Developmental Cell

Supplemental Information

Formin-like 2 Promotes β1-Integrin Trafficking and Invasive Motility Downstream of PKCα

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Supplemental Figure S1

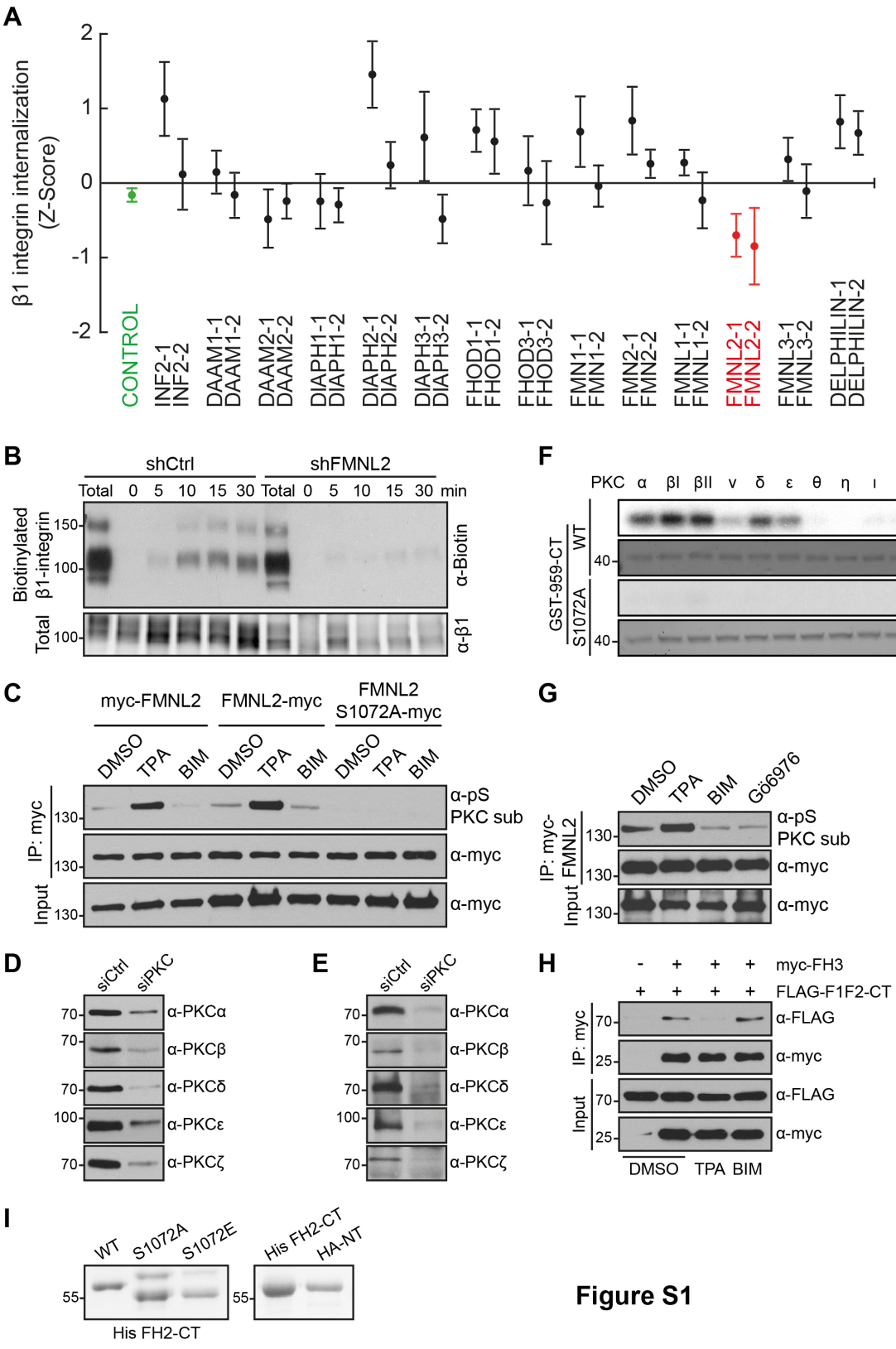

Figure S1

Figure S1. FMNL2 Regulates β1-integrin Endocytosis and Is Phosphorylated by PKCα (Related to Figure 1)

(A) Differential effects of human formins on β1-integrin internalization. After silencing of formin genes, β1-integrin endocytosis was measured using antibody-based β1-integrin endocytosis assay. Endocytosis values are Z-scored among the whole dataset. Error bar: ± SEM (n=6). (B) Representative Western blots showing the immunoprecipitated total β1-integrin or endocytosed biotinylated β1-integrin under shCtrl or shFMNL2 conditions in HeLa cells. (C) myc-FMNL2, FMNL2-myc or FMNL2-S1072A-myc was expressed in HEK293 cells and immunoprecipitated after DMSO, TPA (20 nM) or BIM (2 μM) treatment for 30 min. Phosphorylated FMNL2 was detected by phospho-(Ser) PKC substrate antibody. (D, E) HEK293 cells were transfected with FLAG-FMNL2 and siRNA against different PKC isozymes respectively. siPKCβ targets both PKCβI and PKCβII. Knockdown of different PKC isozymes are shown for Figures 1F and G. (F) In vitro kinase assays using purified PKC isozymes and wild type or S1072A mutant FMNL2 C-terminal fragments (GST-959-CT). Autoradiography and coomassie blue stained gels are shown. (G) HEK293 cells expressing myc-FMNL2 were immunoprecipitated after DMSO, 20 nM TPA, 2 μM BIM or 1 μM Gö6976 treatment. Phosphorylation of FMNL2 by PKC was detected. (H) Immunoprecipitations of HEK293 cells expressing FLAG-FMNL2-F1F2-CT and myc-FMNL2-FH3 after DMSO, 20 nM TPA or 2 μM BIM treatment. (I) Coomassie blue stained gels of the purified proteins used in Figures 1J and 3E. Numbers on the left indicate the molecular weights (kD) (B~I).

Supplemental Figure S2

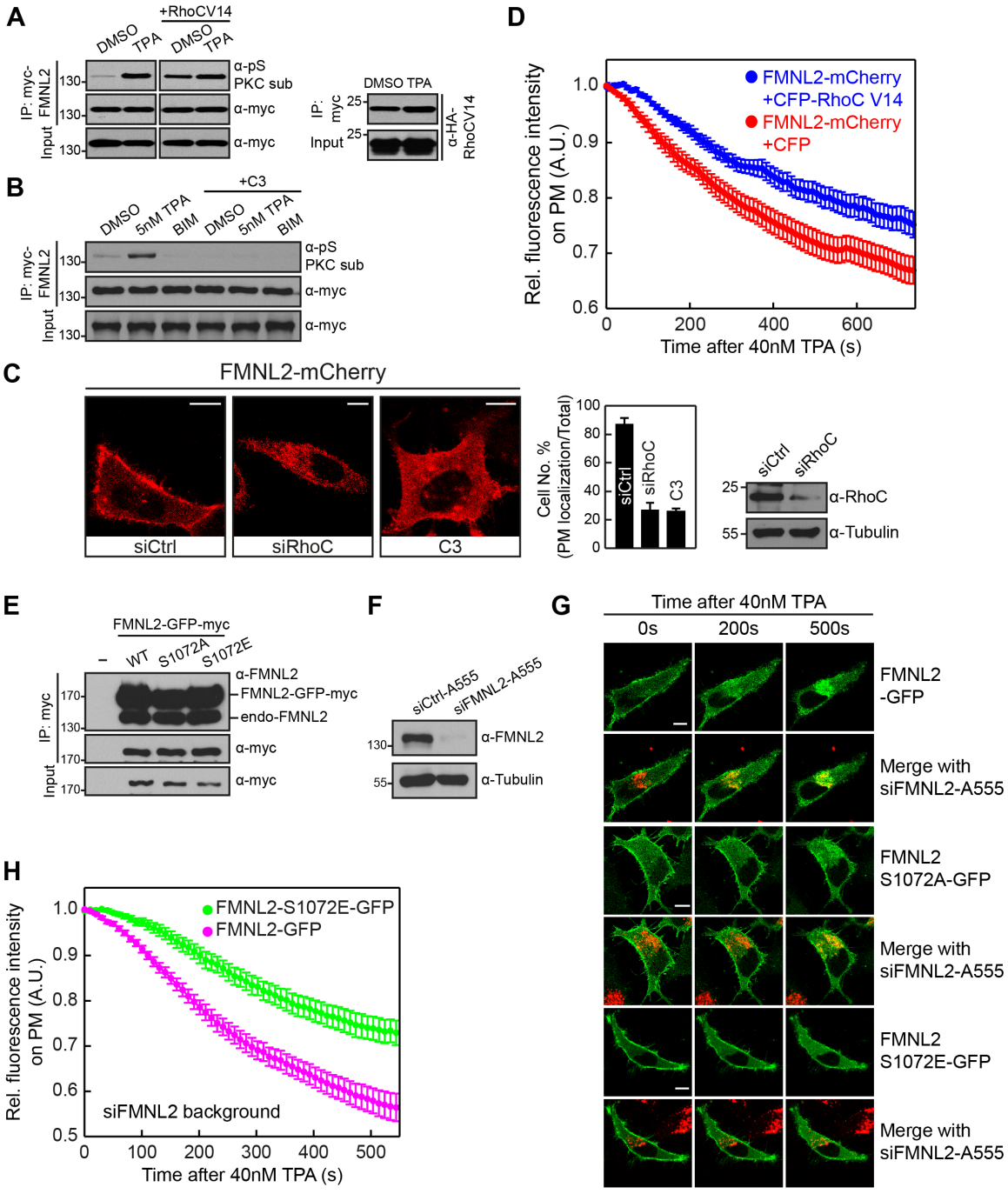

Figure S2

Figure S2. Phosphorylation of FMNL2 Depends on Rho-GTPase Activity and Endogenous FMNL2 Is Knocked-down to Reduce Homodimerization (Related to Figure 2)

(A) FMNL2 and HA-RhoC-V14 were expressed. myc-FMNL2 was immunoprecipitated after DMSO or TPA (20 nM) treatment for 30 min. Phosphorylated FMNL2 and associated HA-RhoCV14 were detected. (B) FMNL2 was co-expressed with or without C3 co-enzyme. myc-FMNL2 was immunoprecipitated after DMSO, TPA (5 nM) or BIM treatment for 30 min. Phosphorylated FMNL2 was detected. (C) FMNL2-mCherry and siCtrl, siRhoC or C3 were co-expressed in HeLa cells. Knockdown of RhoC is shown. Cells expressing FMNL2-mCherry with plasma membrane localization were quantified over the total cell number (in total more than 72 cells per group) counted. Scale bars: 10 μm. Error bar, + SEM (n=3). (D) Quantification of fluorescence intensity changes on the plasma membrane for the mCherry channel in HeLa cells transfected with CFP or CFP-RhoC-V14. Error bar, ± SEM of 8 (with CFP) and 7 (with CFP-RhoC-V14) cells respectively. (E) FMNL2-GFP-myc wild type, S1072A, or S1072E were expressed in HEK293 cells and immunoprecipitated. Endogenous and ectopic FMNL2 were detected by FMNL2 antibodies. (F) Western blot of FMNL2 from HeLa lysates transfected with FMNL2 siRNA labeled with AlexaFluor 555 targeting 3'-UTR. (G) Representative images are shown at indicated time points for Figures 2F and S2H. Green: GFP; Red: AlexaFuor 555. Scale bars: 10 μm. (H) Quantification of fluorescence intensity changes on the plasma membrane for the GFP channel in HeLa cells transfected with FMNL2 siRNA labeled with AlexaFluor 555 targeting 3'-UTR which allowed FMNL2 wild type (FMNL2-GFP) or mutant (FMNL2-S1072E-GFP) DNA expression in the same cell. Error bar, ± SEM of 9 (FMNL2-S1072E-GFP) and 11 (FMNL2-GFP) cells respectively. Numbers indicate molecular weights (kD) in the Western blots. (A~C, & E~F).

Supplemental Figure S3

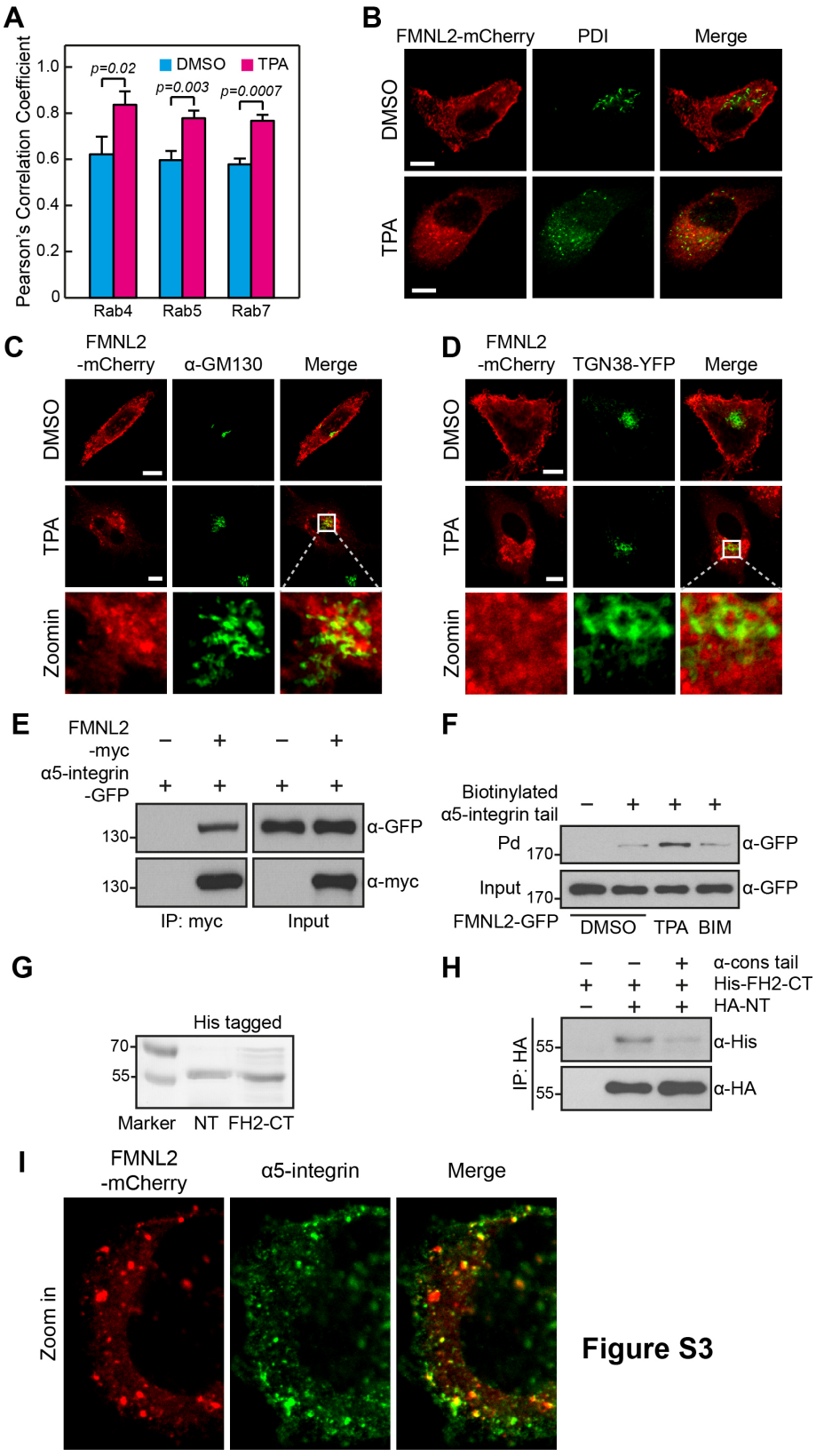

Figure S3

Figure S3. FMNL2 Colocalizes with Endosomal Markers and Interacts with α-integrins (Related to Figure 3)

(A) HeLa cells were transfected with FMNL2-mCherry and Rab proteins respectively. Cells were treated with DMSO or 200 nM TPA for 20 min at 37 °C. Pearson's correlation coefficient was used to quantify the colocalization of FMNL2 and Rab4, Rab5 or Rab7. Error bars: +SD, n=3, with 30 cells each time. P-values are indicated. (B) Representative images of FMNL2-mCherry and ER marker PDI (Protein disulfide isomerase) in HeLa cells with or without TPA treatment. (C) Representative images of FMNL2-mCherry and cis-Golgi marker GM130 in HeLa cells with or without TPA treatment. (D) Representative images of FMNL2-mCherry and trans-Golgi marker TGN38 in HeLa cells with or without TPA treatment. Scale bars: 10 μm. (E) Immunoprecipitation from HEK293 cells showing FMNL2 interacting with α5-integrin in cells. (F) Biotin-streptavidin pull down (Pd) of α5-integrin cytoplasmic tail from HEK293 cell extracts expressing FMNL2 constructs after DMSO, 20 nM TPA, 2 μM BIM treatment for 30 min. (G) Coomassie blue stained gels of the indicated purified proteins in Figure 3D. (H) Immunoprecipitation of purified HA-FMNL2-NT (15 μg) and His-FMNL2-FH2-CT (30 μg) with or without α-integrin conserved cytoplasmic tail (7 μg). (I) Enlarged images of different channels related to Figure 3G. Numbers indicate the molecular weights (kD) (F~H).

Supplemental Figure S4

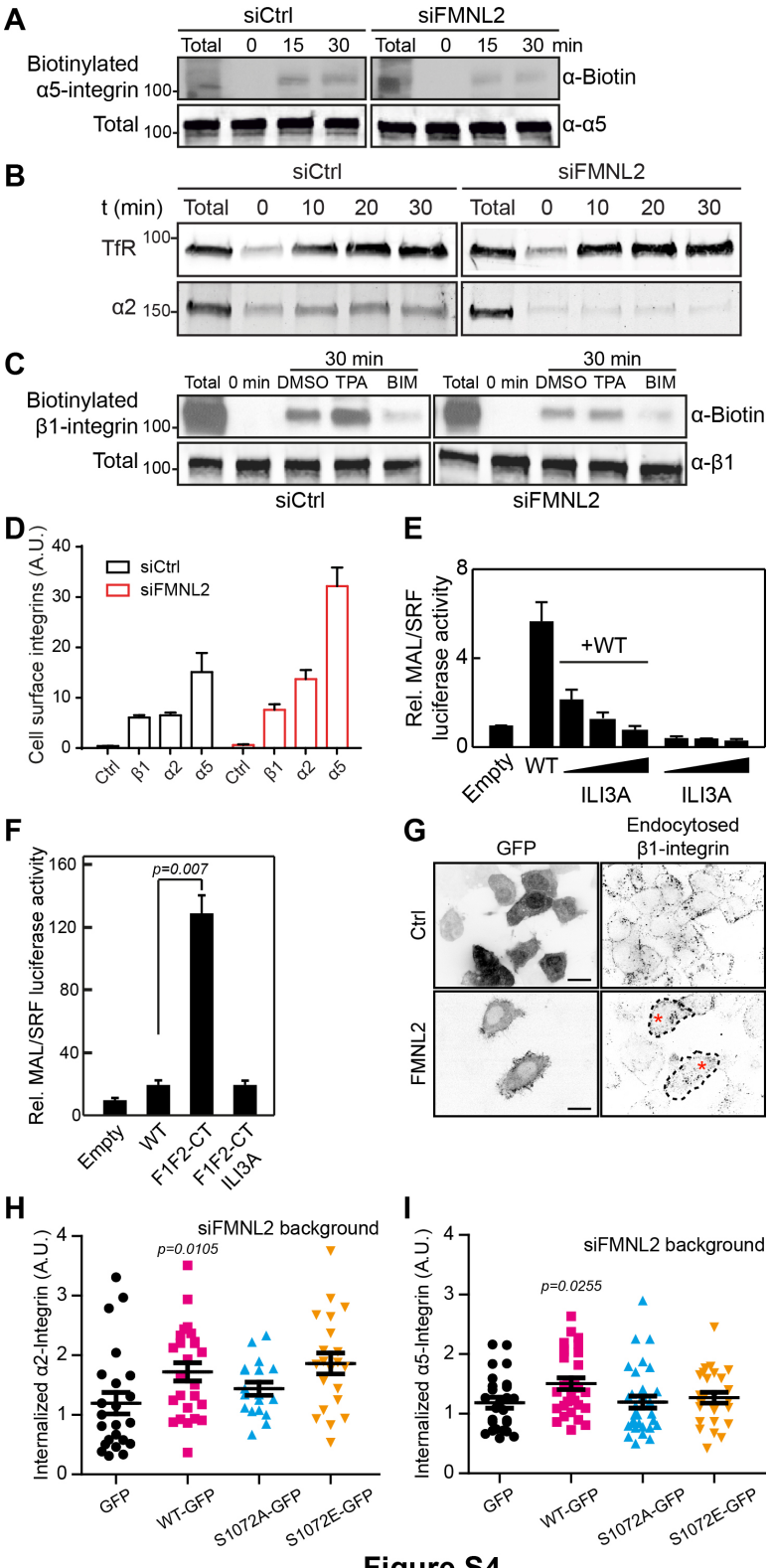

Figure S4

Figure S4. Characterization of FMNL2 Actin Assembly Mutant and Regulation of β1-integrin Internalization by FMNL2 (Related to Figure 4)

(A, B) Representative Western blots showing the immunoprecipitated total or endocytosed biotinylated α2- and α5-integrins or TfR under siCtrl or siFMNL2 conditions in HeLa cells. (C) Representative Western blots showing the immunoprecipitated total β1-integrin or endocytosed biotinylated β1-integrin in control or FMNL2 silenced HeLa cells treated with DMSO, 200 nM TPA or 2 μM BIM 30 min before endocytosis. (D) Cell surface levels of α2-, α5- and β1-integrins were measured in control or FMNL2 silenced HeLa cells. Error bars: + SEM (n=3). (E, F) HEK293 cells were transfected with FMNL2-GFP (WT), FMNL2-ILI3A-GFP, FMNL2-F1F2-CT-GFP, or FMNL2-F1F2-CT-ILI3A-GFP as indicated, together with MAL/SRF reporter 3DA.Luc and Renilla luciferase pRLTK. Black triangle indicates the increasing amount of DNA transfected. SRF activity was measured 24 h after transfection. Error bar: + SEM (n=3). P-value is indicated. (G) Representative images of image-based β1-integrin endocytosis assay. Confocal mid-sections of HeLa cells with transfected FMNL2-GFP in siFMNL2 background are shown here. Dashed lines indicate transfected cells. Red asterisk marks cells with increased β1-integrin endocytosis. Scale bars: 20 μm. (H, I) Quantified levels of endocytosed α2- and α5-integrins in HeLa cells expressing GFP, or GFP-tagged FMNL2 mutants under endogenous FMNL2 silenced background by imaging-based endocytosis assays. Graph shows mean line with error bar, ± SEM (n=3). Each point presents mean value of endocytosed integrin in one field of view. Indicated p-values are compared to the GFP control.

Supplemental Movie Legends

Movie S1. FMNL2 relocates upon PKCα activation (Related to Figure 2). Red: FMNL2-mCherry; Green: GFP-PKCα. Acquisition of images started when 200 nM TPA was applied to the cells. Images were acquired every 10 s. Scale bar: 10 μm.

Movie S2. FMNL2 relocation is inhibited when PKCα activity is inhibited by BIM-1 (Related to Figure 2). Red: FMNL2-mCherry; Green: GFP-PKCα. Cells were pre-treated with 2 μM BIM-1 for 30 min and then treated with 200 nM TPA. Acquisition of images started when TPA was applied to the cells. Images were acquired every 10 s. Scale bar: 10 μm.

Movie S3. The relocation of FMNL2 is reversible (Related to Figure 2). Red: FMNL2-mCherry; Green: GFP-PKCα. Acquisition of images started when 8 μM A23187 was applied to the cells. Images were acquired every 5 s. Scale bar: 10 μm.

Movie S4. FMNL2 relocates upon PKCα activation and localizes to α5-integrin positive vesicles (Related to Figure 3). Red: FMNL2-mCherry; Green: α5-integrin-GFP. Acquisition of images started when 200 nM TPA was applied to the cells. Images were acquired every 10 s. A small part of the upper movie is enlarged in the lower movie to show details of the co-trafficking of FMNL2 and α5-integrin. Scale bars: 10 μm.

Supplemental Experimental Procedures

Plasmids

Expression plasmids were generated and sequence-verified following standard cloning procedures. FLAG-FMNL2, FLAG-FMNL2-F1F2-CT, FLAG-mDia1 and HA-RhoCV14 were described previously (Kitzing et al., 2010; Vaillant et al., 2008). myc-FMNL2, FMNL2-myc, and myc-FMNL2-FH3 (aa 262-484) were sub-cloned into EFpLink with N-terminal or C-terminal myc-tag as indicated. GST-FMNL2-959-CT for protein purification was cloned into pGEX-6P-1 (GE Healthcare). HA-FMNL2-NT (aa 23-484) was cloned as GST-HA-FMNL2-NT into pGEX-6P-1, and the GST tag was cleaved during purification. His-FMNL2-NT was cloned into pET20 (Novagen) and His-FMNL2-FH2-CT was cloned into pET30 (Novagen). FMNL2 was cloned into pmCherryN1 (Clonetech), pEGFPN1 (Clonetech) or pWPXL vectors. Point mutants FMNL2-S1072A, S1072E and ILI3A (I704A, L1028A, and I1029A) were generated by site-directed point mutation. α5-integrin-GFP, GFP-PKCα and GFP-PKCα-T497A were described (Mostafavi-Pour et al., 2003). GFP-PKCα-CA was generated by deleting the first 30 amino acids (Soh and Weinstein, 2003) and subcloned into pEGFPC1 vector (Clonetech).

Cell Lines, Drug Treatments and Transfection

HEK293, HEK293T, HeLa and A375M2 (kind gift from Eric Sahai) cells were maintained in DMEM supplemented with 10% fetal bovine serum at 37°C in a 5% CO_2 environment. When needed, cells were treated with 5 nM to 200 nM TPA (Calbiochem), 2 μM BIM-1 (Calbiochem), 1 μM Gö6976 (Calbiochem) or 8 μM A23187 (Sigma). DNA plasmids were transfected with calcium phosphate methods for HEK293 and HEK293T cells, and with Fugene (Promega) for HeLa cells. siRNA (Qiagen) was transfected with HiperFect (Qiagen). A375M2 stable cell lines were generated by lentiviral transduction. Viruses were produced by transfecting HEK293T cells with packaging plasmid pSPAX2, envelope plasmid pMD2G and expressing plasmids pWPXL-GFP, -FMNL2-GFP, -FMNL2-S1072A-GFP, -FMNL2-S1072E-GFP,

or -FMNL2-ILI3A-GFP. Supernatants were harvested 48 h after transfection and A375M2 cells were infected. HeLa stable cell lines expressing shFMNL2 (V2LHS_384851, Open Biosystems) or shControl (a kind gift by Thorsten Stiewe) were generated similarly with lentiviral shRNAs.

Reagents and Antibodies

Cell culture reagents were purchased from PAA/GE Healthcare. Chemicals were from Sigma. α-phospho-(Ser) PKC substrate, α-PKCα, α-PKCδ, α-PKCε, α-PKCζ, α-biotin-HRP and α-tubulin were obtained from Cell Signaling Technology. α-PKCβ was from Santa Cruz. α-FMNL2 was purchased from Atlas Antibodies. α-α5 integrin (CD49e) was from AbD serotec. α-β1 integrin (CD29) antibody K20 was purchased from Beckman Coulter, 610468 was from BD Biosciences, 12G10 and EP1041Y were from Abcam. α-α2 integrin (MAB1998Z) was from Millipore. α-TfR (ab84036) was from Abcam. α-HRP-conjugated FLAG, myc and HA antibodies and HRP-conjugated secondary antibodies were from Sigma. Alexa Fluor conjugated secondary antibodies were from Invitrogen.

DNA Primers and siRNA Sequences

Point mutants FMNL2-S1072A, S1072E and ILI3A (I704A, L1028A, and I1029A) were generated by site-directed point mutation using the following primer pairs: 5'-AGGAGAGCCGTCAGGCGG-3' and 5'-CCTGACGGCTCTCCTCAC-3', 5'-AGGAGAGAGGTCAGGCGG-3' and 5'-CCTGACCTCTCTCCTCAC-3', 5'-CTTGCCGCCACTTTAAGG-3' and 5'-TAAAGTGGCGGCAAGATT-3', 5'-CAAGAGGCCGCAGCAGAATTA-3' and 5'-TTCTGCTGCGGCCTCTTGCTG-3', respectively. The actin assembly deficient mutant was generated according to (Heimsath and Higgs, 2012; Thompson et al., 2013). siRNA targeting sequences were: 5'-CACCATGGCTGCAATCCGAAA-3' (human RhoC), 5'-CTGCGGGCACATTCCCATAAA-3' (Hs_FMNL2_9, targeting 3'-UTR of human FMNL2, 3'-AlexaFluor 555 modified when indicated), 5'-AACCATCCGCTCCACACTAAA-3' (human PKCα),

5’-CCGGATGAAACTGACCGATTT-3’ (human PKCβI and PKCβII), 5’-AACTCTACCGTGCCACGTTTT-3’ (human PKCδ), 5’-CCCGACCATGGTAGTGTTCAA-3’ (human PKCε), and 5’-CGGAAGCATGACAGCATTAAA-3’ (human PKCζ).

Screening for the Effects of Formins on β1-integrin Endocytosis by Cell Spot Microarray

Cell Spot Microarrays (CSMA) were conducted as previously described (Rantala et al., 2011). Formin targeting siRNAs Hs_C14orf173_2 and 5 (INF2), Hs_DAAM1_6 and 7, Hs_DAAM2_7 and 8, Hs_DIAPH1_2 and 4, Hs_DIAPH2_1 and 4, Hs_DIAPH3_8 and 9, Hs_FHOD1_6 and 7, Hs_FHOD3_6 and 7, Hs_FMN1_7 and 8, Hs_FMN2_11 and 12, Hs_FMNL1_8 and 9, Hs_FMNL2_8 and 9, Hs_FMNL3_6 and 7, Hs_GRID2IP_5 and 6 (Delphilin), and control siRNA were purchased from Qiagen. Before plating HeLa cells on CSMA, the arrays where blocked with Lutrol F108 (Univar AB) for 15 min at room temperature. Cells were washed and harvested using HyQTase, then plated on CSMA. Endocytosis was measured 72 h after the reverse siRNA transfection. Antibody based integrin β1 endocytosis assay was carried out as described(Arjonen et al., 2012). In short, non-functional blocking β1-integrin antibody (K20) was labeled using AlexaFluor 488 protein labelling kit (Invitrogen). Cell surface β1-integrins of the CSMA with live cells were labeled with K20-AlexaFluor 488 for 1 h on ice. CSMA was then washed and the labeled β1-integrin was allowed to be endocytosed for 30 min at 37°C. The residual cell surface fluorescence was quenched using anti-AlexaFluor 488 antibody (Invitrogen) for 1 h on ice and fixed using 4% paraformaldehyde. Cells were then permeabilized and incubated with anti-mouse AlexaFluor 555 secondary antibody to label both internal and cell surface K20 pools (total β1-integrin). Endocytosed β1-integrin was normalized against the total β1 integrin. Nuclei were labeled using Syto60 (Invitrogen). Fluorescence intensities were measured using Laser microarray scanner (Tecan LS400) and ArrayPro Analyzer v4.5 software.

Protein Purification

The expression plasmid was transformed into E. coli BL21 (DE3). Bacteria were cultured in LB medium at 37°C until OD = 0.6 and induced with 200 μM IPTG at 22°C for 16 h or 12.5 g/L lactose at 30°C for 16 h. GST fusion protein was purified using Glutathione Sepharose 4B beads (GE Healthcare) as described before (Brandt et al., 2009). Proteins were dialyzed in 20 mM Hepes (pH 7.4), 25 mM $MgCl_2$, 5 mM EDTA and 5 mM DTT. The GST tag was cleaved during dialysis using Precision Protease (GE Healthcare) when necessary and the cleaved protein was recycled by running through the Glutathione Sepharose 4B once more. 6×His fusion protein was purified using Ni-NTA agarose beads (Qiagen) as shown before (Brandt et al., 2007).

In vitro Kinase Assay

Purified recombinant GST fusion PKC protein isozymes were purchased from ProQinase GmbH. 20 ng of each PKC isozyme and 1 μg substrate protein were incubated in 50 mM Hepes (pH 7.4), 150 mM NaCl, 5 mM EDTA, 5 mM DTT, 25 mM $MgCl_2$, 0.02% Triton X-100, 1 mM ATP and 5 μCi ^{32}P-γ-ATP at 30°C for 15 min. 1 μM Staurosporine (Sigma) was used when indicated. Kinase reaction was stopped by adding the SDS sample buffer. After running SDS-PAGE and fixation of the gel, autoradiography was performed with different exposure times.

Internalization Assay

Integrin or transferrin receptor (TfR) internalization assays were performed as described (Pellinen et al., 2008). 24 h after seeding, cell surfaces were labeled for 30 min with 0.5 mg/mL EZ-Link Sulfo-NHS-SS-Biotin (Pierce Chemical). Internalization was followed for the indicated times. The residual biotin was then removed from the cell surface by MesNa reduction (60 mM, 30 min), followed by iodoacetamide quenching (100 mM, 15 min). Total proteins were immunoprecipitated using the integrin or TfR antibodies. Internalized proteins were detected using Western blot with the HRP-linked biotin antibody. Total proteins were detected by integrin or TfR antibodies. Student's T-test was used to calculate the p-values.

Immunoprecipitation and Biotin-Streptavidin Pull-down

Cells were harvested and lysed in lysis buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 0.1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, and complete protease inhibitors (Roche). Supernatants were collected after centrifugation and incubated with FLAG/myc/HA-conjugated agarose beads (Sigma) or Protein A/G beads (Santa Cruz) together with antibodies for 1 to 5 h at 4°C. For pull-downs, biotinylated α5/β1-integrin cytoplasmic tail peptides were pre-incubated with cell lysates respectively for 4 h (Pellinen et al., 2008). The peptide sequences were: α5-integrin cytoplasmic tail: Biotin-YKLGFFKRSLPYGTAMEKAQLKPPATSDA; β1-integrin cytoplasmic tail: Biotin-HDRREFAKFEKEKMNAKWDTGENPIYKSAVTTVVNPKYEGK; α-integrin conserved cytoplasmic tail: Biotin-WKLGFFKRKYEKM. Following a 1 h incubation with Streptavidin sepharose beads (GE Healthcare), samples were washed. Western blotting were performed as described previously (Brandt et al., 2009).

Membrane Flotation Assay

Membrane flotation assays were performed as described (Pan et al., 2012). Cells were lysed in cold TNE buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, protease inhibitors and phosphatase inhibitors) and passed through 25G syringe 20 times. After centrifugation, supernatants were mixed with 67% Optiprep (Invitrogen) and loaded to SW60 centrifuge tubes. Each sample was overlaid with 2.5 mL 28% Optiprep and 600 μL TNE buffer. Ultracentrifugation was performed with SW60 rotor at 35,000 rpm for 3 h at 4°C (Beckman Coulter). 500 μL fractions were collected with the 2nd and 8th as the membrane and soluble fractions respectively.

Imaging-based Integrin Endocytosis Assay

HeLa cells were transfected with Qiagen Hs_FMNL2_9 siRNA (targeting 3'UTR) and EGFP or different FMNL2-GFP plasmids. Endocytosis was carried out as described with modifications (Gu et al., 2011). Cell surface β1-integrins were stained with primary and secondary antibodies for 1 h on ice and cells were allowed to endocytose the labeled

β1-integrins 30 min at 37°C. Cells were acid stripped on ice with stripping buffer, pH-restored with culture media and fixed. 3D confocal image stacks of fixed cells were acquired using Zeiss Axiovert 200M, Yokogawa CSU22 spinning disc microscope and 63X/1.4 oil objective. Images were analyzed using ImageJ. GFP positive cells were outlined by threshold-mask and the intensity of endocytosed β1-integrin was thresholded, background subtracted and quantified using ImageJ Analyse Particles and Integrated Density command. GraphPad Prism and Mann-Whitney test was used to calculate p-values for the endocytosis under different conditions.

Inverted Transwell Invasion Assay and Image Analysis

Invasion assays were performed as described (Kitzing et al., 2010). Cells were transfected with siRNAs 48 h before analysis. Cells were invading for 48 h before fixation, permeabilization and subsequent staining with DAPI and rhodamine phalloidin (Invitrogen). Confocal z-stacks of 100 μm were acquired for five random imaging fields of each insert with LSM 700 confocal microscope (Zeiss), using the 20X/0.75 objective. Quantification of the invaded and non-invaded cell number was done using the ImageJ Analyze Particle function. Student's T-test was used to calculate the p-values.

Expression of Cell Surface Integrins

HeLa cells were silenced 72h. Cells were harvested using HyQTase (Fisher Scientific) spinned down, lifted on ice and washed with ice-cold PBS. Cells were blocked using 2% BSA in PBS for 30min at 4°C and stained against α2, α5 and β1-integrins. Cells were washed in PBS and counterstained using Alexa-647 secondary antibodies for 30min at 4°C. Cells were washed in PBS and analysed with Accuri FACS.

MAL/SRF Luciferase Assay

MAL/SRF luciferase assay was performed as described (Baarlink et al., 2013). HEK293 cells were transfected with genes of interests, p3DA.luc and pRLTK reporter plasmids. 24 h after transfection, cell media were removed and cells were washed once with PBS. 200 μL 1× Passive lysis buffer (Promega) was added per 6-well. Cells were lysed on ice

for 20 min, scraped and centrifuged at 13,000 rpm for 15 min at 4°C. From 5 to 20 μL cell lysates were used for each assay. Firefly and Renilla luciferase signals were recorded sequentially by Luminoscan Ascent Microplate Luminometer (Thermo Scientific) using the Ascent software (Thermo Scientific) with 900 ms integration time. Firefly signal was normalized to Renilla signal for each sample.

Supplemental References

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