Supplementary Materials and Methods

Plasmids
GFP-Talin was a gift from Sue Monkley. Mutations within this construct were introduced by Quikchange mutagenesis (Stratagene). PH-PLCδ-Cherry was subcloned from PH-PLCδ-GFP kindly provided by Volker Hauke. GFP-Kindlin2 was previously published (Ussar et al, 2006). GST-talin head was a gift from David Critchley. GST-Kindlin2 was generated by subcloning Kindlin2 from GFP-Kindlin2 into the pGEX-4T3 vector. PIPKIγ_i2 was cloned in two stages. The 87 kDa isoform was cloned from Image clone 4459567. The missing C-terminal part was amplified from Image clone 6848587 using CCAGGCCCTGGCCAAAGGG as forward primer and AATTGAAATTCTTATGTGCTCTCGC as reverse primer. A kinase-dead mutation (D253A) in PIPKIγ_i2 and talin mutations were introduced by Quikchange mutagenesis. All in-house constructs were verified by DNA sequencing.

Antibodies
The following antibodies were used: mouse anti-talin and rabbit anti-actin (Sigma); rabbit anti-ILK (Cell Signaling Technologies); mouse anti-paxillin and mouse anti-synaptoplanin1 (Transduction Laboratories); rat anti-tubulin, mouse anti-cortactin and rat α5β1 blocking antibody MAB2575 (Millipore); rabbit anti-myosin light chain (Santa Cruz). PanPIPKIγ- and PIPKIγ_i2-specific antibodies were obtained by immunizing rabbits against a KLIH-conjugated peptide corresponding to amino acids 637-656 of the mouse PIPKIγ90 isoform (TDERSWVYSLHYSARPASD) or amino acids 536-550 recognized by all major isoforms (ERPSDTSEQPRYRR). Fluorophore-conjugated secondary antibodies for immunofluorescence were from Jackson Immunochemicals Laboratories, Inc and HRP-conjugated secondary antibodies were from Biorad. Phalloidin conjugated to Alexa488 was used to visualize F-actin (Invitrogen).

Migration assays
Cell migration was measured in three ways. The standard scratch/wound closure assay was performed by creating a wound in a confluent monolayer with a flame-treated capillary pipette. Scratches were photographed every 10 minutes for 12 hours in an environmental chamber using the 20x objective of a Zeiss Axiovert 200M equipped with Metamorph software. The distance between the edges of the wound were measured and used to calculate migration rates. Alternatively, single cell migration was tracked by taking pictures of a sparse culture every 5 minutes for 6 hours and tracking single cell movements using the ImageJ Chemotaxis tool plugin. Chemotaxis assays were performed on transwell chambers, on which the bottom surface was coated with 5 μg/ml FN. 50 000 cells in DMEM were seeded into the top chamber, and DMEM containing growth factors or 1% BSA was added to the bottom chamber. Cells were incubated at 37°C and 5% CO2 for 4 hours, fixed and stained with 0.1% crystal violet in 20% EtOH, and cells on the underside of the filter were microscopically imaged and counted.

Cell adhesion and spreading assays
To measure adhesion, sectors of a 96-well plate were coated with 2 μg/ml FN or 5 μg/ml VN and blocked with 1% heat denatured BSA. A single cell suspension of 5x10^4 cells/well was plated for the indicated times. To control for integrin dependence, 100 μM cilengitide was included in some experiments. Wells were washed with PBS, fixed with 95% EtOH and stained with 0.1% crystal violet. Stained cells were lysed with 0.1% Triton X-100. Absorbance at 595 nm was measured with a Sunrise-Basic plate reader equipped with Magellan5 software (Tecan). Absorbance in matrix-containing wells was blanked against wells containing BSA. To measure spreading, cells were plated in serum-free medium onto FN-coated plastic dishes and monitored at 37°C and 5% CO2 using the 20x objective of a
Zeiss Axiovert 200M controlled by Metamorph. Pictures were taken every 5 minutes for two hours, and the time at which a cell first appears in the field of view was taken as the 0 time point for that cell. Area was calculated in ImageJ using manually-drawn regions around the borders of cells.

**Collagen gel contraction assay**

Single cell suspensions of $3 \times 10^4$ cells were mixed with a final concentration of 1.6 mg/ml Collagen 1 and 20 μg/ml FN in 1x MEM in a final volume of 150 μl. The entire volume was transferred as a drop to a suspension culture plate (Greiner bio-one) and incubated for one hour in a cell culture incubator to harden. Once hardened, 1 ml of DMEM + 10% FBS was added to the well and photographs were taken at intervals. Top-down areas were calculated with ImageJ by drawing regions around the collagen plugs.

**Purification of GST-tagged proteins**

A plasmid encoding GST-Talin head was transformed into JM109 cells. One colony was picked into a 5ml starter culture, which was used to inoculate a 50 ml starter culture, which then inoculated a 500 mL culture. All incubations were carried out at 37°C. Once the 500 ml culture entered log phase (OD$_{600}$=0.8), induction was carried out for 4 hours with 0.5 mM IPTG. Cells were pelleted, washed in GST bind/wash buffer (4.3 mM Na$_2$HPO$_4$, 1.47 mM KH$_2$PO$_4$, 136 mM NaCl, 2.7 mM KCl, pH 7.3), and lysed in 20 ml GST bind/wash buffer containing protease inhibitors (aprotinin, leupeptin, pepstatin, PMSF) +0.01% Triton X-100 +1 mg/ml lysozyme (Sigma). Suspensions were incubated at room temperature for 30 minutes, and subjected to sonication with a tip sonicator on setting “6” for 3x7 seconds, until the lysate was no longer viscous. Lysate was centrifuged at 10k x g for 20 minutes and passed through a 0.45 μm syringe filter before loading onto a 0.5 ml glutathione Sepharose column (GST-Bind resin, Novagen). GST-tagged protein was bound and eluted by gravity according to manufacturer's instructions.

A plasmid encoding GST-Kindlin2 was transformed into Rosetta2 cells. Cultures were grown and induced as for GST-talin, except all liquid cultures were maintained at 18°C and induction was carried out for 20 hours. Lysis and purification were conducted as for GST-talin.

**Liposome sedimentation assay**

Lipids (POPS=1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine; POPC=1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC); PIP=1-α-phosphatidylinositol-4-phosphate; PIP2=1-α-phosphatidylinositol-4,5-bisphosphate; PIP3=1,2-dioleoyl-sn-glycero-3-phospho-(1′-myo-inositol-3′,4′,5′-trisphosphate) were purchased from Avanti Polar Lipids, and dissolved in MeOH:CHCl$_3$: 1:1. Lipid films containing POPS:POPC 1:4, PIP:POPC 1:19, PIP2:POPC 1:19, PIP3:POPC 1:19 were prepared and stored at -20°C under argon until use. Vesicles were prepared at a concentration of 5 mg/ml by resuspending lipid films in 12 mM HEPES, pH 7.4, 0.2 mM EGTA and 176 mM sucrose at 42°C for 30 minutes, followed by a 1 minute incubation in a bath sonicator, and extrusion through a 0.1 μm polycarbonate membrane (Whatman). Vesicle suspensions were prepared fresh. Proteins were diluted to a final concentration of 0.15 mg/ml with 20 mM Tris-Cl, pH 7.4, 0.1 mM EDTA, 15 mM β-mercaptoethanol and prespun in a TLA100 rotor (Beckman) at 150k x g for 30 minutes at 4°C. 25 μl of liposomes were added to 175 μl of protein and incubated at room temperature for 30 minutes. Samples were spun at 150k x g for 30 minutes at 25°C and separated into pellet and supernatant fractions. The pellet was solubilized in 200 μL of SDS-PAGE loading buffer and equal volumes of supernatant and pellet were resolved by SDS-PAGE and visualized by Coomassie staining.

**Integrin activation and FACS assay**

To determine surface integrin expression levels, $1 \times 10^6$ cells were suspended in FACS buffer (PBS + 2% FCS + 2 mM EDTA) and incubated with PE-labelled anti-β1 (BioLegend), anti-β3 (eBioscience), anti-
αv (BD) or anti-α5 (Pharmingen) antibodies, or isotype controls at 1:200 dilution for 30 minutes on ice. Samples were washed in FACS buffer, resuspended and sorted on a FACScalibur (Beckton Dickson). Data was plotted using Cellquest Pro software.

To observe integrin activation by FACS, 5x10⁵ cells were incubated in the presence or absence of 0.2 mM RGD or RAD peptide (Biomol) for 30 minutes on ice in activation buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 1% BSA, 1 mM MgCl₂, 1 mM CaCl₂) prior to the addition of 9EG7 (activated β1) or MAB1977 (conformation non-specific β1) (1:100) and a further incubation for 30 minutes on ice. Cells were washed and primary antibodies were detected with 1:200 anti-Rat-Alexa488. Cells were sorted on a FACScalibur (Beckton Dickson) and data was analyzed using Cellquest Pro software. % activated β1 integrin was determined by dividing the signal from the 9EG7 antibody by the signal from the MAB1977 antibody.
Supplementary Figure Legends

Figure S1: Genetic deletion of PIPKIγ exon 17. (A) Exon 17 deletion scheme. Wild type allele shows the 3’ region of the murine PIP5KIC gene, encoding PIPKIγ. Exon 17 was targeted by homologous recombination to give the targeted allele, consisting of a neomycin resistance cassette (neo) flanked by frt sites (ovals) and exon 17 flanked by LoxP sites (triangles). The floxed allele, lacking the neo cassette, was obtained by breeding mice harboring the targeted allele with mice expressing flp recombinase. Cells isolated from these mice are designated as wild type throughout this study. PIPKIγΔE17 cells were obtained by transducing floxed cells with Cre recombinase. Bars labelled 1-3 on the floxed allele represent PCR primers used for genotyping. (B) Southern blot of a wild type (WT) mouse and a mouse carrying two copies of the floxed exon 17 (fl/fl). Genomic DNA was digested with EcoRI and probed with a sequence excised from exon 18. Cells derived from a fl/fl mouse were used for all experiments and were genotyped by PCR. (C) Genotyping results for wild type (WT) and PIPKIγΔE17 (ΔE17) fibroblasts using the primers depicted in A. Wild type cells give a 430 bp product when primers 1 and 2 are used, whereas ΔE17 cells give no product as the binding site for primer 2 is deleted in these cells. Asterisks denote additional products arising from primer annealing to other regions of the genome. When primers 1 and 3 are used, WT cells give a 1050 bp product whereas ΔE17 cells give a 700 bp product, reflecting the deletion of 350 bp of intervening sequence.

Figure S2: Colocalization of GFP-talin and Cherry-tagged PIPKIγ constructs in ΔE17 fibroblasts. Cells coexpressing GFP-talin and Cherry-tagged PIPKIγ constructs were examined for colocalization using confocal microscopy. Boxed regions denote the locations chosen for the insets.

Figure S3: Representative data from primary mouse embryonic fibroblasts (MEFs). (A) Talin incorporation assay measured from 7 WT and 8 ΔE17 MEFs expressing GFP-talin. (B) Calculated cell areas from 77 WT and 78 ΔE17 MEFs plated onto 5 μg/ml FN-coated plastic culture dishes for the indicated times. (C) Migration rate calculated from scratch wound closure experiments, showing data from two independent cell preparations.

Figure S4: Kindlin2 incorporation and phosphoinositide-binding specificity of kindlin 2. (A) TIRF time-lapse images were collected from the leading edge of GFP-kindlin 2-expressing wild type or ΔE17 fibroblasts. The incorporation of kindlin 2 into new adhesions (arrows) was monitored by quantifying the rate of increase in GFP epifluorescence. (B) GST, GST-talin head or GST-kindlin 2 were incubated with large unilamellar vesicles containing the indicated phosphoinositide and specific interaction was detected by cosedimentation. Supernatant (S) and pellet (P) fractions were analysed by Coomassie staining. PS, phosphatidylserine; PIP, PtdIns(4)P; PIP2, PtdIns(4,5)P2; PIP3, PtdIns(3,4,5)P3.

Figure S5: Representative AFM force curves. (A) A wild type cell incubated in the absence of RGD peptide (left curve) or in the presence of 0.2 mM peptide (right curve) was attached to a tipless cantilever and brought into contact with a FN-coated surface for 10 seconds before retraction. The approach curve (light red, >0 pN) and retraction curve (dark red, <0 pN) are shown. The peak force of detachment (ΔF) was calculated to determine the strength of adhesion, and the integrin-dependent component is indicated. (B) Individual cells were attached to a tipless cantilever and brought into contact with a 2 μg/ml FN-coated surface for defined time intervals. To separate integrin-specific binding to the substrate from non-specific binding, force curves were obtained after 10 seconds or 30 seconds of contact in the presence or absence of linear GRGDNP peptide (RGD). Data is mean ± S.E. of 15 cells per time point.

Figure S6: Plate-and-wash assay on 2 μg/ml vitronectin. WT (filled circles) and ΔE17 cells (open
circles) were plated onto 2 μg/ml vitronectin (VN), followed by colourimetric detection with crystal violet. Data are mean ± S.E.M. for three experiments. Rate constants were derived from exponential rise to max curves.

**Figure S7: Spinning disc cell numbers and supplemental experiments.** (A) The number of cells counted on cover slips coated with 3 μg/ml FN following a 5 minute spin (n=6). (B) The result of a spinning disc experiment on cover slips coated with 5 μg/ml FN. Data is mean ± S.E.M. for three independent experiments, conducted in duplicate. (C) The number of cells counted on the cover slips for the experiment presented in panel C (n=6). (D) The result of a spinning disc experiment in which the cells were plated onto 2 μg/ml vitronectin-coated cover slips. Data is mean ± S.E.M. for three independent experiments, conducted in duplicate. (E) The number of cells counted on the cover slips for the experiment presented in panel E. (F) The number of cells counted on cover slips treated as in A, but in the presence of 100 μM cilengitide (n=4). For panels A, B, D and E data are mean ± S.E.M. Statistical significance: *, p<0.05; **, p<0.005.

**Figure S8: Attachment of the actin cytoskeleton to paxillin-rich FAs.** Fibroblasts were plated onto 5 μg/ml FN-coated cover slips for the indicated times and immunostained for paxillin (red). Actin was stained with Phalloidin-Alexa488 (green).

**Figure S9: Examination of the surface expression and activation status of β1 integrin.** (A) Surface integrin expression was examined by FACS analysis. Wild type (WT) fibroblasts (blue lines) and ΔE17 fibroblasts (red lines) were assessed for β1, β3, α5 and αv expression, and data were compared against isotype control (purple area). (B) β1 integrin activation on suspended cells was assessed using the conformation-specific 9EG7 antibody. Wild type (WT; black bars) and ΔE17 (grey bars) cells were incubated with 9EG7 in the absence of peptide, or in the presence of 0.2 mM GRGDNP peptide or control GRADSP peptide. Activation is expressed as a percentage of total surface β1 integrin expression. Data is mean ± S.E.M. of three independent experiments. (C) Cells were plated for one hour, and incubated with an antibody against β1 integrin or with the 9EG7 antibody to label activated β1 integrin. Cells were then fixed and processed for immunofluorescence. In this way, only surface integrins were recognised.

**Figure S10: Scratch wound closure by fibroblasts.** Confluent monolayers of fibroblasts were scratched with a pipette tip and closure of the wound was monitored by live cell microscopy. The scale bar at 0 minutes post scratch is 200 μm.

**Movie 1: Talin recruitment measured by TIRF microscopy.** WT (left) and ΔE17 (right) fibroblasts were transfected with GFP-talin1 and incorporation of talin into new adhesions was measured by TIRF microscopy. Black and white images are fluorescence images and colourized images have been processed by adding a ratio LUT via ImageJ to better visualize pixel intensities.

**Movie 2A: Direct visualization of actin flow in a WT fibroblast.** Images were taken every 2 seconds and processed for enhanced phase contrast imaging of the actin cytoskeleton. The black bar crossing the lamellipodium denotes the position chosen for kymograph analysis in Fig. 7B.

**Movie 2B: Direct visualization of actin flow in a ΔE17 fibroblast.** Images were taken every 2 seconds and processed for enhanced phase contrast imaging of the actin cytoskeleton. The black bar crossing the lamellipodium denotes the position chosen for kymograph analysis in Fig. 7D.
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A
Rate constant (min⁻¹)

WT  ΔE17

B
Cell area (µm²)

WT  ΔE17  WT  ΔE17
30 minutes  60 minutes

C
Migration rate (µm/hr)

WT1  WT2  ΔE17-1  ΔE17-2

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A

B

C

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