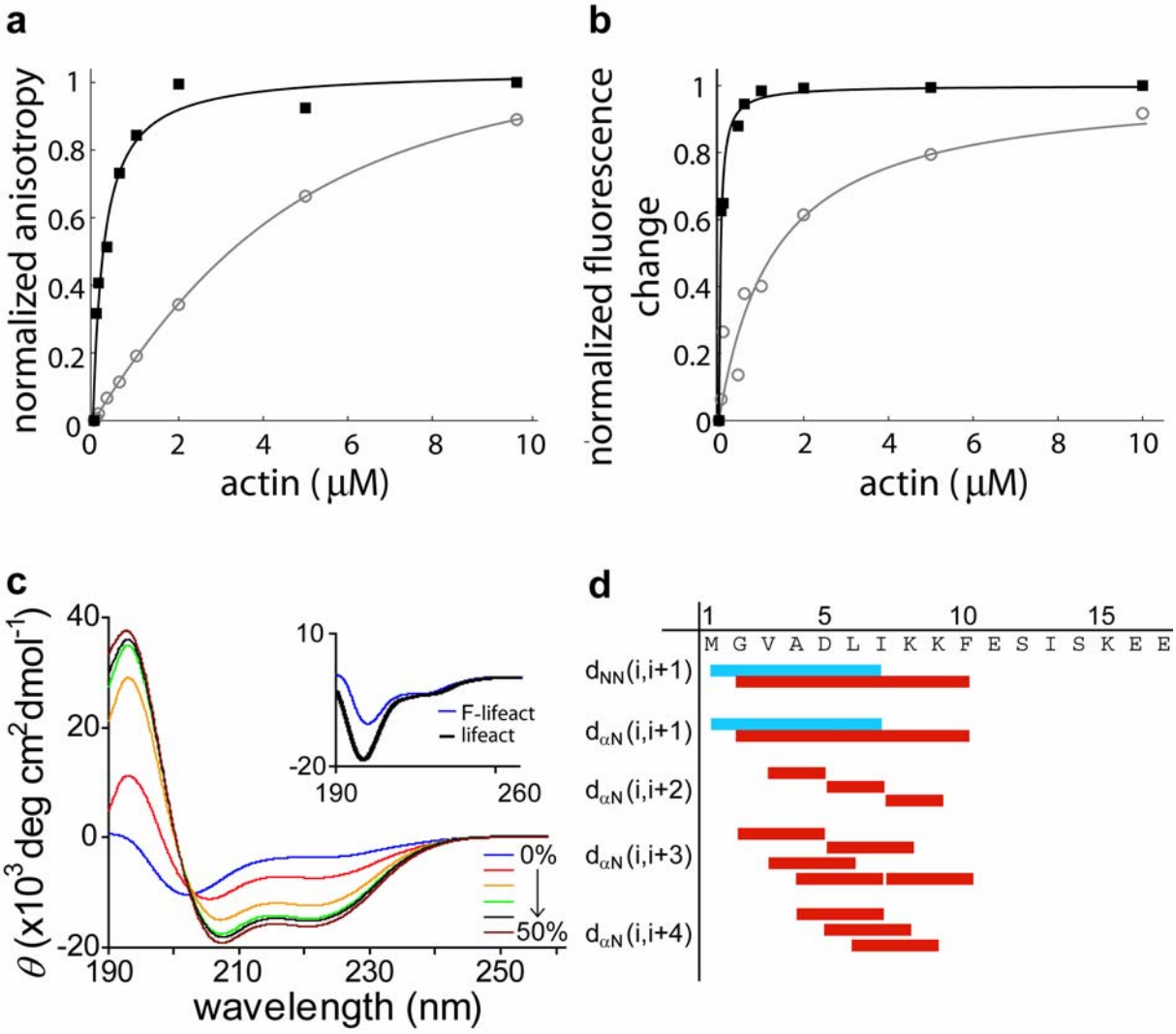
Supplementary Figure 1 Biochemistry of Lifeact.



Supplementary Figure 1 Biochemistry of Lifeact. (**a, b**) Measurement of F-Lifeact binding to G-actin (filled black squares) and F-actin (open grey circles) by monitoring (**a**) changes in the anisotropy of the FITC moiety. Values were normalized to the maximum anisotropy observed. Solid lines: fits to the quadratic expansion of the binding polynomial (see methods) and (**b**) fluorescence enhancement of FITC. The fluorescence was normalized to the maximum observed. Solid lines: fits to the hyperbolic binding isotherm. (**c**) Circular Dichroism (CD) measurements on F-Lifeact upon titration with 0-50% trifluorethanol (TFE). Inset: CD on F-Lifeact (in PBS) and Lifeact (in 10% acetic acid, pH 3) without TFE. (**d**) Short and medium range NOE connectivities involving the NH and $C^{\alpha}H$ protons. Blue bars represent measurements on F-Lifeact at pH 7.1, red bars represent 2D NOESY NMR spectra of Lifeact at pH 3.0 in the presence of 15% (v/v) HFP-d_2.

Supplementary Methods

Yeast strains and plasmid constructions. Yeast cells were grown at 30°C and observed from logarithmically growing cultures (OD < 0.8). Standard yeast media and procedures were used. To determine the minimal actin binding domain of Abp140 various domains fused to GFP were expressed in the S288C yeast background from plasmids based on the pRS315 backbone (LEU2, CEN). Expression was driven by the native Abp140 promoter (300bp) and a short GS linker was generated between Lifeact and GFP. For expression in mammalian cells from the CMV promoter the Lifeact sequence was cloned into pEGFP-N1 (Clonetech) or the respective plasmid with mRFPruby replacing GFP. In both cases the linker GDPPVAT was generated between Lifeact and the fluorescent protein. Actin-GFP and GFP-utrophin were expressed from the same plasmid backbone.

Microscopy and image acquisition. TIRF images were captured on an iMIC-stand from Till photonics with an 1.45 NA 100x objective from Olympus. A 300 mW Argon laser and a 20 mW DPSS 561nm laser were selected through an AOTF. A 2-axis scan head (Yanus II) was used to adjust the TIRF angle. Images were collected with a cooled Imago QE CCD camera. Acquisition was controlled by the TILLvisION software package. Confocal images of tissue samples were collected on a standard Leica SP2 setup. Epifluorescence images were collected on a Zeiss axiovert 200M stand equipped with a climate control chamber from EMBL.
Fluorescence Recovery After Photo-bleaching (FRAP) was done on a DeltaVision Core microscope (Applied Precision, Issaquah, WA) equipped with CO_2 and temperature controllers. The beam width of the bleaching laser was 0.74 μm as determined on fixed, homogenously labelled and thin samples of lamellipodia. Analysis was carried out with custom image analysis software written in MATLAB (The MathWorks, Natick, MA) using raw image stacks.

Image processing and data analysis. All image processing steps were performed in Metamorph (Molecular Devices). For visualization purposes sequences were routinely processed by sequential application of a local background subtraction filter and a 3x3 gauss low pass filter. Color overlays and kymographs were created with the respective functions in Metamorph.

Statistics. For all quantifications and graphs average values and standard deviation were given. Sample size was 3 if not given otherwise. Comparison of Lifeact-GFP and actin-GFP treated cells against control cells (lamellipodial flow, neuronal polarization, Fig. 2l, n) were performed as One-way ANOVA with Dunnetts posthoc test. Migration speed of actin-GFP

and Lifeact-GFP transfected cells was compared to the speed of untreated cells from the same cell preparation by paired t-tests of the means from at least 3 independent experiments with > 40 cells each (Fig. 2m) All presented *P* values are two-tailed. Statistical tests were performed with Prism 4 (GraphPad).

Proteins. Rabbit muscle actin, α-actinin, rabbit skeletal muscle Myosin, human recombinant profilin and pyrene actin were purchased from Cytoskeleton, Inc. Actin was always prepared freshly from lyophilized material and stored at 4 °C for no more than 2 days. Actin was diluted to working concentrations in G buffer (2 mM Tris HCl, pH 8.0, 0.2 mM ATP, 0.1 mM $CaCl_2$, 0.5mM DTT).

Actin binding. Polymerization of actin was induced by addition of 0.1volume 10x KMEI buffer (50 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 10 mM imidazole HCl, pH 7.0) and incubation for > 1h at room temperature. 44 μM of F- Lifeact was incubated 30min with F-actin and then spun 30 min at 350,000 x g at room temperature. The supernatant was removed and the pellet resuspended in 100 μl of 1x KMEI buffer. The amount of peptide was measured in a Cary Eclipse Fluorescence Spectrophotometer with excitation/emission set for FITC at 485 nm/520 nm. For anisotropy measurements, the set of automatic polarizers from Varian® was used. The bound/total ratio was calculated as the signal from the pellet divided by the total signal. Binding to G-actin was determined from the fluorescence increase of pyrene-actin (365ex/407em) in response to titration with F-Lifeact. The K_d was obtained by fitting to a hyperbolic curve, except for anisotropy where the full quadratic expansion of the binding polynomial was used: $\left(K_d+[\mathrm{Lf}]+[\mathrm{A}]\right)-\sqrt{\left(K_d+[\mathrm{Lf}]+[\mathrm{A}]\right)^2-\left(4[\mathrm{Lf}][\mathrm{A}]\right)}\Big/2[\mathrm{Lf}]$, where K_d is the dissociation constant and [Lf] and [A] are the F-Lifeact and actin concentration, respectively. For competition assays with actin side binding proteins α-actinin and myosin were incubated 30min with F-actin, followed by the addition of varying amounts of F-Lifeact. The mix was then pelleted at low speed (10.000 g, for the bundling protein α-actinin) and high speed (100.000 g, for both). The amount of F-Lifeact present in the supernatant and pellets was measured by 485ex/520em. The samples were then incubated in loading buffer (225 mM Tris HCl, 50% Glycerol, 5% SDS, 250 mM DTT and 0.05% of Bromophenol Blue in ddH_2O) and run on 4-12% NuPAGE® (Invitrogen) SDS Gels, using MES buffer (50 mM MES, 50 mM Tris Base, 0.1% SDS and 1 mM EDTA at pH 7.3 in ddH_2O), at 110V for 45min.

Actin polymerization and depolymerization. For polymerization assays, 20% pyrene-labelled actin was centrifuged at 350,000 x g for 30 min at 24°C to remove any nucleation

seeds. Ca to Mg exchange was done adding 0.1 Vol of 10x ME buffer (50 μM $MgCl_2$, 0.2 mM EGTA) for 2 min. Polymerization was promoted by addition of 0.1V 10x KMEI buffer. The final volume was 100 μl. Pyrene fluorescence was monitored spectroscopically (excitation 365 nm, emission 407 nm). To test the effect of F-Lifeact on polymerization different amount of F-Lifeact were added to pyrene-actin after centrifugation and incubated for 5 min. Depolymerization was measured by monitoring pyrene fluorescence after diluting 100% pyrene-labelled F-actin in 1x KMEI buffer to < 0.2 μM. To test its effect on depolymerization the indicated concentrations of F-Lifeact were pre-incubated with F-actin for 5 min before dilution.

Far UV CD Spectroscopy. CD measurements were performed on a Jasco J-715 spectropolarimeter with a peptide concentration of 4.4 μM.

NMR sample preparation and spectroscopy. For NMR F-Lifeact was dissolved in PBS pH 7.1. Unlabelled Lifeact was dissolved in PBS at pH 3. In order to stabilize secondary structure of the peptide, 15% (v/v) of 1,1,1,3,3,3-hexafluoro-2-propanol-d_2 (HFP-d_2) was added to the sample of the unlabeled peptide. 10% of D_2O (v/v) was added to all samples. NMR measurements were carried out at 600MHz on a Bruker DRX-600 spectrometer equipped with a cryoprobe at 300 K. 2D nuclear Overhauser effect (NOESY) spectra were collected with mixing time of 100 ms, and total correlated spectroscopy (TOCSY) spectra were recorded with DIPSI2 mixing sequence of 35 ms and 80 ms duration (for the unlabelled peptide in alcohol/water and labelled peptide in PBS)[1]. Water suppression was carried out using the WATERGATE sequence. Sequence specific resonance assignments were carried out as in [2]. Amino acids spin systems were identified by analysis of TOCSY spectra. NOESY spectra[1] were used to observe contacts < 5 Å.

Cell culture. Mouse primary dendritic cells were generated from flushed bone marrow suspension as described previously[3]. At day 8-10 cells were nucleofected using the primary mouse T cell kit and the Amaxa nucleoporator according to the manufacturer's recommendations and immediately after transfection 200 ng/ml LPS were added over night and cells were subsequently sorted into GFP positive and negative fractions by using fluorescent activated cell sorting. MDCK cells and mouse embryonic Fibroblasts were grown according to standard procedures and transfected using lipofectamine**.**

Chemotaxis assays. PureCol™ gels containing mature dendritic cells were cast as described previously[4]. Gels were overlayed with 50 μl of 0.6 μg/ml CCL19 (R&D Systems) in RPMI, 10% FCS and imaged on an inverted Axiovert 40 (Zeiss) microscopes, equipped with custom

built climate chambers (5% CO2, 37°C, humidified). Under agarose dendritic cell migration was performed as described[31]. Briefly, dendritic cells were added into wells punched into an agarose layer. Recombinant CCL19 was added into the adjacent well and, following the chemokine gradient, the cells entered the space between agarose and cover slip. Chemotaxing cells were subsequently imaged with TIRF microscopy**.**

Culturing, transfection and classification of primary hippocampal neurons. Primary hippocampal neurons were isolated from rat embryos as previously described[5]. Briefly, hippocampi derived from E18 rats were dissected, trypsinized, and dissociated. Directly after dissociation, $5x10^5$ hippocampal neurons were transfected by the Amaxa nucleofector system using 3 μg of highly purified plasmid DNA of pEGFP-Lifeact, pEGFP-actin or pEGFP-N2. The neurons were then immediately plated onto poly-L-lysine-coated glass coverslips in 6 cm petri dishes containing minimal essential medium (MEM) and 10% heat-inactivated horse serum. The cells were kept in 5% CO_2 at 36.5°C. 6-12 hrs after plating the glass coverslips were then transferred into 6 cm petri dishes containing astrocytes in MEM and N2 supplements and kept in culture for 2DIV.

Neurons were categorized into three stages: neurons with a single axon, multiple axons or no axon. A neurite length > 35 μm was used as threshold to define the axon. Developmental stages were observed by live cell microscopy as described[6].

Human neutrophils and IC induced spreading

Human peripheral blood neutrophils were isolated by density centrifugation using a Pancoll™ gradient. Briefly, 10 ml blood containing EDTA was diluted in 10ml PBS and layered on 10 ml pancoll. After 30 min centrifugation at 500 g neutrophil were separated from the erythrocyte rich pellet by dextran sedimentation. Residual erythrocytes were eliminated by hypertonic lysis and after washing in PBS, neutrophils were resuspended in RPMI containing 0.5% low endotoxin bovine serum albumin. Neutrophil purity was routinely ~95% as assessed by forward and side scatter with flow cytometry as well as by morphological analysis.

To form ICs *in vitro,* glass slides were coated with 5 mg/ml ovalbumin in PBS overnight at 4°C followed by washing and incubation in rabbit anti-ovalbumin serum at 50 μg/ml specific IgG for 2 h at room temperature. F-Lifeact loaded neutrophils were subjected to ICs in the presence of 10 ng/ml tumour necrosis factor-α to study actin reorganization in response to ICs.

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