METHODS

Cells

The following cell lines were used in this study: MDA-MB-231 (human breast adenocarcinoma) maintained in DMEM containing 1% MEM nonessential amino acids, 1% L-glutamine and 10% foetal bovine serum (FBS); NCI-H460 (human non-small cell lung carcinoma) in RPMI-1640 (R5886 Sigma) containing 10% FBS, 1% L-glutamine, 1% sodium pyruvate, 1% HEPES (1M) and 1% glucose (45%) and; TIFFs (human telomerase-immortalized foreskin fibroblasts, a gift from Jim Norman) maintained in DMEM containing 1% L-glutamine, 2% HEPES (1M) and 20% FBS; CHO (Chinese hamster ovary cells) in Alpha-MEM (M4526, Sigma) containing 1% L-glutamine and 5% FBS; GD25-β1A (mouse fibroblasts, described in [40]) cultured in DMEM containing 1% L-glutamine, 10% FBS and 10 μg/ml puromycin and FAK-/- and FAK+/+ MEFs (mouse embryonic fibroblasts provided by D. Schlaepfer UCSF, described in [41]) cultured in DMEM containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 1% MEM nonessential amino acids, 1% sodium pyruvate and 1% L-glutamine. DMEM was from Sigma (D5796). Unless otherwise indicated, the cell lines were obtained from American Type Culture Collection (ATCC). The cell lines used in this study are not found in the ICLAC database for cross-contaminated or otherwise misidentified cell lines, and were not authenticated. All cells were serum starved for 24 h before all experiments unless otherwise indicated. All cells were routinely tested for mycoplasma contamination. All experiments were repeated at least three times unless otherwise indicated.

Antibodies & reagents:

A detailed list of used primary antibodies is provided in Supplementary Table 3. Phalloidin-Atto 647N (65906-10NMOL) was purchased from Fluka; AlexaFluor-conjugated secondary antibodies (488, 555, 647 conjugated anti-mouse, rabbit and rat antibodies) were purchased from Life Technologies; Mega-520 (rabbit) and Atto-647 (mouse) secondary antibodies for STED were from Sigma; secondary antibodies for detecting immunoblots with Odyssey (DyLight 680 and 800

conjugated anti-mouse, rabbit and rat antibodies) were from Thermo Scientific. Live-cell dyes, Far red DDAO-SE and green CMFDA were obtained from Molecular Probes. Dynasore monohydrate (D7693), DMSO (D2650) and collagen type I solution (C8919) were obtained from Sigma. Dyngo4a was from Abcam (ab120689) and FAK-inhibitors PF-562271 (S2890) and PF-573228 (S2013) were obtained from Selleckchem. CellPlayer 96-Well Kinetic Caspase-3/7 Apoptosis Assay Kit (Nucview, 4440) was purchased from Essen Bioscience, LY294002 (PHZ1144) and AlexaFluor568 conjugated transferrin (T-23365) from Life Technologies, fibronectin bovine plasma (341631) from Merck and Polybead® Microspheres 6.00 µm (07312-5) from Polysciences. FAK inhibitor 14 (3414) and Human Phospho-RTK Array (ARY001) were from R&D Systems. Active (0165-0000-3) and non-activated (0165-0000-1) recombinant FAK were purchased from ProQinase. pET15b-FNIII (7-10) was a gift from Reinhard Fässler. HyQTase (SV30030.01) from HyClone was used to detach cells for re-plating before start of experiment.

siRNAs and plasmids

The following siRNAs and their target sequences were used (all from Qiagen): Allstars negative control siRNA (1027281); siEEA1, ATGGATAACATGACCTTGGAA; EEA1 smart pool (three siRNAs), AGCCGCTATATTAGACTTGGA, AAGCTAAGTTGCATTCCGAAA, CCCGGCACAGAATGTGAGTTA; siβ1-integrin, CCCGACATCATCCCAATTGTA, and CTGGTCCATGTCTAGCGTCAA; siRab21, AAGGCATCATTCTTAACAAAG (3'-Alexa Fluor 555) and siAPPL1, CAGGACAATCTCGGCCACCGA. The following plasmids were used: pEGFP-C1 (Clontech), GFP-Rab5-Q79L and GFP-Rab21 [9], pIRES-GFP-α2 WT/-α2-AA [28], GFP-Dyn2K44A [42], GFP-EEA1 (Addgene plasmid 42307 [43]), GFP-FAK wt, GFP-FAK FERM (1-402) and GFP-FAK FAT from David Schlaepfer. Lipofectamine 2000 (11668-019, Life Technologies) and HiPerfect (301705, Qiagen) were used for transient transfections and siRNA silencing according to manufacturer's protocol.

Subcellular fractionation

The protocol was modified from [23]. Briefly, adherent cells were scraped on ice into hypotonic lysis buffer (10 mM HEPES-KOH pH 7.2, 0.25 M sucrose, 1 mM EDTA, 1 mM MgOAc and protease & phosphatase inhibitors (PhosSTOP and Complete mini tablets from Roche). Cell membranes were fragmented with French Press and nuclei removed with 10 min 1000 x g centrifugation. Plasma membrane fraction was collected with 10 min 10,000 x g centrifugation and endosomal/cytoplasmic fractions with 1 h 100,000 x g centrifugation. Membrane fractions were washed at least once with lysis buffer and cytoplasmic fractions were centrifuged twice. All fractionation steps were performed at +4°C or on ice. All fractions were dissolved in sample buffer for immunoblotting. Direct protein binding to isolated endosomes was assessed with recombinant proteins. Therefore, the endosomal fraction derived from FAK -/- MEFs was resuspended in buffer containing 20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT and protease & phosphatase inhibitors and subsequently incubated with 0.5 µg of either phosphorylated or non-phosphorylated FAK (ProQinase) or GST for 2 h at room temperature. In order to assess whether FAK can be activated on endosomes in vitro, GST or non-phosphorylated FAK were incubated for 1.5 h with the purified endosomes and then 10 µM ATP was added or not for another 30 min. Following 100,000 x g centrifugation for 1 h, the total soluble (supernatant) and non-soluble endosomal (pellet) fractions were analysed by SDS-PAGE followed by western blotting with the indicated antibodies.

In-situ Proximity ligation assay (PLA)

PLA was done according to manufacturer's protocol (Duolink, Olink BioScience) in serum starved TIFFs plated on collagen for 45 min +/- dynasore followed by 4% PFA + 1 mM $MgCl_2$ fixing.

Replating assay: integrin activation by ligand engagement

The same protocol was used in all experiments where integrin signalling was activated by integrin ligand engagement at different time points. Cells were serum starved for 24h, detached with

HyQTase, washed and kept in suspension in serum-free medium for 1 h to stop adhesion signalling. Dynasore (80 μM [44, 45]), LY294002 (40 μM), Dyngo4a (10 μM) or equal volumes of DMSO were added to cells 15 min before replating on collagen type I or fibronectin-coated (5 μg/ml) dishes or coverslips for different time points at +37°C. Cells were washed with cold PBS and collected on ice for immunoblotting.

Inhibitor assay

Adherent unstarved cells were treated with FAK inhibitors (10 μM of FAK-14, 1 μM of PF271 or 1 μM of PF228) for 3h at +37°C after which the cells were either fixed and stained for immunofluorescence or collected in sample buffer for immunoblotting.

Immunoblotting

Immunoblotting was performed by using standard western blotting techniques and either ECL detection or Odyssey LICOR imaging system. The level of phosphorylated proteins was quantified by measuring the integrated band intensities with NIH ImageJ (1.45s) and unless otherwise indicated, band intensities of phospho-proteins were normalised to the corresponding tubulin band and to the sum of all time points inside an experiment.

Flow cytometric assay

Cells were treated as described in replating assay with the exception that in the end cells were detached with HyQTase, washed and fixed. Fixing and staining of the cells was performed as described in [46].

Bead assay

Beads (6 μm, Polybead® Microspheres,) were coated with 10 μg/ml collagen in 2% BSA for 2 h at +37°C and blocked with 2% BSA/PBS for 1h at RT. Serum-starved cells were treated with DMSO or dynasore as described in the replating assays, mixed with coated beads in low-attachment 6-well

cell culture dishes (Corning Costar Ultra-Low attachment 6 well plate, CLS3471, Corning) and incubated at +37°C for 45 min. Cold PBS was added to the wells, cells with beads were collected on ice, centrifuged at 210 x g for 3 min at +4°C and resuspended in sample buffer for immunoblotting.

Anoikis assay

Adherent, serum-starved cells were treated with 80 μM dynasore, 3 or 10 μM dyngo4a or equivalent amounts of DMSO for 1 h at +37°C. Cells were maintained at +37°C for 5 h or detached with HyQTase, washed and suspended in 5 ml of serum-free medium containing the same concentrations of dynasore or Dyngo4a and kept in suspension for 5h at +37°C. Adherent cells were collected with HyQTase and all cells were then analysed for the Caspase-3/7 positivity using Nucview Apoptosis Assay according to manufacturer's protocol (Essen Bioscience).

Immunofluorescence, microscopy, image and co-distribution analysis

Cells plated on Ibidi dishes (Integrated Biodiagnostics) or on acid washed coverslips were fixed with 4% paraformaldehyde (PFA) containing 1mm $MgCl_2$ for 15 min at RT and permeabilised with 0.2% Triton-X-100 in 30% horse serum/TBS for 15 min at RT. Samples were blocked and antibodies were diluted in 30% horse serum/TBS. Primary antibodies were used at predetermined concentrations (5-10 μg/ml) and AlexaFluor secondary antibodies as 5 μg/ml. Unless otherwise stated, all images were acquired using the spinning disc confocal microscope as described [7] with the exception of 100x/1.3 Oil (Pha3, EC Plan Neofluar) objective being used. The microscope, image acquisition and related data analysis for the crossbow-shaped micropatterns is described [18]. Quantitative analysis for all other images was performed using NIH ImageJ (1.45s). Following background subtraction, β1 integrin and 555-Trf endocytosis was calculated from mid-slices by measuring the integrated density within the cell as a percentage of the total integrated density

(integrin endocytosis) or by measuring the mean intensity of the cell (Trf endocytosis). Leica TCS SP5 Stimulated Emission Depletion (STED) laser scanning microscope was used to acquire images in super-resolution level (Leica Microsystems GmbH, Mannheim, Germany), where approximately 65 nm resolution in x,y -axis was achieved. Leica STED IR corrected objective 100x/1.4 was used. Mega-520 fluorophore was excited at 532 nm wavelength (PicoQuant, Berlin, Germany) and Star-635 at 635 nm wavelength (PicoQuant, Berlin, Germany). The channels were scanned sequentially and emission was detected by avalanche photodiode detectors at the emission range of 685/40 (Leica Microsystems GmbH, Mannheim, Germany). Leica LAS software (Leica Microsystems GmabH, Mannheim, Germany) was used do perform background subtraction and deconvolution in all images. In the deconvolution process a Lorentzian PSF was generated by using the measured PSF value of 62 nm, which was exploited to signal energy based deconvolution algorithm. GFP-Rab5-CA was imaged with confocal resolution, by using 488 nm laser line for excitation, and emission was detected at 500-530 nm. In the deconvolution process a Gaussian PSF was generated for the confocal channel by using the measured PSF value of 230 nm, which was exploited to signal energy based deconvolution algorithm. STED imaging was performed at the Laboratory of Biophysics, University of Turku. Image colocalisation analysis was done by using the Intensity Correlation Analysis plugin for ImageJ to create PDM (product of the differences from the mean) images, where PDM = (red intensity−mean red intensity)×(green intensity−mean green intensity). Analysis of co-distribution between active β1-integrin and pFAK puncta on microdomains of either Rab5-, Rab21- or EEA1-positive endosomes, was performed as described in [15]. The distance between pFAK and FAK was measured by selecting a random non-endosomal pFAK puncta and measuring the distance to closest neighbour pFAK puncta.

Micropatterns and probabilistic density maps

Micropatterns were produced on glass coverslips as described in [47]. Cells were seeded on micropatterns in serum-free medium for either 3h or 45 min (cells on spots) before fixing. Samples

were stained as described above. The microscope, image acquisition and creation of the probabilistic density maps are described in [18].

Mice

Eight weeks old female Hsd:Athymic Nude - Foxn1nu mice were used for animal experiments. Studies were performed under a valid animal license (license number 4199/04.10.07/2014) and conducted according to the Finnish act on animal experimentation-guidelines.

Lung extravasation

Control and Rab21 or EEA1 siRNA transfected MDA-MB-231 cells were labelled with live cell dyes (siCtrl: Far red DDAO-SE; siRab21/EEA1: green CMFDA) according to the manufacturer's instructions. siControl- and siRab21/siEEA1-treated cells (0.75-1x10^6 cells each) were injected in a 1:1 ratio in the tail vein of 15 mice and lungs were collected 48 h after injection. This animal number has previously been sufficient for statistical significance in a similar experimental set-up [48, 49]. The left lung of each mouse was formalin fixed and processed for frozen sections to be analysed by fluorescence microscopy and right lungs were processed into a cell suspension by Collagenase I treatment as described in [48, 49] and the number of fluorescently labelled cells was analyzed by flow cytometry using LSRFortessa™ from BD Biosciences. The analyses were blinded such that the investigator analyzing the data was unaware of the identity of the labelled cell populations. The quantitation shows the ratio of siCtrl- and siRab21- or EEA1-treated cells in each lung. No blinding or randomization was performed as both cell types are co-injected in the same recipient animals simultaneously and analysed from the same samples using flow cytometry.

Mass spectrometry

Protein samples obtained from subcellular fractionation of WT MEFs were separated by SDS-PAGE and allowed to migrate 20 mm into a 4–12% polyacrylamide gel. Following staining with InstantBlue (Expedeon), gel lanes were sliced into ten 2-mm bands and subjected to in-gel digestion with trypsin as described previously [50] with modifications. Tryptic peptides were evaporated to dryness, dissolved in 1% HCOOH and 5 µl was submitted to LC-MS/MS analysis. The LC-MS/MS analysis was performed on a nanoflow HPLC system (EasyNano, Thermo Fisher Scientific) coupled to the LTQ Orbitrap Velos Pro mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a nano-electrospray ionization source. Peptides were first loaded on a trapping column and subsequently separated inline on a 15 cm C_{18} column (75 µm x 15 cm, Magic 5 µm 200 Å C_{18}, Michrom BioResources Inc., Sacramento, CA, USA). The mobile phase consisted of water/acetonitrile (98:2 (v/v)) with 0.2% formic acid (solvent A) or acetonitrile/water (95:5 (v/v)) with 0.2% formic acid (solvent B). A linear 50 min gradient from 5% to 35% B was used to elute peptides.

MS data was acquired automatically using Thermo Xcalibur software (Thermo Fisher Scientific). An information dependent acquisition method consisted of a TOF MS survey scan of mass range 300-2000 m/z. The data files were searched for protein identification using Proteome Discoverer (1.4) connected to in-house Mascot (v. 2.4) software. Data was searched against the SwissProt database (release 2014_08). Carbamidomethylation of cysteine was set as a fixed modification and oxidation of methionine was allowed as a variable modification. Only tryptic peptides were considered, with up to one missed cleavage permitted. Monoisotopic precursor mass values were used, and only doubly and triply charged precursor ions were considered. Data were validated in Scaffold (version 3.6) using a threshold of identification of at least 50% probability at the peptide level, at least 99% probability at the protein level and assignment of at least two unique, validated

peptides. These acceptance criteria resulted in an estimated protein false discovery rate of 0.1% for all datasets.

Data were converted using PRIDE Converter 2 [51], validated using PRIDE Inspector (version 2.5) [52]. Details of all identified proteins are provided in supplementary material Tables S1.

Proteins identified in the various cellular fractions and described to be components of the Geiger Adhesome [29] were hierarchically clustered on the basis of uncentred Pearson correlation using Cluster 3.0 (C Clustering Library, version 1.50) [53] and visualised using Java TreeView (version 1.1.6r2) [54]. Protein-protein interaction (PPIs) network analysis was performed using Cytoscape (version 3.2) [55]. Proteins were mapped onto a merged human interactome consisting of PPIs reported in the Protein Interaction Network Analysis platform Homo sapiens network (May 2014) [56]. Identified proteins were also compared to the proteins identified in various published dataset of isolated adhesion complexes [57-61].

Accession numbers

The proteomic data are deposited in the PRIDE database (http://www.ebi.ac.uk/pride) [62] under project accession no. PXD001870 and project DOI: 10.6019/PXD001870.

Statistics

The data sets with sufficient n numbers to run the D'Agnostino & Pearson Omnibus were all found to be normally distributed, therefore these data were analysed using unpaired Student's t-test. For data sets that the n numbers were too small for D'Agnostino & Pearson Omnibus normality test, we assumed normal distribution based on the appearance of the data. Statistical significance was analysed using Student's t-test with normal distribution and equal variance. $P < 0.05$ = *, $p < 0.01$ = **, $p < 0.005$ = ***. No statistical method was used to predetermine sample size.

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