (which is 100%) from four independent Western blots. MM, molecular mass.

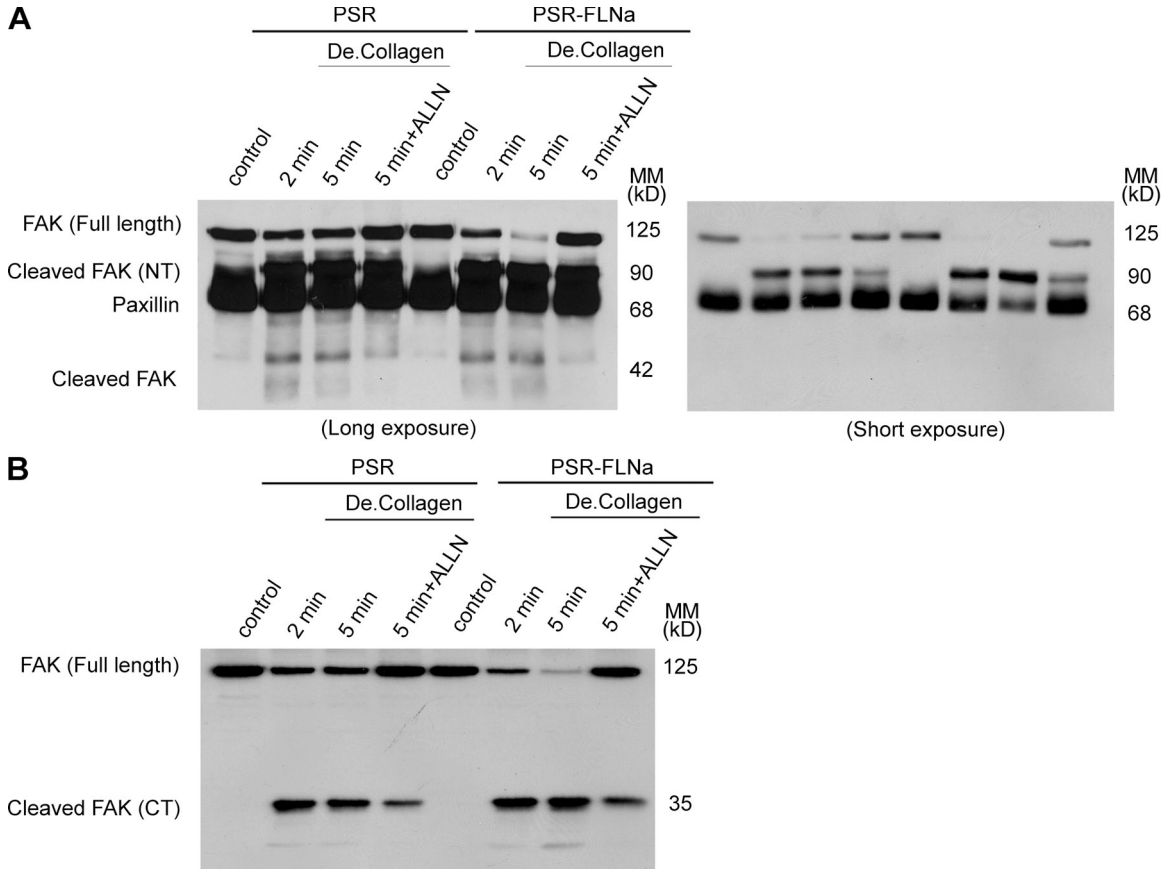


Figure S4. Cleavage of FAK in response to treatment with degraded type I collagen. (A) Total cell lysates were prepared from cell lysates and prepared and separated by SDS-PAGE and immunoblotted with antibodies recognizing the N terminus (NT) of FAK. The location and approximate molecular masses (MM) of native (125 kD) FAK, putative cleavage fragments of 90 kD, and 42-kD fragment (which was generated by further cleavage of the 90-kD fragment) are indicated. The membrane was immunoblotted with paxillin antibody first and followed with FAK antibody blotting (without stripping), so 68 kD of the paxillin band is also indicated. (B) A polyclonal antibody to the C terminus (CT) was used. Note the presence of both native FAK and the cleaved 35-kD fragment. De.Collagen, degraded type I collagen.

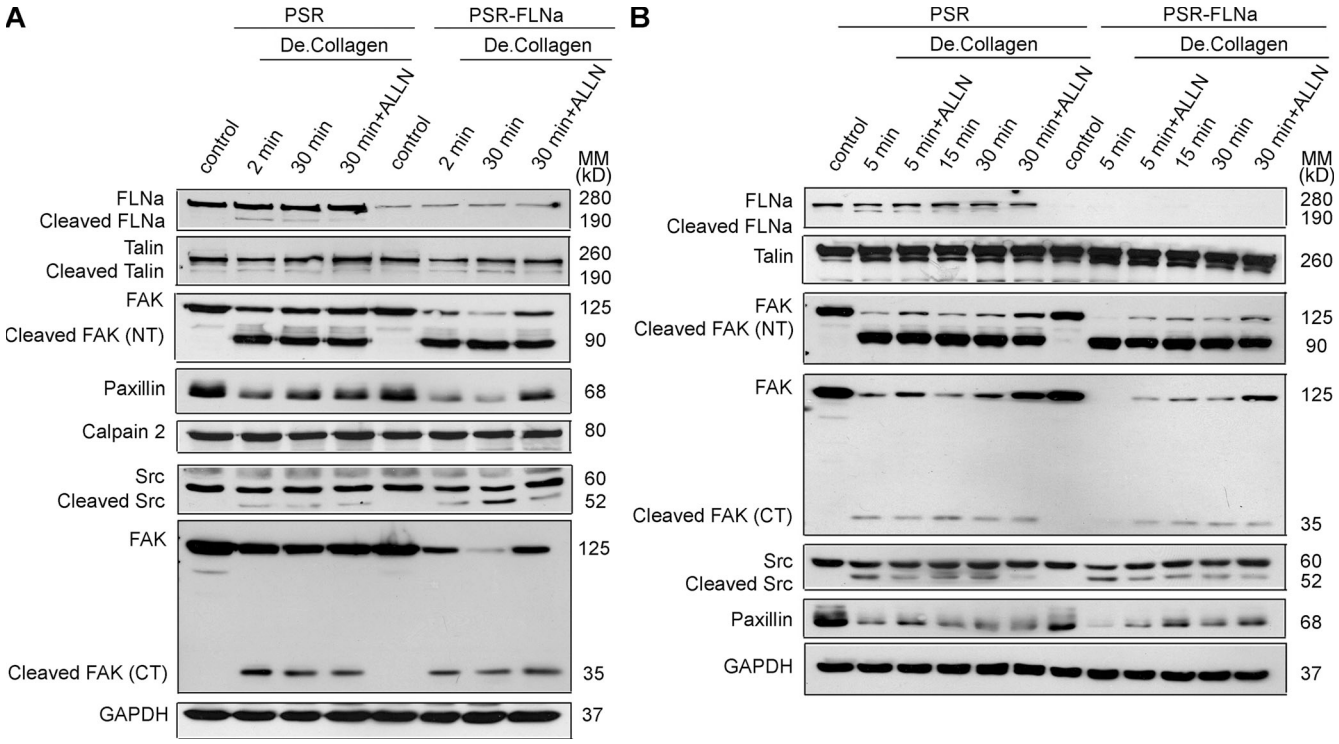


Figure S5. Effect of FLNa inhibition on calpain activity. (A) Control (PSR) or FLNa-silenced (PSR-FLNa) MDA-231-ErbB2 cells were cultured on 10-cm dishes and stimulated or not with degraded type I collagen (De.Collagen) for the indicated times. Where indicated, cells were treated with 100 µM of calpain inhibitor ALLN (4 h). Lysates were prepared at the indicated time points and analyzed by Western blotting using the corresponding antibodies. Control indicates unstimulated. (B) Control (PSR) or FLNa-silenced (PSR-FLNa) BT-20 cells were cultured on 10-cm dishes and stimulated or not with degraded type I collagen for the indicated times. Where indicated, cells were treated with 100 µM of calpain inhibitor ALLN (4 h). Lysates were prepared at the indicated time points and analyzed by Western blotting using the corresponding antibodies. Control indicates unstimulated. CT, C terminus; MM, molecular mass; NT, N terminus.

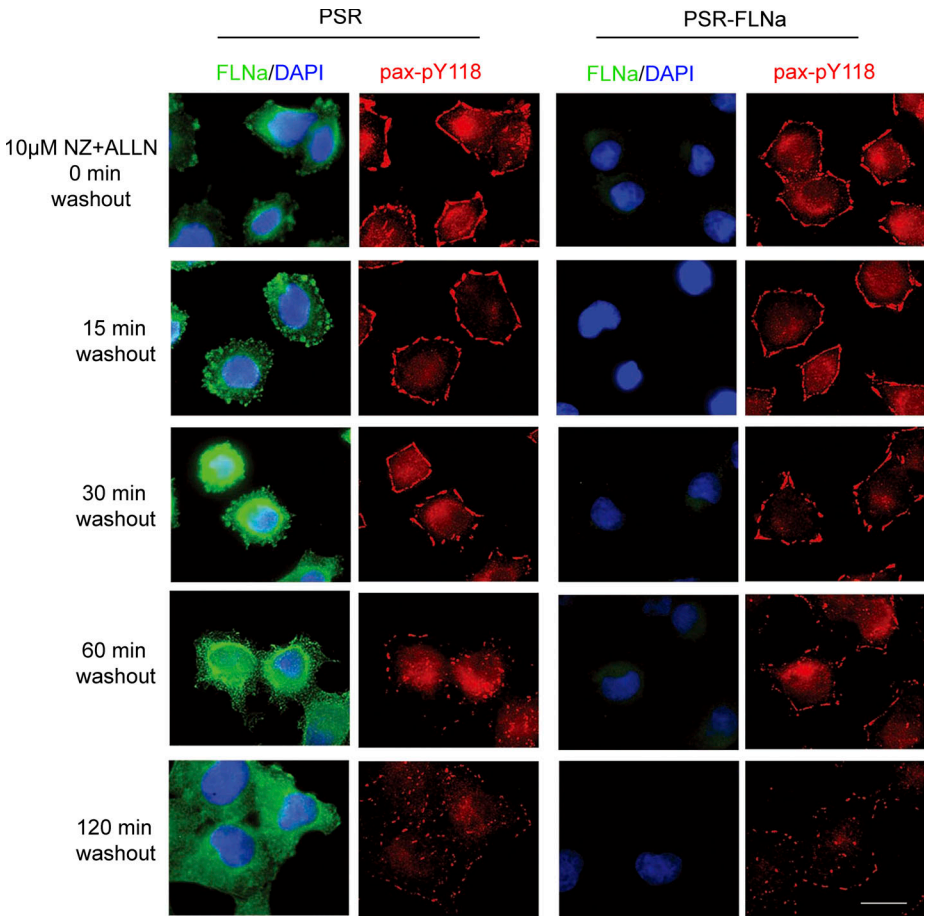


Figure S6. Calpain inhibitor ALLN abolishes the effect of FLNa in regulation of FA disassembly. Serum-starved BT-20 cells were incubated for 4 h with 10 μM nocodazole (NZ) plus 100 μM ALLN. Drugs were then removed, and cells were cultured in normal conditions. At the indicated time after drug removal, cells were fixed and immunostained with anti–paxillin-pY118 and anti-FLNa antibodies. Bar, 20 μm.

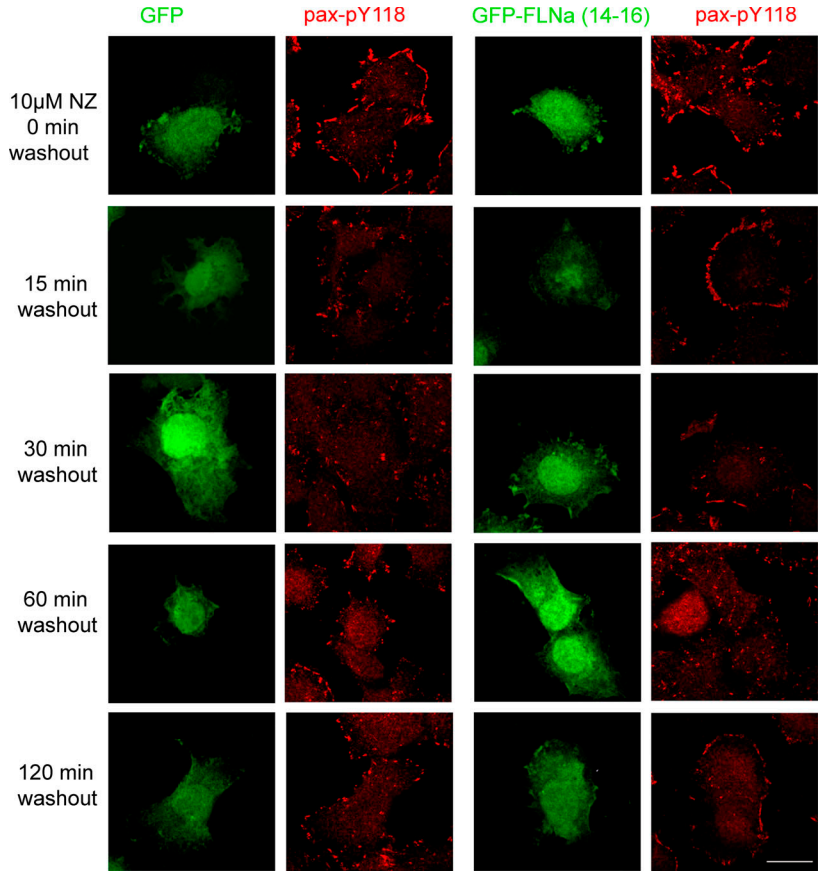


Figure S7. Expression of FLNa 14–16 repeats partially rescues the effect of FLNa knockdown in regulation of FA disassembly. Serum-starved FLNa-silenced BT-20 cells were incubated for 4 h with 10 μM nocodazole (NZ). Drugs were then removed, and cells were cultured in normal conditions. At the indicated time after drug removal, cells were fixed and immunostained with anti–paxillin-pY118 antibody. Bar, 20 μm.

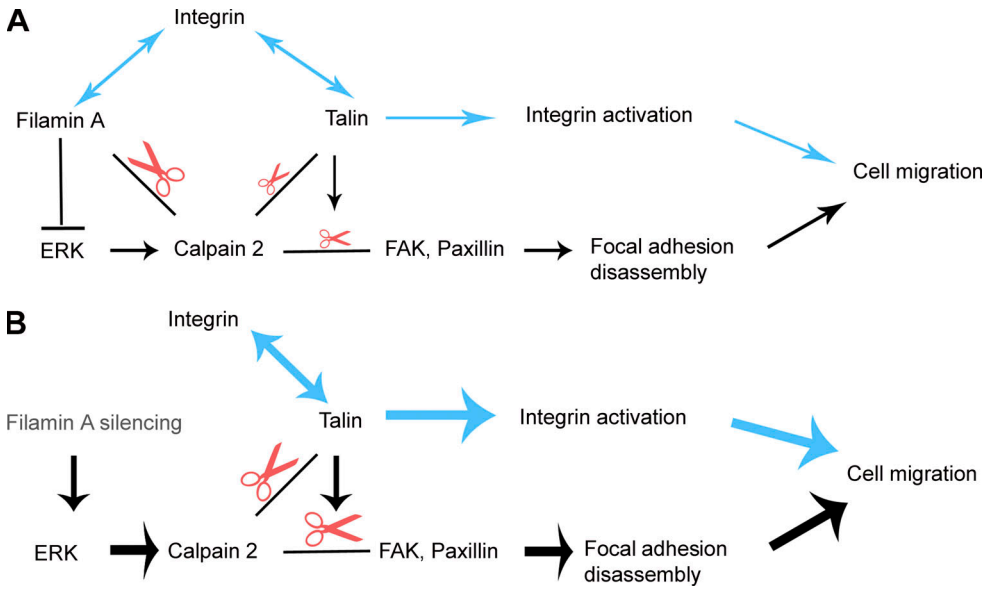
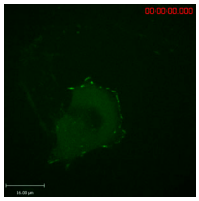
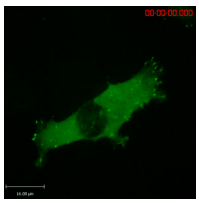


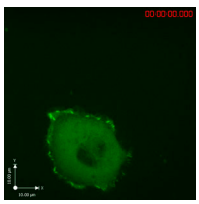
Figure S8. Schematic representation of FLNa regulation of cell migration. (A) FLNa and talin competitively bind with integrin. FLNa-integrin binding stabilizes membrane and inhibits cell migration, whereas talin-integrin binding promotes integrin activation, which eventually activates cell adhesion, cell migration, and extracellular matrix assembly. FLNa and talin may also compete for calpain 2. Cleavage of FLNa regulates cell spreading, whereas cleavage of talin by calpain 2 is a rate-limit step for FA turnover. (B) Knocking down of FLNa results in increasing of integrin activation with increased talin binding caused by the release of an FLNa-mediated inhibition. Moreover, FLNa silencing may possibly increase ERK activity, which leads to activation of calpain 2. Upon significant reduction of FLNa level, calpain 2 cleavage of other substrates such as talin, FAK, and paxillin is increased, possibly enhancing FA turnover and cell migration.



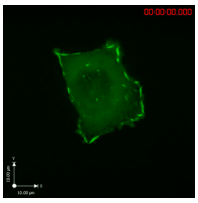
Video 1. FA dynamics of BT-20 control cells. BT-20 control cells (PSR) transfected with EYFP-paxillin (green) were plated on chambered coverglass and stimulated with EGF. Images were analyzed by time-lapse confocal microscopy using a spinning disk confocal microscope. Video sequences of the cell were monitored at 4-min intervals for >1.5 h. Note that at the extremely high speed, we investigated cells of Videos 1 and 2 in 15-s intervals. This video is shown at 3 frames/s.



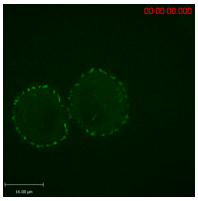
Video 2. FA dynamics of BT-20 FLNa-silenced cells. BT-20 FLNa-silenced cells (PSR-FLNa) transfected with EYFP-paxillin (green) were plated on chambered coverglass and stimulated with EGF. Images were analyzed by time-lapse confocal microscopy using a spinning disk confocal microscope. Video sequences of the cell were monitored at 4-min intervals for >1.5 h. Note that at the extremely high speed, we investigated cells of Videos 1 and 2 in 15-s intervals. This video is shown at 3 frames/s.



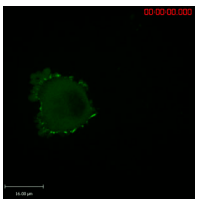
Video 3. FA dynamics of BT-20 calpain 2–silenced cells. BT-20 control cells (PSR) stably expressing EYFP-paxillin (green) transfected with calpain 2 siRNA were plated on chambered coverglass and stimulated with EGF. Images were analyzed by time-lapse confocal microscopy using a spinning disk confocal microscope. Video sequences of the cell were monitored at 4-min intervals for >1.5 h. Note that at the extremely high speed, we investigated cells of Videos 3 and 4 in 15-s intervals. This video is shown at 3 frames/s.



Video 4. FA dynamics of BT-20 cells with FLNa and calpain double knockdown. BT-20 FLNa-silenced cells (PSR-FLNa) stably expressing EYFP-paxillin (green) transfected with calpain 2 siRNA were plated on chambered coverglass and stimulated with EGF. Images were analyzed by time-lapse confocal microscopy using a spinning disk confocal microscope. Video sequences of the cell were monitored at 4-min intervals for >1.5 h. Note that at the extremely high speed, we investigated cells of Videos 3 and 4 in 15-s intervals. This video is shown at 3 frames/s.



Video 5. FA dynamics of ALLN-treated BT-20 control cells. BT-20 control cells (PSR) stably expressing EYFP-paxillin (green) treated with the calpain inhibitor ALLN were plated on chambered coverglass and stimulated with EGF. Images were analyzed by time-lapse confocal microscopy using a spinning disk confocal microscope. Video sequences of the cell were monitored at 4-min intervals for >1.5 h. Note that at the extremely high speed, we investigated cells of Videos 5 and 6 in 15-s intervals. This video is shown at 3 frames/s.



Video 6. FA dynamics of ALLN-treated BT-20 FLNa-silenced cells. BT-20 FLNa-silenced cells (PSR-FLNa) stably expressing EYFP-paxillin (green) treated with the calpain inhibitor ALLN were plated on chambered coverglass and stimulated with EGF. Images were analyzed by time-lapse confocal microscopy using a spinning disk confocal microscope. Video sequences of the cell were monitored at 4-min intervals for >1.5 h. Note that at the extremely high speed, we investigated cells of Videos 5 and 6 in 15-s intervals. This video is shown at 3 frames/s.