

Supporting Figures

Visible light-induced specific protein reaction delineates early stages of cell adhesion

Rolle Rahikainen^a, Susan K. Vester^{b,†}, Paula Turkki^a, Chasity P. Janosko^c, Alexander Deiters^c, Vesa P. Hytönen^a and Mark Howarth^{b,d}

^aFaculty of Medicine and Health Technology, Tampere University, Arvo Ylpön katu 34, 33520, Tampere, Finland and Fimlab Laboratories, Biokatu 4, 33520, Tampere, Finland.

^bDepartment of Biochemistry, University of Oxford, South Parks Road, Oxford, OX1 3QU, UK.

^cDepartment of Chemistry, University of Pittsburgh, Pittsburgh, PA 15260, United States.

^dDepartment of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge, CB2 1PD, UK.

[†]Present address: Randall Centre for Cell and Molecular Biophysics, King's College London, New Hunt's House, London, SE1 1UL, UK.

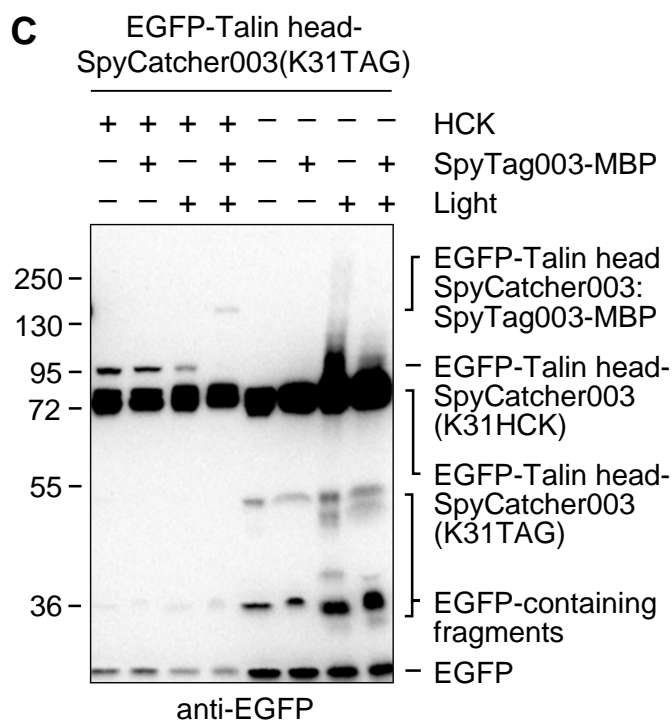
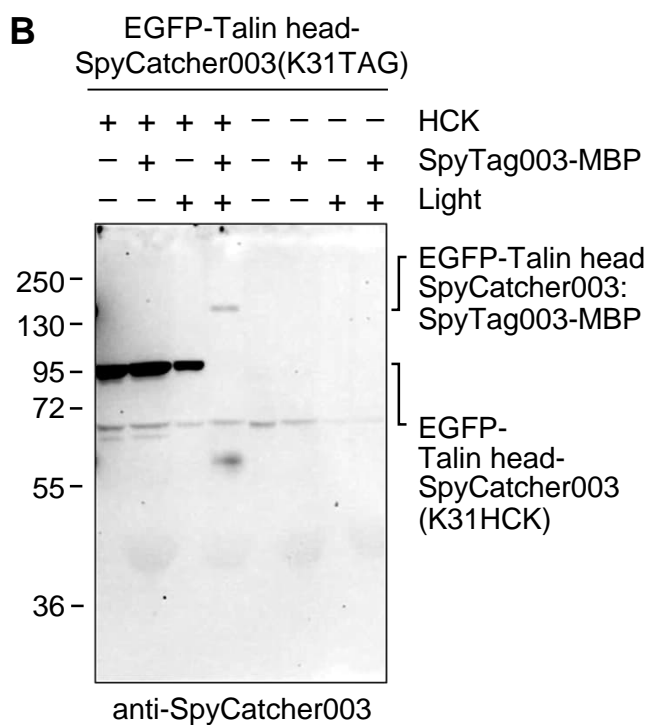
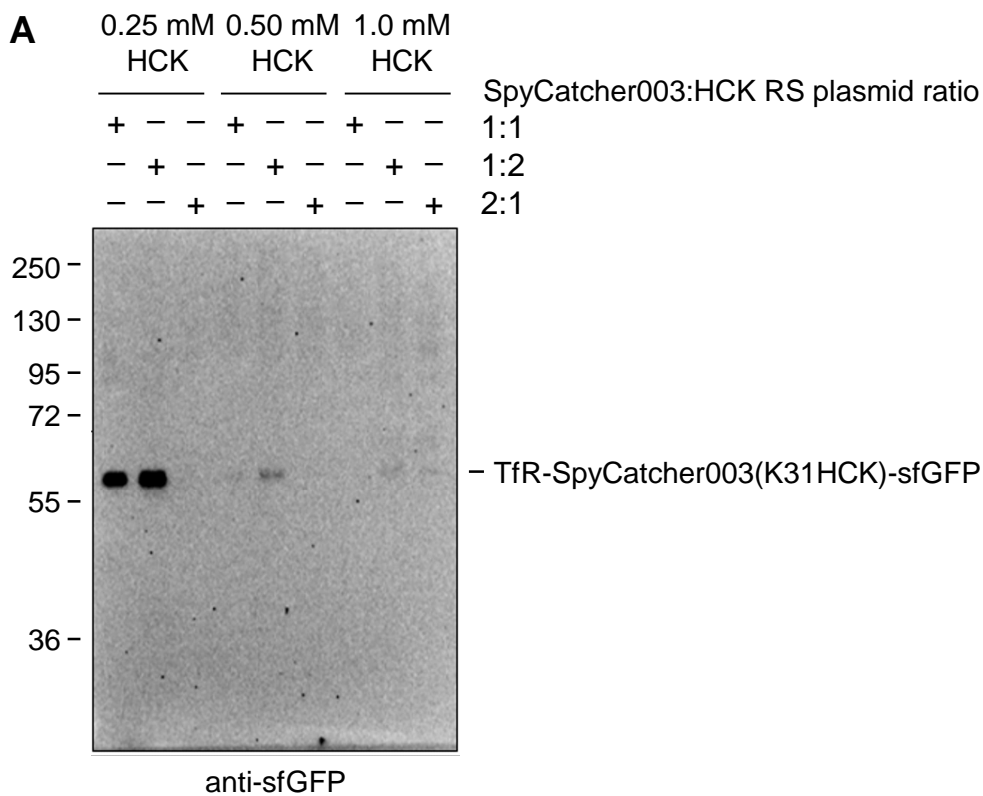


Figure S1. Validation of SpyCatcher003(K31HCK) photoactivation. (A) Titration of HCK concentration and plasmid ratios. HEK293T cells were transfected with different molar ratios of TfR-SpyCatcher003(K31TAG)-sfGFP and HCK RS plasmids and cultured in the presence of 0.25 mM – 1.0 mM HCK. Cell lysates were blotted for sfGFP, indicating successful incorporation of HCK in the polypeptide chain. (B) Photoactivation of covalent reactivity in EGFP-Talin head-SpyCatcher003(K31TAG) in cell lysates. Lysates of HEK293T cells transfected with EGFP-Talin head-SpyCatcher003(K31HCK) and HCK RS with or without HCK and light activation were mixed with recombinant SpyTag003-Maltose-binding protein (SpyTag003-MBP), before Western blotting with anti-SpyCatcher003. (C) As in panel B, but analyzed by Western blot against EGFP. GFP variants are highly stable to proteasomal degradation, so GFP-linked fragments are commonly observed to accumulate in cells.⁵⁴

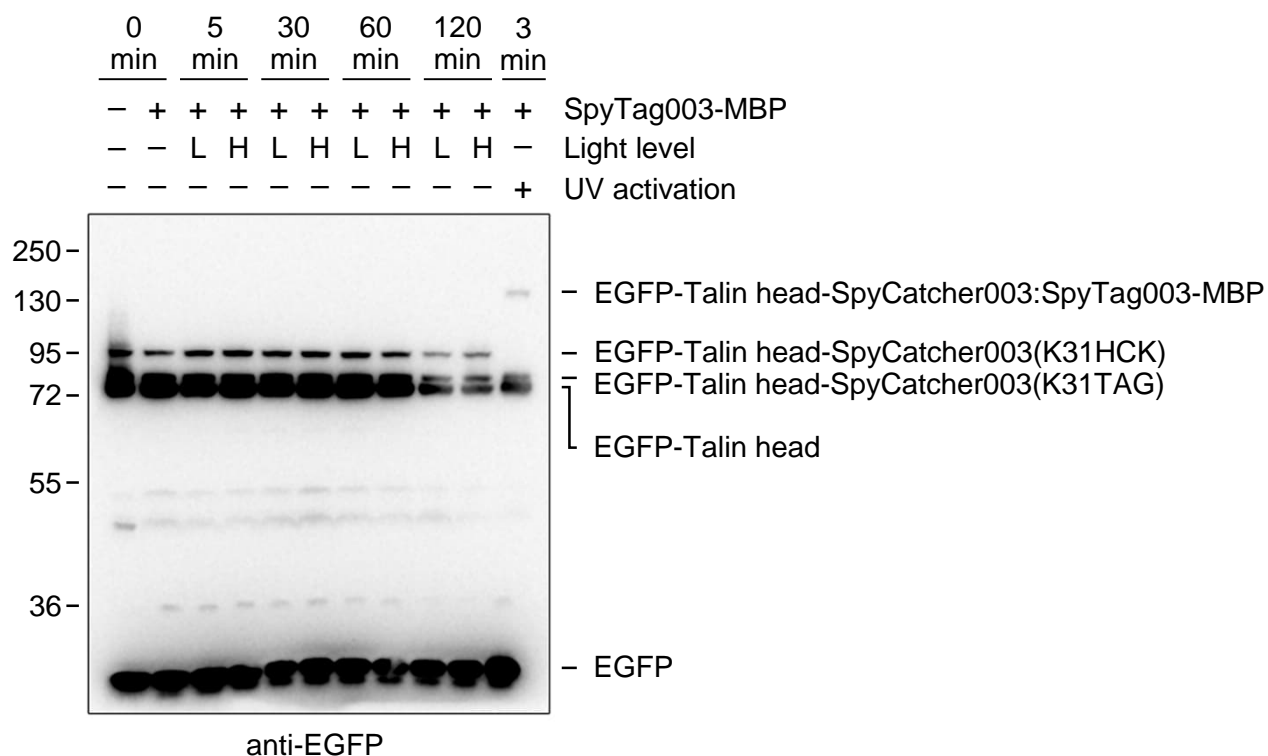


Figure S2. Photostability of SpyCatcher003(K31HCK) in ambient light. Photostability of SpyCatcher003(K31HCK) in typical laboratory lighting. Lysates of HEK293T cells expressing EGFP-Talin head-SpyCatcher003(K31HCK), HCK tRNAs and HCK RS were mixed with 50 μ M SpyTag003-MBP and incubated for the indicated time, either in a dimly lit cold-room (Low intensity, L) or by the laboratory window (High intensity, H). The positive control sample was activated with a UV transilluminator. Analysis by Western blot against EGFP. As in Fig. S1, GFP variants are highly stable to degradation by the proteasome, so GFP-linked fragments are observed to accumulate in cells.⁵⁴

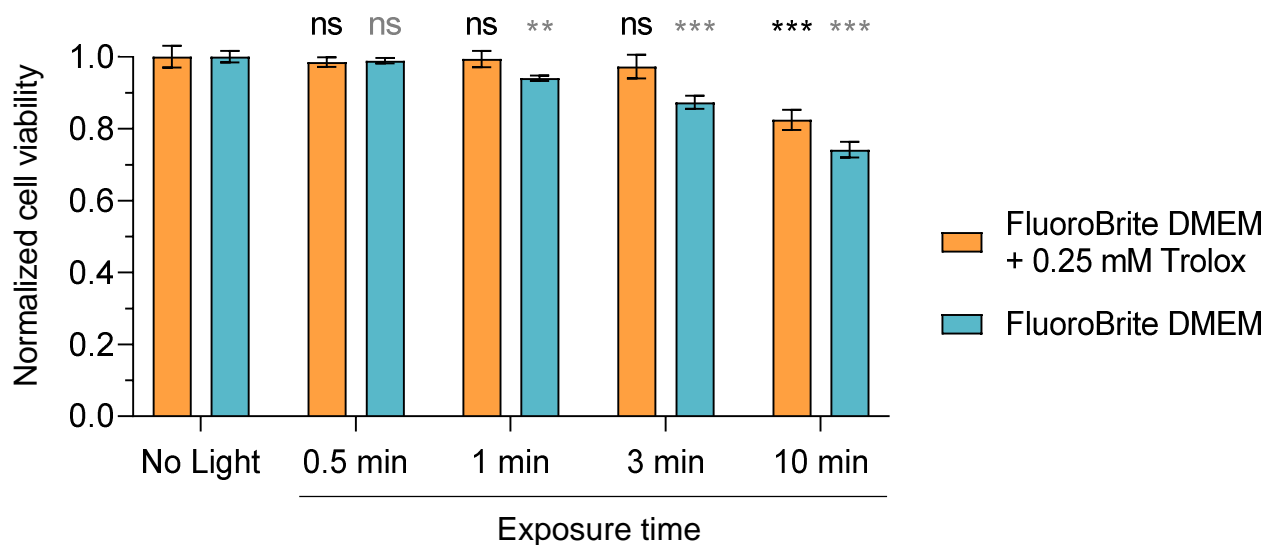


Figure S3. Assessment of potential phototoxicity from 405 nm illumination. Talin knock-out cells were cultured in FluoroBrite DMEM with Trolox (orange bars) or without (blue bars) and exposed to 405 nm wide-field light for the indicated time at 45 mW/cm², before resazurin-based cell viability assay. Viability is normalized to the No Light control, showing mean \pm 1 SD for 3 replicates. Statistical comparisons against No Light control cells are shown for cells cultured with Trolox (black font) or no Trolox samples (gray font) using One-way ANOVA and Dunnett's test: *** $p < 0.0001$, ** $p < 0.001$, ns $p > 0.05$.

A

SpyTag003	RGVPHIVMVDAYKRYK
SpyTag003(V114T, V116T)	RGVPHI T MTDAYKRYK
SpyTag003(M115A)	RGVPHIV A VDAYKRYK
SpyTag003(M115G)	RGVPHIV G VDAYKRYK

Mutated residue

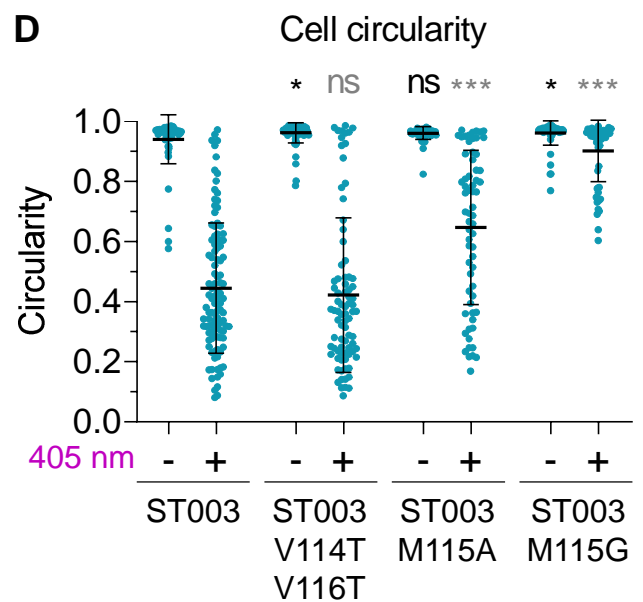
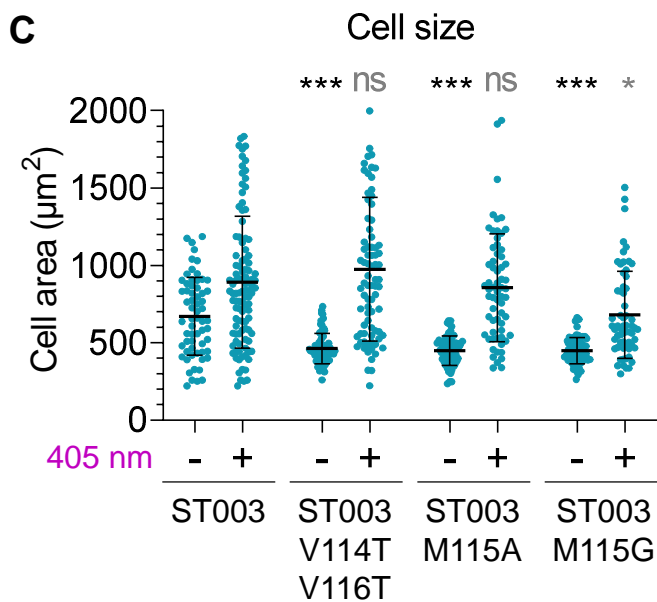
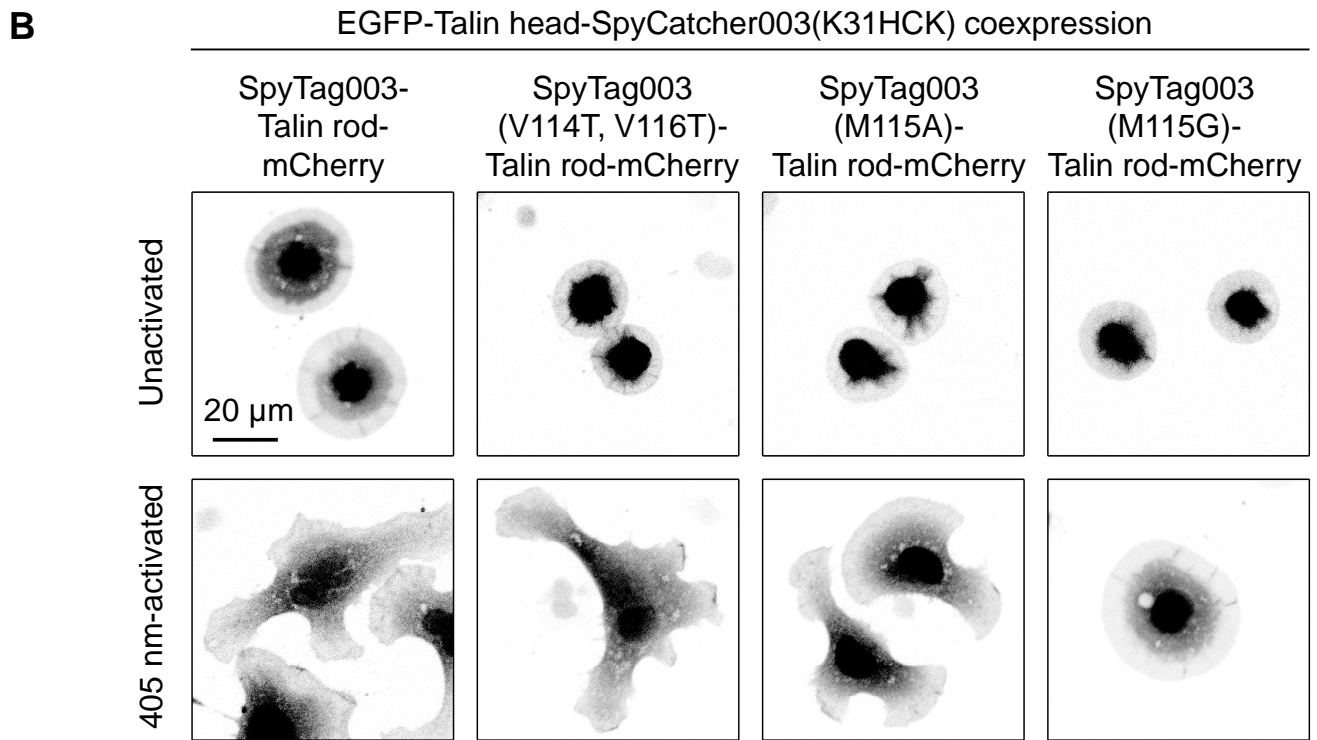


Figure S4. SpyTag003 mutants modify talin reconstitution after photoactivation. (A) SpyTag003 mutants designed to tune SpyCatcher003 covalent reconstitution. (B) Cell spreading and polarization after photoactivation. Talin knock-out cells expressing EGFP-Talin head-SpyCatcher003(K31HCK), variants of SpyTag003-Talin rod-mCherry, and HCK RS were activated for 1 min with 405 nm wide-field illumination, fixed after 90 min spreading, and imaged for EGFP by fluorescence microscopy. (C-D) Quantification of cell size and circularity following talin photoactivation as in B. Each blue circle is one cell, with black lines showing mean \pm 1 SD. Mutants are compared with unactivated (black font) or 405 nm-activated (gray font) SpyTag003 cells using One-way ANOVA and Dunnett's test: *** $p < 0.0001$, ** $p < 0.001$, * $p < 0.05$, ns $p > 0.05$, $n = 62-106$ cells. Data are representative of two experiments.

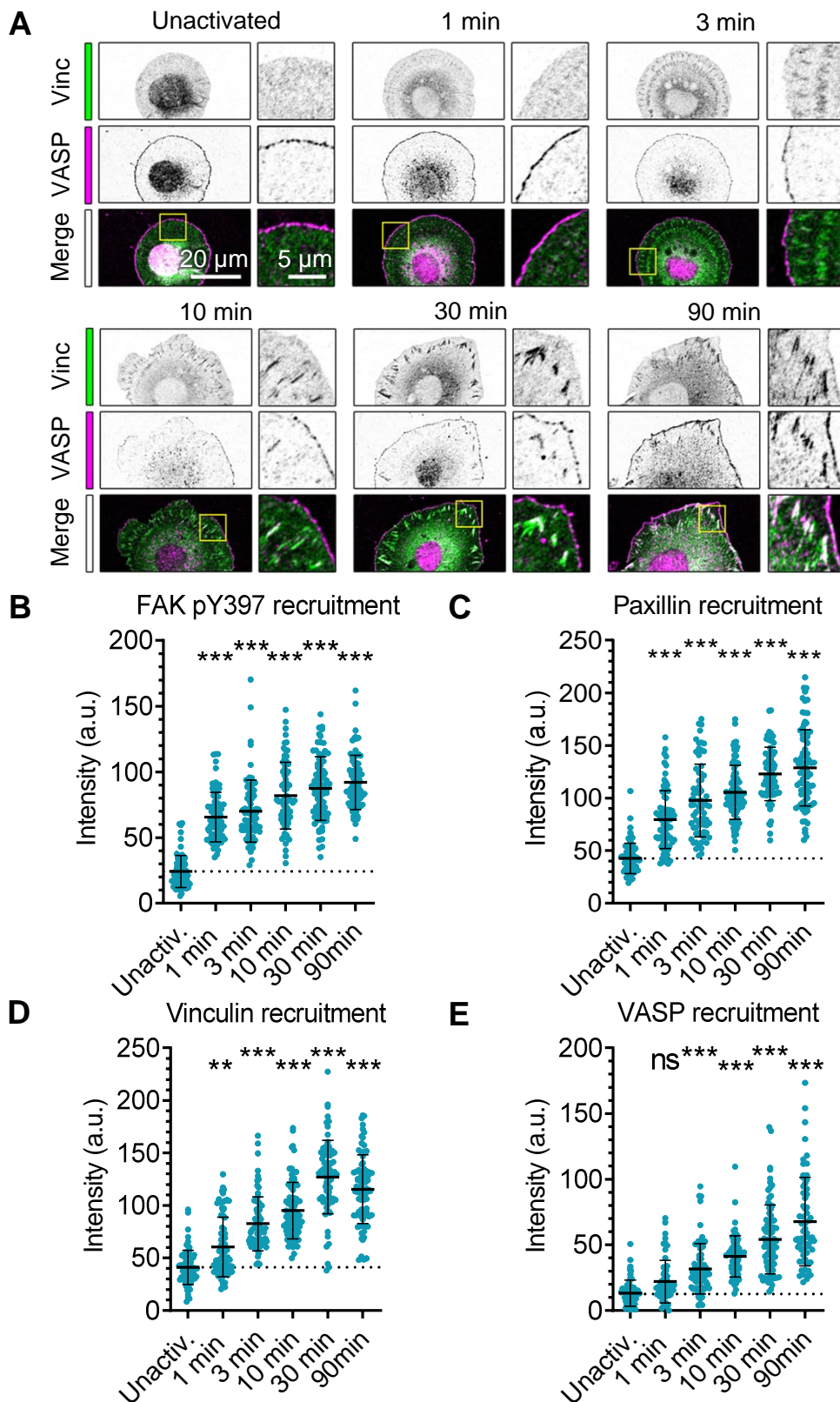


Figure S5. Time-course for adhesion assembly. (A) Vinculin and VASP recruitment after talin photoactivation. Talin knock-out cells were transfected as in Fig. 3E, activated at 405 nm and incubated for the indicated time. Cells were immunostained for VASP and vinculin. The yellow square is expanded in the right image. Vinculin and VASP are shown on their own in grayscale. Overlap of vinculin (green) and VASP (magenta) in the merge shows as white. (B-E) Recruitment of adhesion components following photoactivation. Raw adhesion intensities in arbitrary units (a.u.), to accompany normalized intensity in Fig. 3F. Each dot represents a single adhesion with mean \pm 1 SD indicated as horizontal lines. FAK pY397: $n = 75-81$ adhesions in 15-16 cells. Paxillin: $n = 70-86$ adhesions in 14-17 cells. Vinculin: $n = 74-81$ adhesions in 12-15 cells. VASP: $n = 60-86$ adhesions in 12-15 cells. Results were pooled from two independent experiments. One-way ANOVA with Dunnett's test. *** $p < 0.0001$, ** $p < 0.001$, ns $p > 0.05$

Movie S1 legend.

Movie of split talin photoactivation in live cells. Talin knock-out cells were transfected with LifeAct-mNeonGreen, Talin head-SpyTag003, SpyCatcher003(K31HCK)-Talin rod-mScarlet1 and HCK RS and cultured with 0.25 mM HCK for 16–18 h. Left column shows inverted signal of actin labeling with LifeAct-mNeonGreen, middle column shows bright-field, and right column shows a merge of bright-field (grayscale) with LifeAct-mNeonGreen (green) determined by confocal microscopy. Top row: at 1 min 50 s, a circular 4- μ m region was photoactivated by 405 nm illumination for 5 s, indicated by a magenta circle. Bottom row: cells treated in the same way but without 405 nm illumination. Photoactivation triggers initial local extension of the lamellipodium, followed by global cell spreading as reconstituted talin diffuses throughout the cell. The scale bar is 10 μ m and the movie is shown at 6 frames per s (30-fold sped-up).

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Methods

Plasmids and cloning

We used standard PCR methods with Q5 High-Fidelity 2× Master Mix (New England Biolabs) and Gibson assembly to perform cloning and site-directed mutagenesis. All open-reading frames were validated by Sanger sequencing (Source Bioscience). Residue numbers for SpyCatcher003 variants follow PDB ID: 2X5P.⁴³ Genscript plasmid cloning service was used to synthesize talin expression constructs for experiments in talin knock-out cells.

pcDNA3-mCherry-TAG-EGFP with one copy of *Desulfitobacterium hafniense* pyrrolysyl-tRNA_{CUA} (PylT) with G8U under the control of a U6 promoter (1× U6-PylT) and one copy of *Methanosarcina barkeri* PylT with U25C under the control of a H1 promoter (1× H1-PylT) has been described.⁴⁴ *D. hafniense* PylT G8U mutation restores canonical U8-A14 interaction and *M. barkeri* PylT U25C mutation stabilizes the anticodon stem and slightly increases amino acid incorporation efficiency.^{45,46} pcDNA3-EGFP-Talin head-SpyCatcher003 and pcDNA3-EGFP-Talin head-SpyCatcher003(K31TAG) (GenBank OR711469, Addgene ID 210023) with 1× U6-PylT and 1× H1-PylT were derived from pcDNA3-mCherry-TAG-EGFP and pEGFP-C1-EGFP-Talin head-SpyCatcher003 (GenBank MN527523, Addgene ID 133566),⁴⁷ which have been described. Talin head comprises amino acids 1-433 of mouse talin-1. The amber stop codon TAG instead of the lysine at position 31 of SpyCatcher003 allows for the incorporation of 7-hydroxycoumarin-caged lysine (HCK) when co-expressed with HCK tRNA synthetase (HCK RS) and PylT.

pcDNA3-TfR-SpyCatcher003-superfolder green fluorescent protein (sfGFP) and pcDNA3-TfR-SpyCatcher003(K31TAG)-sfGFP-MycTag-Ctag (GenBank OR711470, Addgene ID 210021) with 1× U6-PylT and 1× H1-PylT were derived from pcDNA3-mCherry-TAG-EGFP and pENTR4-TfR-sfGFP-SpyCatcher003 (GenBank MN433890, Addgene ID 133451),⁴⁷ which have been described. TfR comprises the Transferrin receptor transmembrane domain and cytosolic region with Y20C and F23A mutations to block internalization and allows for mammalian cell surface expression of SpyCatcher003.⁴⁷ In this context, sfGFP should only be expressed when HCK has successfully been incorporated in the growing polypeptide chain.

pcDNA3-LifeAct-mNeonGreen-IRES-Talin head-SpyTag003 (GenBank OR711471, Addgene ID 210024) with 1× U6-PylT and 1× H1-PylT was derived from pcDNA3-EGFP-Talin head-SpyCatcher003 and encodes mouse codon-optimized LifeAct-mNeonGreen, followed by encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) and Talin head-SpyTag003. pcDNA3-Talin head-SpyTag003 was derived from pcDNA3-LifeAct-mNeonGreen-IRES-Talin head-SpyTag003 by removing the cassette encoding LifeAct-mNeonGreen and IRES. pEGFP-C1 SpyCatcher003(K31TAG)-Talin rod (434-2541)-mScarletI (GenBank OR711472, Addgene ID 210022), pEGFP-C1 SpyTag003(V114T, V116T)-Talin rod (434-2541)-mCherry (Addgene ID 210027), pEGFP-C1 SpyTag003(M115A)-Talin rod (434-2541)-mCherry (Addgene ID 210025) and pEGFP-C1 SpyTag003(M115G)-Talin rod (434-2541)-mCherry (Addgene ID 210026) were derived from pEGFP-C1 SpyTag003-Talin rod (434-2541)-mCherry (GenBank MN527524 and Addgene ID 133567) previously described.⁴⁷ In the context of pEGFP-C1 SpyCatcher003(K31TAG)-Talin rod (434-2541)-mScarletI, mScarletI should only be expressed when HCK has successfully been incorporated in the growing polypeptide chain.

pE323-HCK RS contains the engineered HCK tRNA synthetase with five copies of PylT from *Methanosarcina mazei* with U25C under the control of a U6 promoter (5× U6-PylT) and has been described.⁴⁸ pET28a-SpyTag003-MBP (GenBank MN433888, Addgene ID 133450) has been previously described.⁴⁷

Hydroxycoumarin-caged lysine

HCK was synthesized as previously described⁴⁹ and made up at 100 mM in DMSO. HCK stock was stored at -20 °C protected from light and directly added into cell culture medium after transfection. For all No-HCK control samples, an equal volume of DMSO was added. To minimize the possibility of premature photoreaction, handling of HCK was performed under low light conditions in a tissue culture hood (with the hood light off and the room lights dimmed). During assays, HCK-incorporated samples were handled at ambient light but protected with aluminum foil and/or placed in a closed polystyrene ice box during incubations and waiting times.

To avoid possible phototoxicity of reactive oxygen species in the 405 nm activation of HCK in living cells, 0.25 mM Trolox (Sigma-Aldrich) was added as an antioxidant.⁵⁰ 100 mM stock of Trolox was prepared in 99.7 % (v/v) ethanol and stored at 4 °C.

Expression of caged SpyCatcher003 in HEK293T cells

HEK293T cells were maintained in complete Dulbecco's Modified Eagle Medium [DMEM (Sigma-Aldrich) supplemented with 10% (v/v) fetal bovine serum (FBS, Sigma-Aldrich), as well as 50 U/mL penicillin and 50 µg/mL streptomycin (Thermo Fisher)] under humidified conditions at 37 °C and 5% (v/v) CO₂. The day before transfection, 250,000 HEK293T cells were seeded in 2 mL complete DMEM in 6-well plates (Greiner). Cells were transfected with 2 µg plasmid DNA (1 part pcDNA3-EGFP-Talin head-SpyCatcher003 WT/K31TAG and 2 parts pE323-HCK RS) using 2 µL jetOPTIMUS (Polyplus) according to the manufacturer's instructions. HCK was added to a final concentration of 0.25 mM after transfection. For control wells, 0.25% (v/v) DMSO was added. Cells were grown for 48 h, before Western blot analysis with the plates wrapped in aluminum foil.

HEK293T cell lysis

HEK293T cells in 6-well plates were washed three times with 1 mL phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4), then incubated in 200 µL ice-cold lysis buffer [25 mM Tris-HCl pH 7.5 (pH adjusted at 25 °C) + 150 mM NaCl + 5% (v/v) glycerol + 1% (v/v) Triton X-100, supplemented with cComplete Mini EDTA-free Protease Inhibitor Cocktail (Roche) and 1 mM phenylmethylsulfonylfluoride (PMSF, Thermo Fisher)] for 10 min on ice in a closed ice box. The cells were scraped off, transferred to 1.5 mL tubes and centrifuged at 20,000 g for 10 min at 4 °C. The supernatant was used immediately or stored at -20 °C. Protein concentrations were determined by bicinchoninic acid assay (BCA Protein Assay Kit, Thermo Fisher) in 96-well format using 5 µL cell lysate diluted with 5 µL lysis buffer and correcting for lysis buffer, with bovine serum albumin (BSA) standards at 0, 125, 250, 500, 750, 1000, 1,500 or 2,000 µg/mL. Incubation with 200 µL BCA working solution was performed at 37 °C for 30 min. A₅₆₂ measurement was performed using a FLUOstar Omega plate reader with FLUOstar Omega software version 5.10 R2 (both BMG Labtech) at 25 °C.

Talin knock-out cell lysis

Tln1^{-/-}*Tln2*^{-/-} mouse kidney fibroblasts were a kind gift from Prof. Carsten Grashoff, University of Münster, and have been previously described.⁵¹ Briefly, mice carrying *loxP* flanked *Tln1* alleles (*Tln1*^{fllox/fllox}) were crossed with a mouse strain with nullizygous *Tln2* alleles (*Tln2*^{-/-}).

Kidney fibroblasts were isolated from the resulting *Tln1*^{flox/flox} *Tln2*^{-/-} mice and immortalized by retroviral transduction of SV40 large T antigen. Adenoviral transduction of Cre recombinase was used to delete the floxed *Tln1* alleles.⁵¹ Talin knock-out cells were maintained in complete FluoroBrite medium [FluoroBrite DMEM (A1896701, Thermo Fisher) supplemented with 10% (v/v) FBS (10270106, Gibco) and 1% (v/v) GlutaMax (35050061, Thermo Fisher)] under humidified conditions at 37 °C and 5% (v/v) CO₂. The cell-line was regularly tested for mycoplasma contamination.

For immunoblotting in Fig. 1E, Talin knock-out cells cultured on 60 mm dishes were briefly washed with PBS and lysed with 150 µL of 5× SDS-PAGE sample buffer: 250 mM Tris-HCl, pH 6.8, 10% (w/v) SDS, 30% (v/v) glycerol, 0.02% (w/v) bromophenol blue, 25 mM tris(2-carboxyethyl)phosphine (TCEP). Lysed cells were collected to the dish corner with cell scrapers and the lysate was transferred to 1.5 mL tubes cooled on ice. Samples were heated for 5 min at 95 °C, briefly spun down to collect liquid, and stored at -20 °C before blotting.

Initial optimization of caged SpyCatcher003 expression in HEK293T cells

Optimization was performed in 24-well plates (Greiner). HEK293T cells were seeded at 50,000 cells/mL in 0.5 mL complete DMEM. Cells were transfected with 0.25 µg total DNA using 0.25 µL jetOPTIMUS, at molar ratios 1:1, 1:2 and 2:1 [pcDNA3-TfR-SpyCatcher003(K31TAG)-sfGFP to pE323-HCK RS]. Directly after transfection, HCK was added to a final concentration of 0.25 mM, 0.5 mM or 1 mM. Western blot analysis was performed 48 h after transfection. For lysis, HEK293T cells were washed three times with 0.5 mL PBS, then incubated in 75 µL ice-cold lysis buffer and processed as described above.

Protein purification by Ni-NTA

SpyTag003-MBP was expressed in *E. coli* EXPRESS BL21(DE3) (Lucigen) and purified by Ni-NTA at 4 °C. Bacterial cell pellets of SpyTag003-MBP were resuspended in Ni-NTA buffer (50 mM Tris-HCl pH 7.8 + 300 mM NaCl) supplemented with cOmplete Mini EDTA-free Protease Inhibitor Cocktail (Roche) and 1 mM PMSF. Cells were lysed by sonication on ice at 50% duty cycle for 4 × 1 min with 1 min rest between runs. Cell lysates were clarified by centrifugation at 30,000 g for 30 min at 4 °C, then incubated with Ni-NTA agarose (Qiagen) for 1 h at 4 °C with rolling. Resin was pelleted by centrifugation, then washed with 20 column volumes (CV) of Ni-NTA buffer and Ni-NTA wash buffer (50 mM Tris-HCl + 300 mM NaCl + 10 mM imidazole at pH 7.8). The resin was transferred to Econo-Pac Chromatography Columns (Bio-Rad) and washed with a further 20 CV of Ni-NTA wash buffer by gravity flow. Elution was performed with Ni-NTA elution buffer (50 mM Tris-HCl + 300 mM NaCl + 200 mM imidazole at pH 7.8) by incubating the resin with 4 × 1 CV for 5 min. SpyTag003-MBP was dialyzed into PBS in three dialysis steps using 3.5 kDa molecular weight cut-off (MWCO) dialysis tubing (Spectrum Labs). Protein concentrations were determined by A₂₈₀ measurement on a NanoDrop One (Thermo Fisher) using NanoDrop One software version 1.4.2, with extinction coefficients predicted by ExPASy ProtParam.⁵²

Intracellular 405 nm uncaging for Western blot

Talin knock-out cells were transfected with pcDNA3-EGFP-Talin head-SpyCatcher003(K31TAG), pEGFP-C1 SpyTag003-Talin rod (434-2541)-mCherry and HCK RS plasmids as described below, and plated on a 60 mm dish at 2 × 10⁶ cells per dish. After an initial 2 h recovery phase at 37 °C and 5% (v/v) CO₂, FluoroBrite DMEM was replaced with FluoroBrite DMEM supplemented either with 0.25 mM HCK (100 mM stock in DMSO) and 0.25 mM Trolox or with 0.25 % DMSO and 0.25 mM Trolox. Incubation at 37 °C and 5% (v/v) CO₂ was continued for 17 h, after which cells were washed twice with FluoroBrite DMEM supplemented with 0.25 mM Trolox and incubated at 37 °C and 5% (v/v) CO₂ for 15 min. For

intracellular SpyCatcher003(HCK) uncaging, culture dishes were placed for 1 min on 2 × 6 W 405 nm LED panels (Sovol) with total intensity of 45 mW/cm² at the plane of cells. Activated cells were allowed to recover at 37 °C and 5% (v/v) CO₂ for 5 min and the activation cycle was repeated two more times for a total activation time of 3 min. Cells were incubated at 37 °C and 5% (v/v) CO₂ for 1h 30 min and lysed for Western blot as described below.

Uncaging and reaction of SpyCatcher003 in cell lysates

Equal amounts of protein lysates as determined by BCA assay were transferred to PCR tubes. To test spontaneous uncaging by ambient light, samples were incubated exposed to room light in a cold room or next to a window on ice for the indicated amounts of time. For uncaging, designated samples were irradiated on a ChemiDoc XRS+ using the Ethidium Bromide setting for the indicated amounts of time at 25 °C. Samples were transferred back to ice to chill and incubated with 50 μM SpyTag003-MBP at 4 °C for 1 h. Reaction was stopped by mixing with 6× SDS loading buffer [234 mM Tris-HCl pH 6.8, 24% (v/v) glycerol, 120 μM bromophenol blue, 234 mM SDS, supplemented with 60 mM dithiothreitol (DTT) for reduced samples] and heating at 95 °C for 5 min in a Bio-Rad C1000 thermal cycler.

Western blot

SDS-PAGE in Fig. 1E was performed using Bio-Rad Mini-PROTEAN Tetra Vertical Electrophoresis Cell with Bio-Rad 4-15% Mini-PROTEAN TGX Precast gels. Gels were run with in 24 mM Tris base, 192 mM glycine, 3.5 mM SDS at 120 V. Proteins were transferred to nitrocellulose membranes using Bio-Rad Trans-Blot Turbo Transfer System at 1.3 A and 25 V for 10 min. Membranes were blocked with Bio-Rad EveryBlot blocking solution for 1 h 30 min at 25 °C. Anti-EGFP antibody (SICGEN ab0020) was diluted 1:1,000 and anti-mScarlet (also recognizing mCherry) antibody (SICGEN ab9088) was diluted 1:2,000 directly into the blocking solution. Primary antibodies were incubated for 16 h at 4 °C with gentle agitation. Membranes were washed four times in 0.05% TBST [50 mM Tris-HCl, 150 mM NaCl, pH 7.4 + 0.05% (v/v) Tween 20] for 5 min at 25 °C. Secondary antibody detection was performed by staining for 1 h 30 min at 25 °C with Licor IRDye 680RD donkey anti-goat IgG (Licor 926-68074) diluted 1:20,000 into Bio-Rad EveryBlot blocking buffer. Membranes were washed five times in 0.05% TBST before a final wash with TBS. Membranes were imaged using Licor CLx imager with 84 μm resolution and medium image quality.

SDS-PAGE in Fig. S1A-C and Fig. S2 was performed using an XCell SureLock system (Thermo Fisher) with 10% (w/v) polyacrylamide gels. Gels were run in 24 mM Tris base, 192 mM glycine, 3.5 mM SDS at 200 V. Proteins were transferred onto nitrocellulose membranes by dry transfer using an iBlot 2 gel transfer device (Thermo Fisher) at 25 V for 10 min. Membranes were blocked for 1 h at 25 °C in 5% (w/v) skimmed milk in PBS. The primary antibody was diluted in 2.5% (w/v) skimmed milk in 0.05% PBST [PBS + 0.05% (v/v) Tween 20], with mouse anti-GFP (Thermo Fisher, MA5-15256, clone GF28R) at 1:2,000 or mouse anti-SpyCatcher003 serum⁵³ at 1:300. Incubation was performed overnight at 4 °C. Membranes were washed three times in 0.1% PBST [PBS + 0.1% (v/v) Tween 20] for 5 min at 25 °C. Secondary antibody detection was performed by staining with goat anti-mouse IgG Horseradish Peroxidase (Sigma-Aldrich, A4416) at 1:5,000 for 1 h at 25 °C in 2.5% (w/v) skimmed milk in 0.05% PBST. After three further washes in 0.1% PBST and one wash in PBS at 25 °C, membranes were developed at 25 °C with SuperSignal West Pico Plus Chemiluminescent Substrate (Thermo Fisher) and imaged using a ChemiDoc XRS+ imager (Bio-Rad). In immunoblotting with anti-EGFP antibody, we observed EGFP-containing degradation products of EGFP-Talin head-SpyCatcher003(K31TAG), consistent with reported high stability of EGFP in cells.⁵⁴ A colon is used to indicate products linked by Tag/Catcher isopeptide bond formation.

Expression of caged SpyCatcher003 in talin knock-out cells

Talin knock-out cells were transiently transfected with Neon Transfection System electroporator (Thermo Fisher) according to the manufacturer's instructions. Briefly, 10^6 cells in 100 μ L were transfected with 12.5 μ g of total plasmid DNA at 2:2:1 molar ratio of pE323-HCK RS, SpyCatcher003(K31TAG)-Talin rod (434-2541)-mScarletI and Talin head-SpyTag003 plasmids, respectively. The electroporation parameters were 1,400 V, 30 ms and 1 pulse.

Phototoxicity assay for 405 nm wide-field activation

Talin knock-out cells were plated on Nunclon Delta-Treated 96-well plates (Nunc) at 50,000 cells per well. Cells were incubated at 37 °C and 5% (v/v) CO₂ for 1 h in 100 μ L FluoroBrite DMEM (Thermo Fisher) supplemented with either 0.25 mM Trolox (Sigma-Aldrich, 100 mM stock in ethanol) or matching ethanol concentration at 0.25 % (v/v). Cells were exposed to 405 nm light (45 mW/cm²) from a wide-field LED light source for 0.5-10 min and allowed to recover for 15 min at 37 °C and 5% (v/v) CO₂. 20 μ L resazurin-based CellTiter-Blue reagent (Promega) was added per well, before the plates were gently mixed and incubated for 4 h at 37 °C and 5% (v/v) CO₂. Plates were cooled to 21 °C and 80 μ L samples of culture media were transferred to black half-area microplates (Nunc). Media fluorescence at 550 nm excitation wavelength and 590 nm emission wavelength was measured using a Tecan Spark microplate reader. The linearity of CellTiter-Blue fluorescence signal at the used cell density and incubation time was confirmed with control experiments. Media-only controls with FluoroBrite DMEM supplemented with either 0.25 mM Trolox or 0.25% (v/v) ethanol and exposed for 0-10 min at 405 nm were used to correct results for changes in media fluorescence. All results were normalized by the fluorescence of unexposed cells cultured in matching media conditions.

Live-cell imaging and on-stage 405 nm photoactivation

Zeiss high-performance 170 μ m-thick coverslips were washed with 2% (v/v) Hellmanex-III (Sigma-Aldrich) in a bath sonicator at 40 °C for 20 min (Finnsonic), rinsed with deionized water, and sonicated again in 96% (v/v) ethanol. Coverslips were rinsed with deionized water, air-dried, and attached to perforated 35 mm polystyrene dishes (MatTek). Attached coverslips were coated with 25 μ g/mL human fibronectin in PBS for 30 min at 37 °C and washed twice with PBS. Fibronectin was purified from human plasma preparate (Octaplas) using gelatin affinity chromatography (Gelatin Sepharose 4B; GE Healthcare) as previously described.⁵⁵

Transfected talin knock-out cells were plated on glass bottom dishes (166,000 cells per dish) in 120 μ L complete FluoroBrite medium and were incubated at 37 °C and 5% (v/v) CO₂ for 1 h to allow cell attachment. Dishes were filled with complete FluoroBrite medium and the incubation at 37 °C and 5% (v/v) CO₂ continued for 2 h. Media was replaced with 800 μ L of FluoroBrite-Trolox-HCK (complete FluoroBrite medium supplemented with 0.25 mM Trolox and 0.25 mM HCK) and cells were incubated at 37 °C and 5% (v/v) CO₂ for 16-18 h. Before imaging, media was replaced with FluoroBrite-Trolox (complete FluoroBrite medium supplemented with 0.25 mM Trolox) and the dish was mounted to a humidified 37 °C and 5% (v/v) CO₂ incubator on the microscope stage.

For live-cell imaging and on-stage 405 nm photoactivation, we used a Nikon Eclipse Ti2-E inverted microscope equipped with A1R+ laser scanning confocal (Nikon), Perfect Focus System (Nikon) and Nikon SR HP Plan Apo 100 \times /1.35 Silicone immersion objective. Single cells were imaged at 80 nm pixel size and 67.7 μ m pinhole size using 488 nm solid state laser (0.25 - 0.5% laser) for mNeonGreen excitation and 561 nm solid state laser (0.2 - 0.5% laser) for mScarletI excitation. We used 560 nm long-pass dichroic mirror and 525/50 nm band-

pass filter for mNeonGreen emission and 640 nm long-pass dichroic mirror and 595/50 nm band-pass filter for mScarletI emission.

Differential Interference Contrast (DIC) images were captured using transmitted 488 nm excitation light. Images were captured at 5 s intervals for 23 images before photoactivation. For HCK photoactivation, a circular region of 4.0 μm was activated for 5 loops (4.8 s total) with 10.5 μW (5%) 405 nm laser. Imaging was then immediately continued for 73 images at 5 s intervals.

“Unactivated” cells were cultured with HCK but not 405 nm activated. In Fig. 3, “No HCK” cells were cultured without HCK, activated with 405 nm, and allowed to spread for 90 min. When green and magenta images are merged, the overlapping regions appear white.

Lamellipodium extension analysis

Fiji distribution of ImageJ 1.53t⁵⁶ was used to analyze lamellipodium extension after HCK photoactivation. A 10-pixel (0.8 μm) wide line perpendicular to the cell edge was drawn across the cell lamellipodium at the site of photoactivation. A kymograph of mNeonGreen intensity was created along this line, covering all 96 frames of the time-series. Using free-hand selection tool, the movement of the lamellipodium edge over time was traced in the kymograph by following the shift in the position of mNeonGreen intensity. The selection created in this way was used to quantify the number of pixels above the line (inside cell) and below the line (outside cell) for all columns of pixels, each representing a single image captured at 5 s intervals. For relative lamellipodium extension, the results were normalized with the initial position of the lamellipodium edge. To calculate the lamellipodium extension rate, sharp irregularities in the extension data were smoothed by averaging with a 4-frame sliding window and the rate of change over time was calculated.

Actin flow-rate analysis

To improve the clarity of LifeAct-mNeonGreen live-cell imaging data, Huygens Essential 23.04 (Scientific Volume Imaging) with classic maximal likelihood estimation (CMLE) deconvolution algorithm was used. Deconvoluted images were analyzed using Fiji distribution of ImageJ 1.53t.⁵⁶ Briefly, at the site of photoactivation, a 20-pixel (1.6 μm) wide line perpendicular to the cell edge was drawn across the lamellipodium to create 5 parallel kymographs, each representing the mean intensity of 4 pixels in the original image. These kymographs were used to measure manually the slope of actin flow at 30 s intervals (6-pixel spacing in the kymographs) using the line selection tool. Of the 5 parallel kymographs, at each time-point, the one with the best image clarity was selected for analysis. The slopes measured in this way were used to calculate actin flow rate (nm/s) using trigonometry.

Widefield 405 nm activation of talin knock-out cells

Transfected talin knock-out cells were plated on 60 mm dishes (Nunc) in complete FluoroBrite medium and allowed to recover for 3 h at 37 °C and 5% (v/v) CO₂. The media was replaced with FluoroBrite-Trolox-HCK and the incubation at 37 °C and 5% (v/v) CO₂ was continued for 16-18 h. After the addition of FluoroBrite-Trolox-HCK, all subsequent culture steps were performed in low light conditions. Cells were rinsed with PBS, detached using TrypLE Express (Thermo Fisher) and plated in FluoroBrite-Trolox-HCK on fibronectin coated glass-bottom dishes, prepared as described above. After 1 h incubation at 37 °C and 5% (v/v) CO₂, cells were rinsed once with FluoroBrite-Trolox and incubated at 37 °C and 5% (v/v) CO₂ for 15 min to wash out residual HCK. For HCK uncaging, dishes were placed on top of 2 × 6 W 405 nm LED panels (Sovol) with 45 mW/cm² total intensity and photoactivated for 60 s. Photoactivated cells were incubated at 37 °C and 5% (v/v) CO₂ and fixed at defined timepoints. For fixation,

media was aspirated and 4% (w/v) paraformaldehyde in PBS was added for 20 min at 22 °C. Cells were washed 4 times with PBS and stored at 4 °C.

Immunostaining

Fixed cells were permeabilized with 0.2% (v/v) Triton X-100 in PBS for 5 min at 22 °C. Non-specific antibody binding was inhibited with blocking buffer [5% (v/v) fetal calf serum (Gibco), 1% (w/v) bovine serum albumin (Sigma-Aldrich) and 0.05% (v/v) Triton X-100 (Sigma-Aldrich) in PBS] for 30 min at 22 °C. Primary antibodies were diluted in the blocking buffer and incubated for 90 min at 22 °C with 1:400 anti-vinculin V9131 (Sigma-Aldrich, RRID:AB_477629), 1:100 anti-VASP HPA005724 (Sigma-Aldrich, RRID:AB_1858721), 1:200 anti-FAK pY397 ab81298 (Abcam, RRID:AB_1640500), or 1:100 anti-paxillin BD610051 (BD Biosciences, RRID:AB_397463). Cells were washed 4 times for 10 min with PBS. Fluorescently-labeled secondary antibodies were diluted in the blocking buffer at 1:250 and incubated on samples for 90 min: Goat anti-Mouse IgG (H+L) AlexaFluor Plus 488 A32723 (Thermo Fisher, RRID:AB_2633275), Goat anti-Rabbit IgG (H+L) AlexaFluor Plus 488 A32731 (Thermo Fisher, RRID:AB_2633280), Goat anti-Mouse IgG (H+L) AlexaFluor Plus 647 A32728 (Thermo Fisher, RRID:AB_2633277), Goat anti-Rabbit IgG (H+L) AlexaFluor Plus 647 A32733 (Thermo Fisher RRID:AB_2633282). Cells were washed 4 times for 10 min with PBS and covered with a clean coverslip using Prolong Glass antifade mounting media (Thermo Fisher). The mounting media was cured at 24 °C for a minimum of 40 h before imaging.

Cell size and morphology analysis

For cell size and morphology analysis, mScarletI signal of fixed samples was imaged using a Nikon Eclipse Ti2-E inverted microscope equipped with A1R+ laser scanning confocal unit (Nikon) and Nikon Apo 60×/1.40 Oil λ S DIC N2 objective. For *No HCK* samples (Fig. 3B,C) not expressing mScarletI because of polypeptide chain termination, anti-vinculin antibody staining was imaged instead.

Cell area and circularity were analyzed in Fiji distribution of ImageJ 1.53t⁵⁶ using the free-hand selection tool to trace cell boundaries. Cell circularity was calculated with the formula: $\text{circularity} = 4\pi(\text{area}/\text{perimeter}^2)$. Cells with partially detached lamellipodium or extremely high mScarletI signal were excluded from the analysis. For each timepoint, 45 to 110 cells from 2 independent experiments were analyzed.

Adhesion intensity analysis

For adhesion intensity analyses, immunostained cells were imaged using a Zeiss Axio Observer.Z1 equipped with Zeiss LSM800 laser scanning confocal unit and Zeiss Plan-Apochromat 63×/1.40 oil immersion objective. 107 $\mu\text{m} \times 107 \mu\text{m}$ fields were imaged at 70 nm pixel size and 200 nm Z-stack interval, maintaining fixed laser intensities and detector gains for all samples.

Adhesion protein localization to adhesion sites was analyzed using Fiji distribution of ImageJ 1.53t.⁵⁶ The Z-stack slices with the highest adhesion/cytosol contrast were selected for the analysis. For each cell, the 5 adhesions with the highest fluorescence intensity were masked using the freehand selection tool and the mean intensity of each selected region was measured. For each image, the intensity of background control region outside the cell was measured and its intensity value was subtracted from all adhesion intensity values of the same image. For Fig. 3F, adhesion intensity values were normalized by the mean intensity measured at the 90 min timepoint. For unactivated cells, no clear adhesion structures were observed and the reported intensity values represent mean lamellipodium intensity. For each adhesion protein, 60-85 adhesions in 12-17 cells from two independent experiments were analyzed. Statistical

significance of results was tested using one-way ANOVA with Dunnett's post-test in GraphPad Prism 9.0.0 (GraphPad Software). All other samples were compared with the unactivated samples. p-values below 0.05 were considered statistically significant. p-values above 0.05 were considered non-significant (ns). Samples in the same experiment were prepared, imaged and analyzed under uniform conditions.

Visualization of structural models and reaction schematics

The structural model for SpyTag/SpyCatcher is based on PDB ID 4MLI⁵⁷ and visualized in PyMOL version 2.2.2 (Schrödinger). SpyTag/SpyCatcher reaction was drawn in ChemSketch 2022.2.3 (ACD/Labs) and HCK uncaging reaction in ChemDraw 20.1.1.125 (PerkinElmer).

Data availability

Sequences of constructs are available in GenBank, as described in the section "Plasmids and Cloning". Indicated plasmids have been deposited in the Addgene repository (https://www.addgene.org/Vesa_Hytonen/). Further information and request for resources and reagents should be directed to and will be fulfilled by the lead contact, M.H. (mh2186@cam.ac.uk). Requests for unnatural amino acid resources should be directed to A.D. (deiters@pitt.edu).

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