Supplementary Information

Supplementary Methods

Antibodies, fluorescent probes and plasmids

Rabbit anti-human VPS34 antibody, rabbit anti-human integrin β5 and integrin α5 were purchased from Cell Signaling. The antibody against MTMR3 was produced by Genscript by immunizing two rabbits with the peptide CSPDQPSRSHLDDDG. The antibody was affinity-purified from crude sera by the same peptide. The sheep anti-PIKfyve antibody was purchased from Tocris Bioscience. The anti-human integrin α5 antibody for ELISA capture (integrin recycling) was from BD Biosciences. The rabbit anti-FGD1 prestige antibody, mouse monoclonal anti-β-actin, anti-human vinculin and anti-human talin were from Sigma-Aldrich. Anti-human FAK was from BD Transduction Laboratories. Mouse anti-human CD29 (integrin β1) was from BD Biosciences Pharmingen. Mouse anti-GM130 antibody was purchased from BD Transduction Laboratories™. Rhodamine phalloidin and Hoechst 33342 were purchased from Invitrogen Molecular Probes. The plasmids encoding human MTMR3 and the mutated MTMR3 C413S fused to EGFP were a kind gift from Prof. Michael J. Clague (Physiological Laboratory, University of Liverpool, Liverpool, UK). The siRNA-resistant mutants were generated by site-directed mutagenesis (QuickChange, Stratagene). The plasmids encoding IpgD and its mutant IpgD C438S were a kind gift from Dr. Bernard Payrastre (Centre de Physiopathologie de Toulouse Purpan, Toulouse, France).

Cell Culture

The human normal foreskin fibroblast cell line BJ (A.T.C.C.) was cultured in Quantum 333 For Fibroblasts from PAA Laboratories GmbH at 37°C and 5% CO_2 in humidified air.

Cell Migration Assay and siRNA screen for proteins containing PtdIns3P-binding motifs

Cells were seeded in Oris™ 96-well plates as described by the manufacturer (Platypus Technologies). The next day, cells were transfected with the siRNA library (described before [1]) using 100 nM siRNA in combination with Lipofectamine™ RNAiMAX (Invitrogen) according to the manufacturer's protocol. After 3 days, cells were allowed to migrate into the migration zone for 24 hours upon FGF1 stimulation. Cells were at the end fixed with 4% formaldehyde (Sigma-Aldrich) and stained with Hoechst33342. The same non-targeting siRNAs were used. The screen was performed three times. Pictures were taken with the Olympus Scan^R System with a 10x objective and 12 pictures were stitched together and analyzed with the Scan^R analysis program. Nuclei of the captured areas were counted, which represents the total cell number. Based on the size of the migration zone from nocodazole treated cells, the migration zone of every well was cut out and counted again with the Image J

software and the plugin Nucleus Counter to obtain the migrated cell number. Final results are presented as ratio of the migrated cells per total cell number.

RNA interference studies

The SMARTpool ON-TARGET*plus* siRNAs and the single deconvoluted siRNAs (VPS34 siRNA 2: cat. no. D-005250-02; MTMR3 siRNA 1: J-008039-06; siRNA 2: J-008039-07; siRNA 3: J-008039-08; siRNA 4: J-008039-09; PIKfyve siRNA 1: J-005058-13; siRNA 2: J-005058-14; siRNA 3: J-005058-15; siRNA 4: J-005058-16; FGD1 siRNA 1: J-009612-06; siRNA 2: J-009612-07; siRNA 3: J-009612-08; siRNA 4: J-009612-09) and ON-TARGET*plus* and siGENOME Control reagents were purchased from Dharmacon Research. BJ cells were plated one day before transfection in medium without antibiotics and transfected with a mixture of Lipofectamine™ RNAiMAX (Invitrogen) and 50 nM siRNA; either control siRNA or siRNA against VPS34, MTMR3 or FGD1. For VPS34, siRNA 2 gave a strong and specific knockdown as previously described [1]. For MTMR3, as shown in Supplementary figure 2a, siRNA 1 gave the strongest knockdown and was therefore further on used. For PIKfyve, combining two oligonucleotides was necessary for efficient knockdown: siRNA 3 and 4, and a double knockdown with 75 nM total of both siRNAs was performed, as previously described [2]. The cells were plated on 5 cm dishes and replated after 2 days on either MatTek Glass Bottom Dishes (MatTek Corporation) or on new 5 cm dishes for another 2 days. The MatTek dishes were used for analysis in combination with live-cell imaging, whereas the 5 cm dishes were used for Western blotting or for quantitative RT-PCR analysis.

Western blotting

To determine the effect of siRNA treatment, cells from a 5 cm dish were first collected in PBS and then centrifuged for 5 min. at 6000 rpm. The cell pellet was lysed in lysis buffer (0.1 M sodium chloride; 10 mM disodium hydrogen phosphate; 1 mM EDTA; 1% Triton X-100; pH 7.4; with 1:100 Complete Protease Inhibitor Cocktail tablets (Roche Applied Science)) and sonicated for 10 s at 70 volts. 30 µg of protein of the whole cell lysate was loaded for SDS-PAGE (4-20% gradient) and afterwards transferred to Immobilon-P membrane (Millipore) for Western blotting. Blots were developed with the Amersham™ ECL Plus Western Blotting Detection System (GE Healthcare).

Quantitative real-time PCR

Total RNA was isolated using the RNeasy Plus Mini Kit (Qiagen) in combination with the automated sample preparation system QIAcube (Qiagen). RNA concentration was measured with the NanoDrop 2000 (Thermo Scientific) and 1 µg of RNA was employed for cDNA synthesis. cDNA was generated by the iScript^{TM} cDNA Synthesis Kit (BIO-RAD) and the C1000^{TM} Thermal Cycler (BIO-RAD). Quantitative Real-Time PCR reaction was performed with the LightCycler® 480 SYBR Green I Master (Roche Applied Science) and run on a LightCycler® 480 Real-Time PCR System. For internal control the expression of the SDHA gene (Succinate Dehydrogenase) was used. QuantiTect Primer Assay for MTMR3 (QT01678376), PIKfyve (QT00035231), FGD1 (QT00084182) and SDHA (QT00059486) were from Qiagen. The following program was used: Preincubation 5 min at 95°C, amplification 45 cycles (10 seconds denaturation at 95°C; 20 seconds annealing at 60°C and 10 seconds extension at 72°C), followed by melt-curve analysis. Relative quantification was done with the LightCycler® 480 software.

Wound healing assay, rescue experiments and perfusion assay

For wound healing assays, confluent cells were scratched with a 10 µl tip and subsequently recorded for 10 hours. For rescue experiments, cells were transfected with EGFP fusion constructs with the FUGENE-6 reagent (Roche Applied Science) as described by the manufacturer and recorded in random cell migration experiments over a period of 10 hours. For the perfusion assay, the PIKfyve inhibitor YM201636 was added after 4 hours of recording to a final concentration of 800 nM (as described before [3]) with help of the perfusion component in the BioStation IM and cells were recorded for 6 more hours.

Immunofluorescence and confocal microscopy

BJ cells were fixed with 3% paraformaldehyde and permeabilized with 0.05% saponin in PEM buffer (80 mM PIPES, 5 mM EGTA, 1 mM $MgCl_2$ pH 6.8). Primary and secondary antibodies were diluted in PBS containing 0.05% saponin. Confocal images were acquired with a 63X objective on a Zeiss LSM 780 confocal laser-scanning microscope. Cell polarization was determined by previously described criteria [4].

Superresolution imaging using structured illumination

Superresolution 3D SIM imaging was performed on an Deltavision OMX V4 system (Applied Precision, Issaqua, WA) equipped with an Olympus 60x NA 1.42 objective, cooled sCMOS cameras and 405, 488, 568 and 642 nm diode lasers. Z-stacks covering the whole cell were recorded with a Z-spacing of 125 nm. A total of 15 raw

images (5 phases, 3 rotations) per plane were collected and reconstructed using Softworx software. (Applied Precision, Issaqua, WA). Shown are maximum intensity projections of the acquired Z-stacks.

Border cell migration assay during *Drosophila melanogaster* oogenesis

Border cells are a cluster of 6-10 specialized somatic follicle cells that perform directed collective migration during *Drosophila* oogenesis. Early in egg chamber stage 9, the border cells delaminate from the anterior tip of the egg chamber follicle cell epithelium and initiate their posterior migration between the nurse cells toward the oocyte, reaching the oocyte about 6 hours later, early in stage 10 [5]. For RNAi-mediated gene silencing in border cells, the GAL4-UAS system was used to express double-stranded hairpin RNA that is processed by Dicer into siRNAs that in turn direct sequence-specific degradation of the target mRNA [6]. The *Drosophila* driver slbo-GAL4, UAS-GFP (purchased from the Bloomington Stock Centre) that expresses in border cells, centripetal cells, and a few other follicle cells was crossed to three different *Myotubularin-like*, two different *PVR RNAi* lines or a negative control line. Crosses were performed at 25˚C. Young female offspring of the appropriate genotypes were collected and placed in vials together with a couple of males and yeast paste to stimulate oogenesis for 2 days at 29˚C. Ovaries were dissected out, fixed for 30 min in 4% formaldehyde at room temperature and stained with rhodamine phalloidin and Hoechst 33342 as previously described [7]. Egg chambers were mounted in Prolong Antifade mounting medium (Molecular Probes). Images of early stage 10 egg chambers were captured using Zeiss LSM 5 DUO and Zeiss LSM 780 laser scanning confocal microscopes (Carl Zeiss, Inc.) and a Plan Apochromat 20×/0.8 NA objective at 20°C. Measurement of the border cell migration distance was performed using the Zeiss LSM 510 software (Version 3.2, Carl Zeiss, Inc.).

Myotubularin-like (CG3632) RNAi lines: (i) *CG3632-RNAi 1: 3632R-1-RNAi* (chr II) (NIG-FLY), (ii) *CG3632-RNAi 2: 3632R-3-RNAi* (chr III) (NIG-FLY) and (iii) *CG3632-RNAi 3: CG3632-RNAi* (26254, chr II) (VDRC). *PVR RNAi* lines: (i) *PVR-RNAi 1: 8222R-2-RNAi* (chr X) and (ii) *PVR-RNAi 2: 8222R-3-RNAi* (chr II), both from NIG-FLY. Negative control line: *60000*; the isogenic host strain for the RNAi library, from VDRC.

Addition of exogenous PtdIns5*P* to cells

The phosphoinositide (PIP) di-C_{16} and carrier were reconstituted in DMEM (GIBCO). After 1 min bath sonication, carrier and PIP di-C_{16} were combined with a ratio of 1:1 for 10 min at room temperature. The mixture of PIP di-C_{16} and carrier was incubated on the MatTek dishes for a period of 45 min and followed by three times washing with Quantum 333 medium. The final concentration used was 50 μM. For the negative control, DMEM was combined with carrier only and added to the cells.

Colorimetric MTT Assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma) was dissolved in sterile PBS at 5 mg/ml. The stock solution (50 µl to 500 µl medium in a 24-well dish) was added after 4 days of knockdown or after 4 hours of inhibitor treatment. Plates were then incubated at 37°C for 4 hours. DMSO (Dimethyl sulfoxide) was added to all wells and the plate was put into a plate reader (Synergy 2, BioTek). After 5 minutes of shaking, absorbance was measured at 570 nm (test wavelength) and at 630 nm (reference wavelength).

Measurement of Cytotoxicity

MTMR3 or PIKfyve were siRNA-depleted, or in the case of PIKfyve inhibited by YM201636 in BJ cells. The cells were washed in Hepes medium without leucine and then transferred to Hepes medium containing 1 µCi/ml [^{3}H]leucine and incubated for 30 minutes at 37 °C. The cells were then extracted with 5% trichloroacetic acid for 10 minutes followed by a brief wash in 5% trichloroacetic acid and subsequently dissolved in 0.1 mM KOH. The cell-associated radioactivity was measured by scintillation counting as described previously [8].

Integrin recycling assay

Control and MTMR3 siRNA-depleted cells were stimulated with 100 ng/ml FGF1 and heparin (10 U/ml) for a period of 4 hours before integrin recycling measurement. For experiments using the PIKfyve inhibitor YM201636, BJ cells were incubated with 800 nM YM201636 together with 100 ng/ml FGF1 and heparin (10 U/ml) for a period of 4 hours prior to integrin recycling measurement, and YM201636 was also present during the entire recycling period. Recycling of α5β1 integrin was performed as previously described [9]. Briefly, cell-surface proteins were labeled with sulfo-NHS-SS-biotin for 30 min at 4ºC, followed by an internalization period of 30 minutes at 37ºC. Biotin remaining at the cell surface was removed with a solution containing 20 mM MesNa in 50 mM Tris pH 8.6 and 100 mM NaCl for 20 minutes at 4ºC. Recycling was allowed by returning cells to 37ºC for the indicated times. Cells were then placed on ice and biotin was removed by a second reduction with MesNa. Biotinylated integrins were captured by ELISA using 5 µg/ml α5 integrin antibody bound to an ELISA plate. Wells were incubated with streptavidin-conjugated horseradish perodidase, and signal was detected by a chromogenic reaction with ortho-phenylenediamine.

Biotinylation and pull-down assay

Control and knockdown cells were surface-biotinylated with EZ-Link Sulfo-NHS-SS-Biotin (Thermo Scientific) for 15 minutes at 4ºC, washed with 100 mM glycine in PBS, followed by lysis. Pull-down was done with Dynabeads M-280 Streptavidin (Invitrogen) for 90 minutes at 4ºC with constant rotation. After washing,

associated proteins were eluted by boiling at 95ºC for 10 minutes in SDS-sample buffer, resolved by SDS-PAGE, transferred to PVDF membrane and blotted with either β5, β1 or α5 integrin antibody.

Immuno-Electron Microscopy

Cells for immuno-EM were prepared as described earlier [10]. Immunolabeling was done with the mouse-anti-α5-integrin (BD Biosciences), followed by rabbit-anti-mouse (IgG Dako, Carpinteria, CA) and protein A gold 10 nm (Cell Microscopy Center, Utrecht, The Netherlands). Sections were observed at 60–80 kV in a JEOL JEM-1230 electron microscope (Tokyo, Japan). Micrographs were recorded with a Morada digital camera using iTEM (SIS) software (both from Soft Imaging Solutions; Muenster, Germany).

Cell spreading assay

Adhesion assays were performed as previously described [11]. Briefly, coverslips were coated with fibronectin (20 μg/ml) for 1 hour at 37°C and blocked with 10 mg/ml heat-denatured BSA for 30 minutes at room temperature. siRNA-depleted BJ fibroblasts were trypsinized, trypsin-inactivated, spun down and resuspended in serum-free medium. Cells were seeded onto coverslips and allowed to adhere for 60 minutes (37°C, 5% CO2) before being washed, fixed and permeabilized. Cells were stained for actin in order to visualize the cells, and the surface area of cells was assessed using Image J software.

Statistical analysis

Values are given as means and ± s.e.m. in all figures. Based on inspection of the distribution of cell migration data (aggregated within experiments), tests were carried out on the logarithmic scale. The data were found to be free from outliers, justifying the use of analyses of variance. Treatments and experiments were used as factors in the analysis; the tests are then identical to paired t-tests if only control and one treatment are compared. Tests are two-sided, and p-values below 0.05 were considered significant.

Supplemental References

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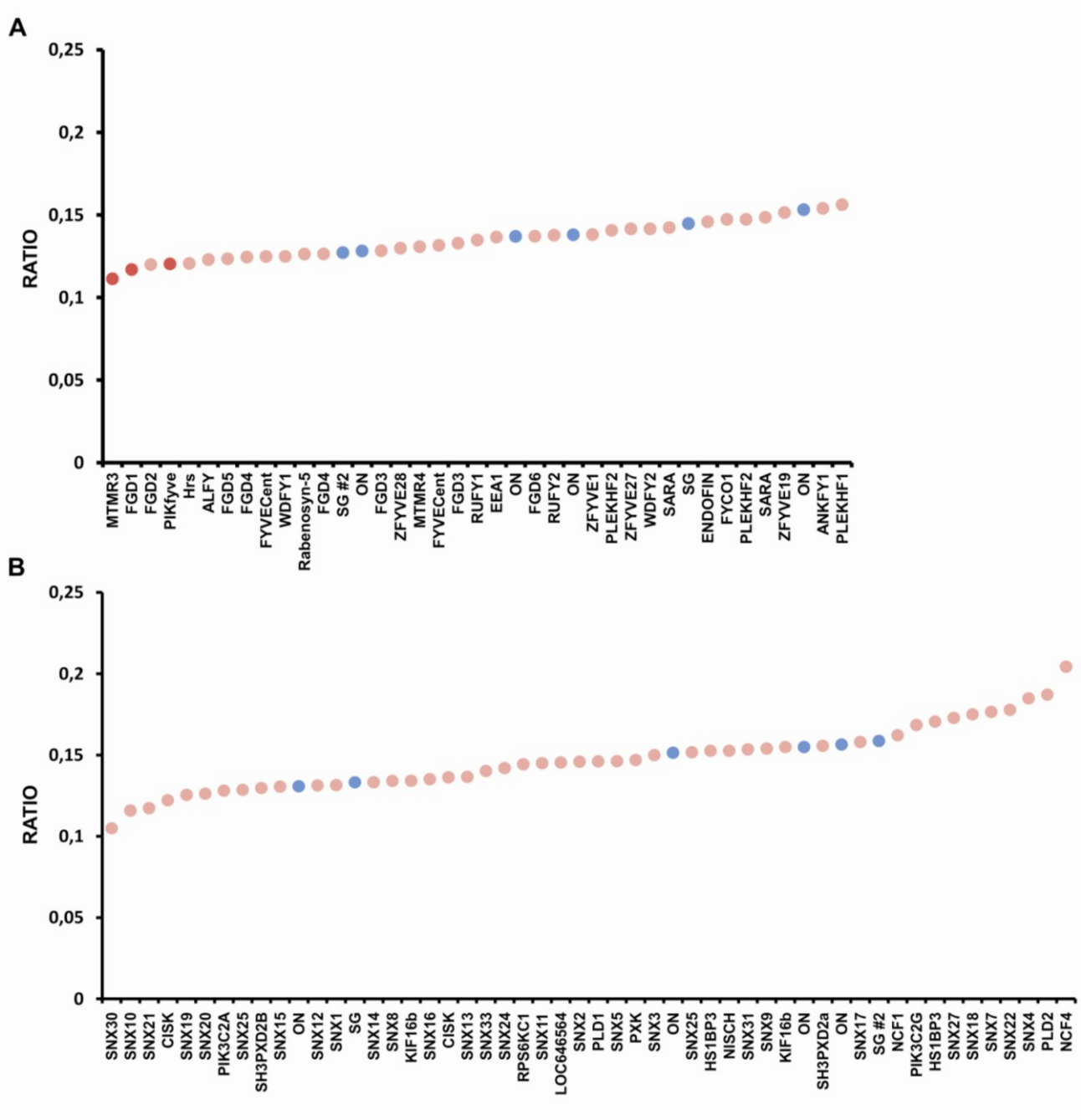
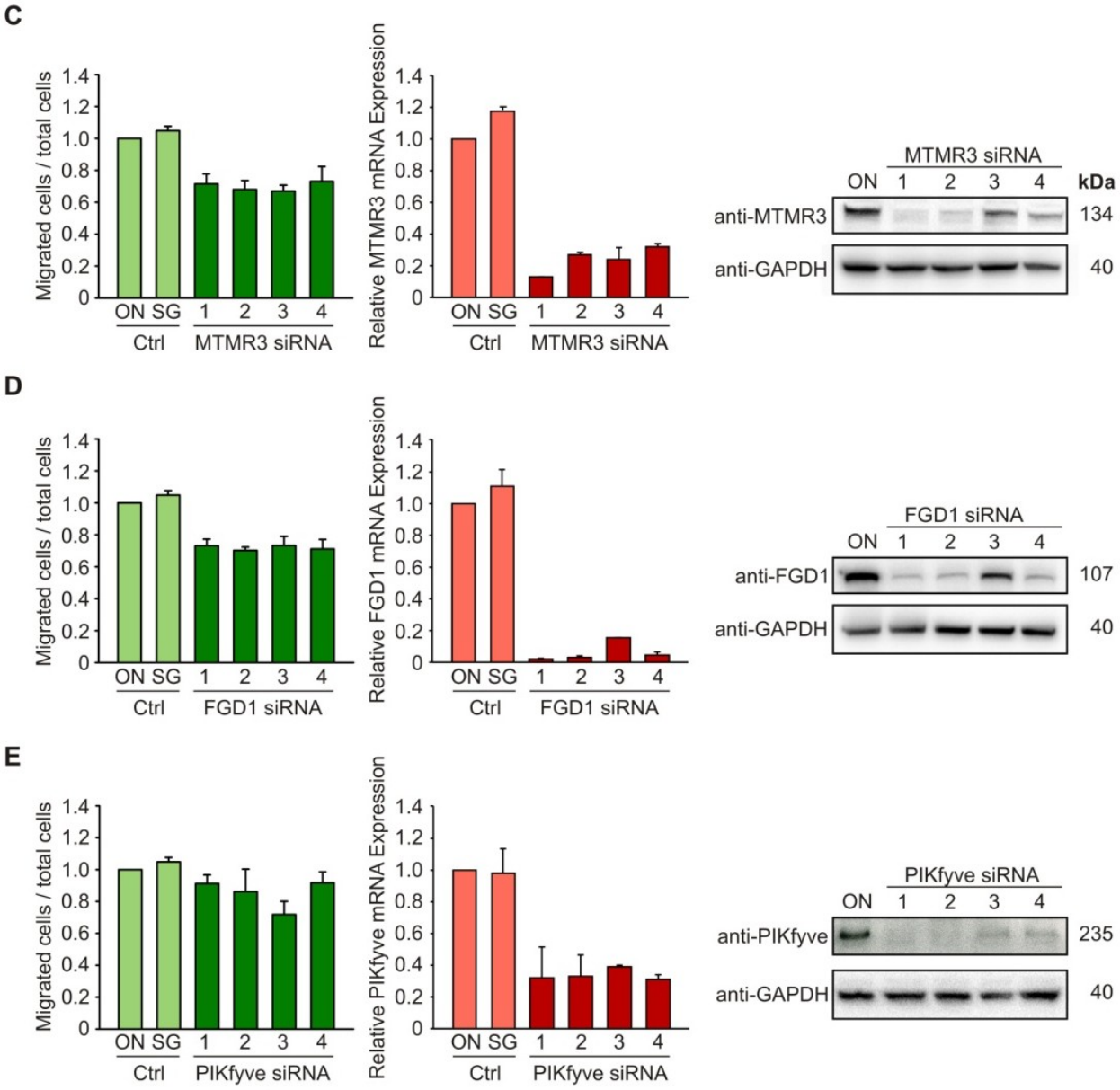
Supplementary Figures

Supplementary Figure S1



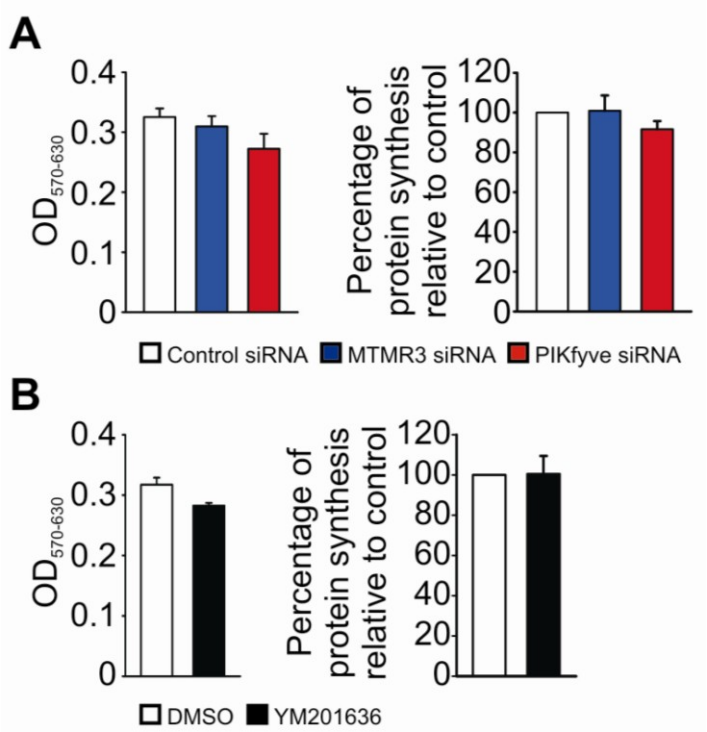


Supplementary Figure S1: siRNA screening for proteins containing PtdIns3*P*-binding motifs in a cell migration assay and validation with single siRNA oligonucleotides.

A-B, Ranked average ratios of migrated cells per total cells in the imaged area. The PtdIns3*P*-binding motif containing proteins are ranked according to increasing number of cells migrated into the migration zone. In **A**, FYVE domain-containing proteins; in **B,** PX domain-containing proteins. The red dots indicate PtdIns3*P*-binding proteins, dark red dots highlight the selected hits, blue dots indicate control siRNA treatment. Average ratios are of three independent experiments. **C-E,** Validation of the four single oligonucleotides from the selected hits from the siRNA screen. Left (green): Migration assay data relative to control. Middle (red): Quantitative real-time PCR data relative to control. Right: a representative Western blot analysis. In **C,** for the four MTMR3 siRNA

oligonucleotides; in **D,** for the four FGD1 siRNA oligonucleotides; in **E,** for the four PIKfyve siRNA oligonucleotides. ON, ON-TARGET*plus* Control siRNA; SG, siGENOME Control siRNA; Ctrl, Control; numbers indicate the number of the oligonucleotide. For all panels and subsequent figures, error bars represent the mean ± s.e.m. of three independent experiments. *$P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; Student's t-test.

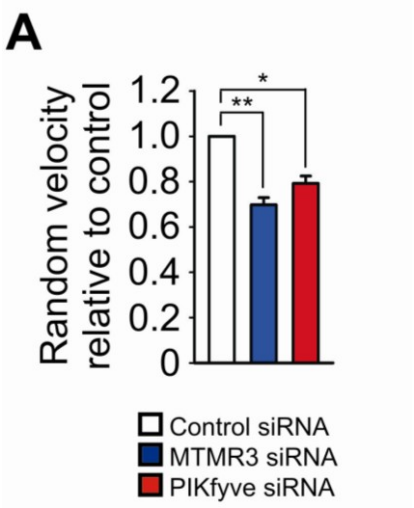
Supplementary Figure S2



Supplementary Figure S2: Cytotoxicity upon knockdown or inhibitor treatment.

In **A,** and **B,** Left panel: MTT colorimetric assay showing no cytotoxic effects upon knockdown of MTMR3 or PIKfyve compared to control siRNA treatment; or upon PIKfyve inhibitor YM201636 treatment compared to DMSO treated cells. Right panel: protein synthesis measured by [^{3}H]leucine incorporation. No significant differences.

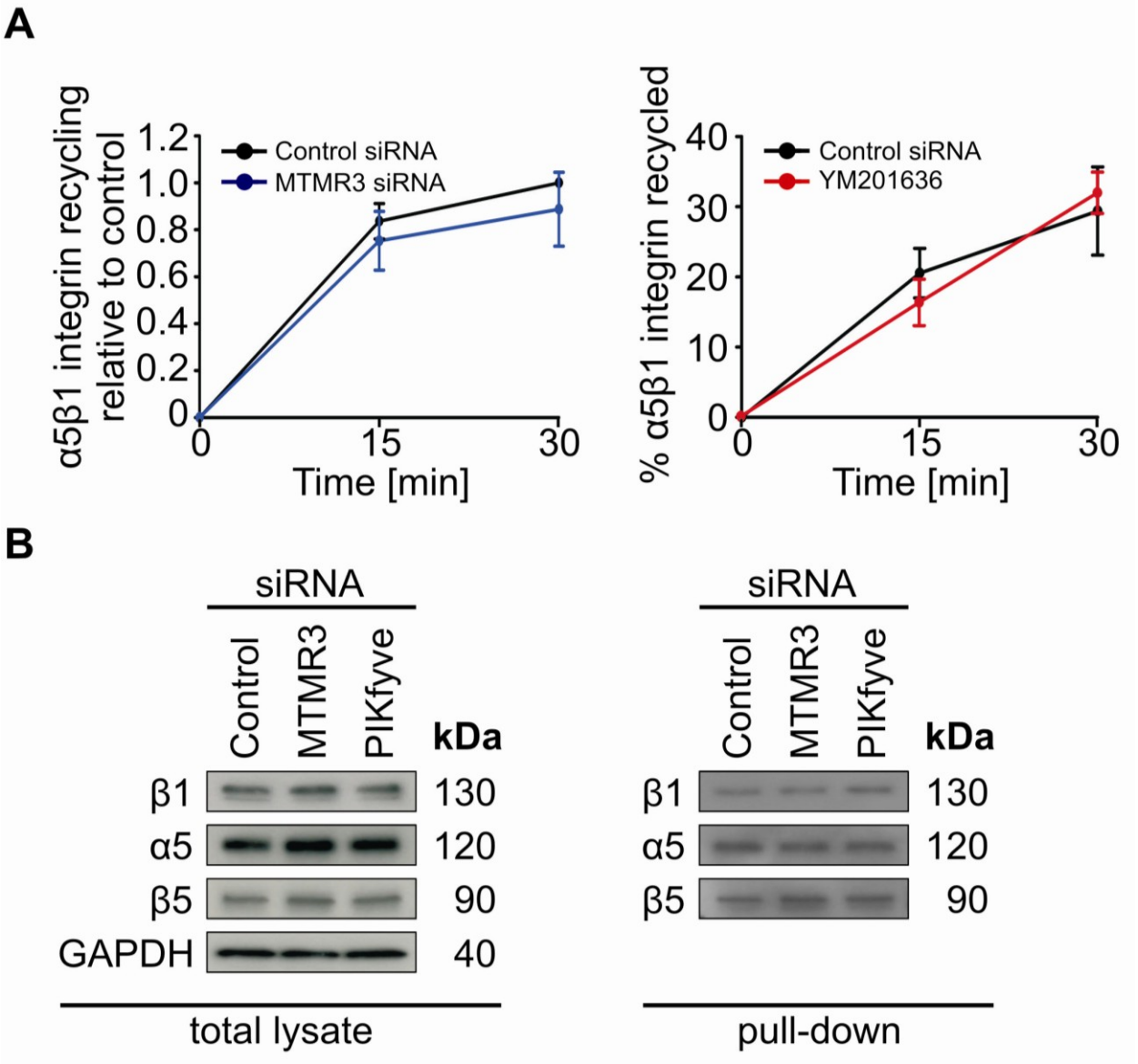
Supplementary Figure S3



Supplementary Figure S3: Random velocity without growth factor stimulation.

A, Velocities of either control siRNA, MTMR3 siRNA or PIKfyve siRNA treated cells without FGF1 stimulation in a random cell migration assay. Cells analyzed in total: 210 (Control siRNA); 235 (MTMR3 siRNA); 230 (PIKfyve siRNA).

Supplementary Figure S4



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Merge with Hoechst
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MVB
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PM
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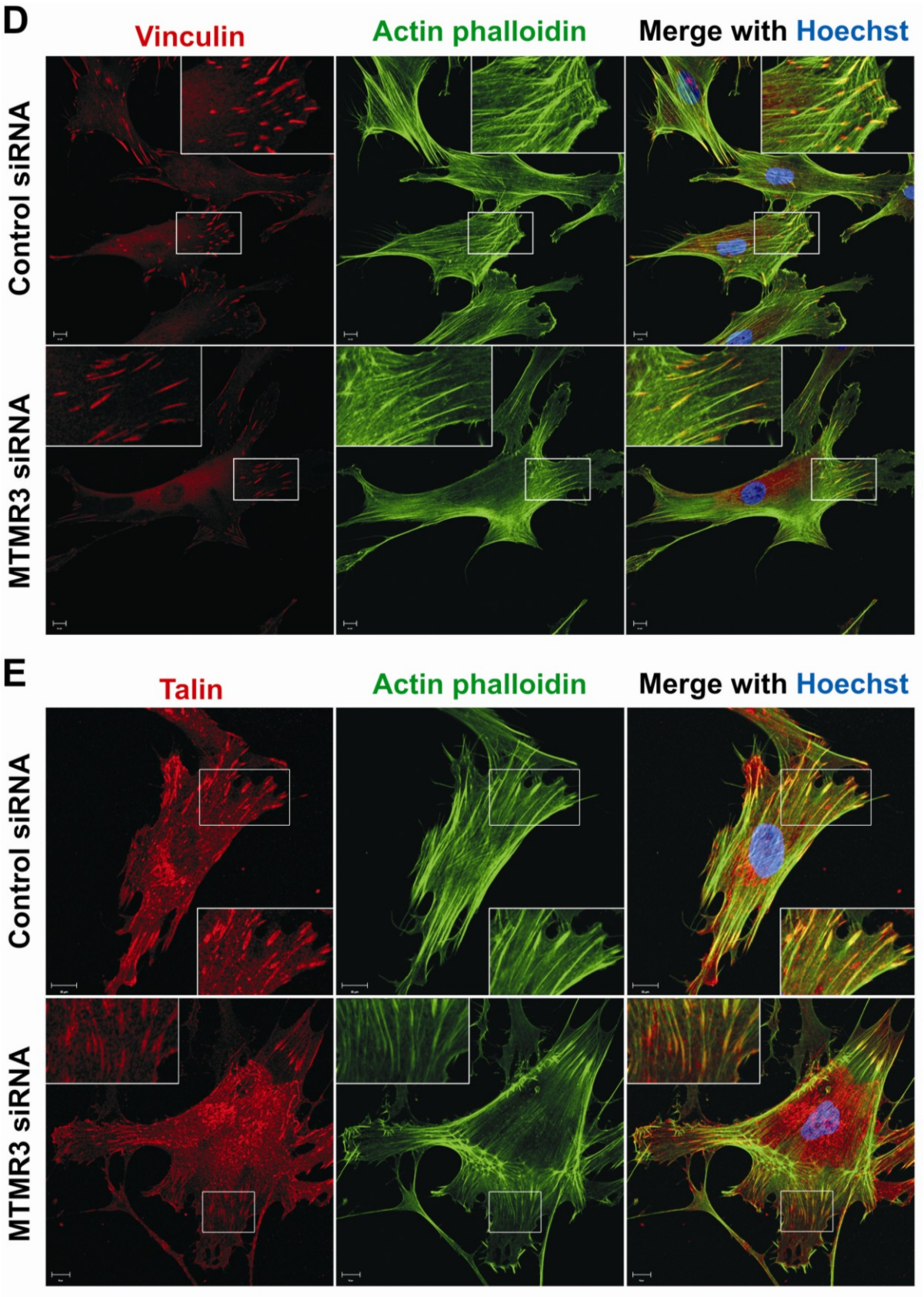
PM
MVB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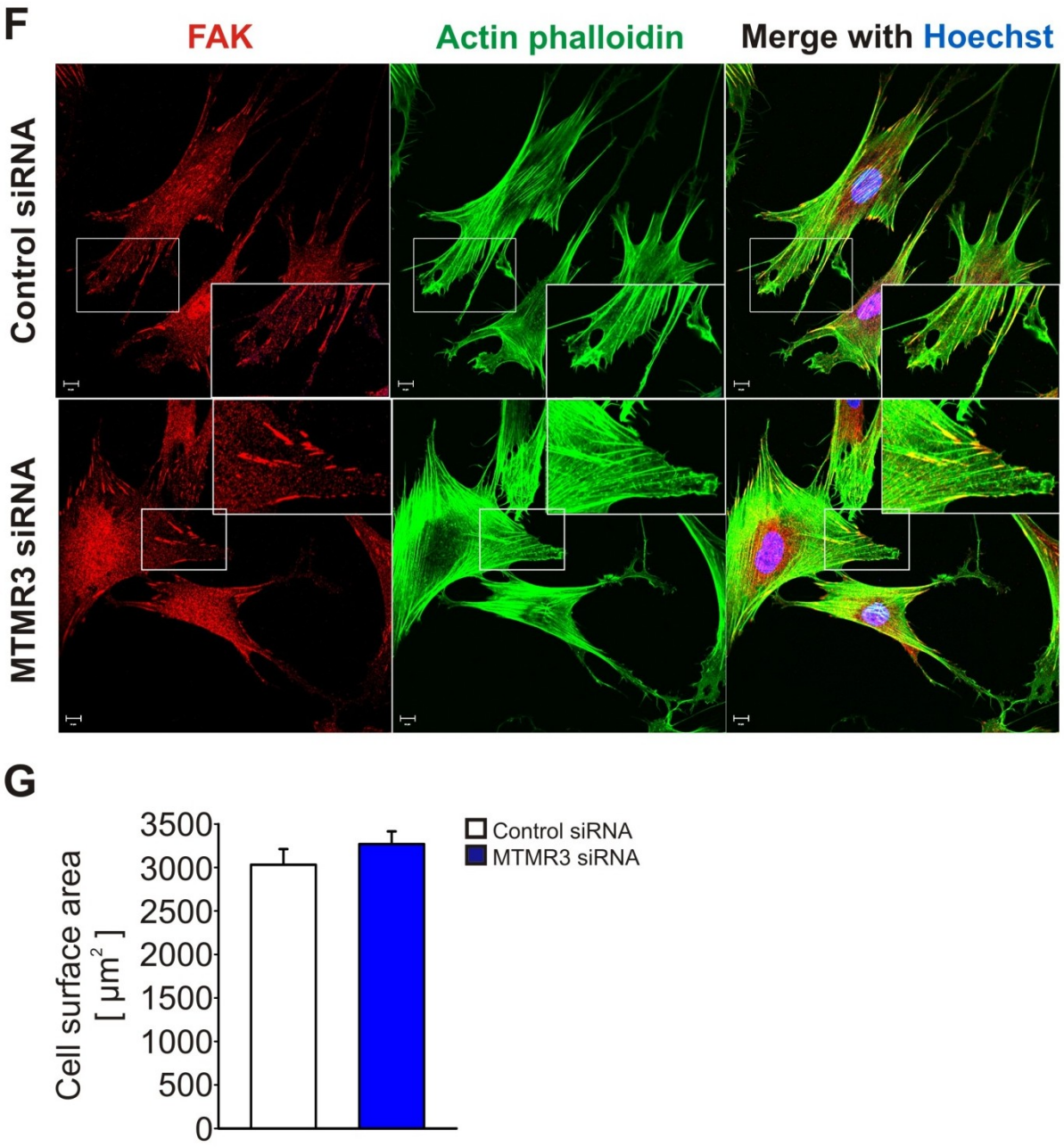
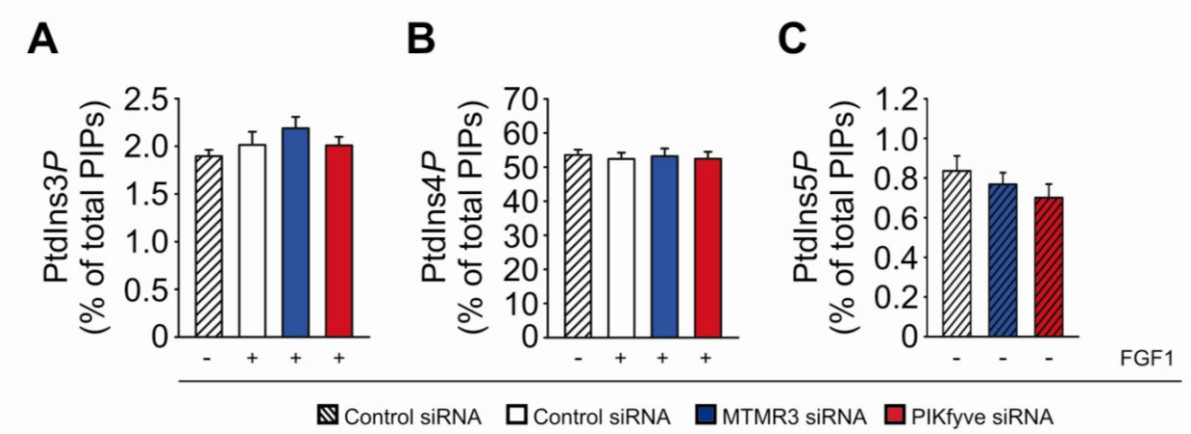


Supplementary Figure S4: MTMR3 does not affect recycling and localization of α5β1 integrin, the integrity of focal adhesion complexes, or the spreading on fibronectin.

A, α5β1 integrin recycling in control and MTMR3-depleted BJ cells stimulated with FGF1 (left panel) and in control siRNA and PIKfyve inhibitor YM201636-treated cells stimulated with FGF1 (right panel). **B,** Western blot analysis of total cell lysates (left panel) and of pull-down experiments showing cell-surface proteins (right panel) from siRNA treated cells as indicated. Immunoblotting as indicated. **C-F,** Control and MTMR3-depleted cells were seeded onto coverslips and stained with anti-α5 integrin and EEA1 (**C**), vinculin and actin (**D**), talin and actin (**E**) and focal adhesion kinase (FAK) and actin (**F**). Scale bars, 10 µm. Lower panel in **C,** Characterization of α5-integrin labeling by immuno-EM. Integrin was found in control cells at the plasma membrane (PM), smaller vesicular structures (upper left panel) and in multivesicular bodies (MVB) (upper right panel). We observed a

similar distribution of α5-integrin in cells depleted for either MTMR3 (lower left panel) or PIKfyve (lower right panel). Arrowheads denote α5-integrin labeling in all micrographs. Scale bar represents 200 nm. **G,** Cell spreading on fibronectin. Control and MTMR3-depleted BJ cells were seeded on fibronectin-coated coverslips and left to adhere for a period of 1 hour. Cells were fixed with actin and cell surface area was quantified using Image J software. Cells analyzed in total: 197 (Control siRNA); 164 (MTMR3 siRNA).

Supplementary Figure S5



Supplementary Figure S5: Phosphoinositide measurements.

A, PtdIns3*P* levels and **B,** PtdIns4*P* levels in percentage of total phosphoinositides in BJ cells, stimulated with FGF1 and different siRNA treatments as indicated. **C,** PtdIns5*P* levels in percentage of total phosphoinositides in BJ cells upon siRNA treatment as indicated without growth factor stimulation. Values are means ± s.e.m. of five independent experiments for all panels.

Supplementary Table 1

Putative *Drosophila* homologues of members of the family of human myotubularin phosphatases.

Homo sapiens	***Drosophila melanogaster***
MTM1/MTMR1/MTMR2	CG9115 (Myotubularin)
MTMR3	CG3632 (Myotubularin-like)
MTMR4 (MTMR6/MTMR7/MTMR8)	CG3530
MTMR9	CG5026
MTMR10/MTMR11/MTMR12	CG14411
MTMR13/MTMR5 (SET-binding factors)	CG6039 (SET domain binding factors)

Putative *Drosophila* homologues of members of the family of human myotubularin phosphatases were identified by sequence homology searches. Members of each group of human myotubularin phosphatases have putative homologues in *Drosophila melanogaster*. Among these, Myotubularin-like (CG3632) shows highest homology to human MTMR3 and contains an N-terminal myotubularin phosphatase domain and a C-terminal FYVE domain. *Drosophila* myotubularin (CG9115) shows high homology to human MTM1/MTMR1/MTMR2. *CG3530* shows high homology to human MTMR4 and contains both a myotubularin phosphatase domain and a FYVE domain, as well as to human MTMR6/MTMR7/MTMR8 within the N-terminal region that contains the myotubularin phosphatase domain. *CG5026* shows high homology to MTMR9, *CG14411* to MTMR10/MTMR11/MTMR12 and *Drosophila* CG6039 (SET domain binding factors) to MTMR13/MTMR5 (SET binding factors).

Supplementary Movies

Movie 1: Shown are movies of FGF1-treated BJ cells in a confluent monolayer, scratched with a pipette tip. To the left, control siRNA-transfected cells; to the right, MTMR3 siRNA-transfected cells. The movie represents a period of 10 hours with 6 frames per hour recorded. It is evident that control siRNA-transfected cells are moving faster and closing the wound. Cell division events seen at the end of the movie indicates that the imaging conditions are non-toxic.

Movie 2: MTMR3 knockdown cells were retransfected with an siRNA-resistant EGFP-MTMR3 cDNA construct. The first picture shows the EGFP signal from the retransfected cell (indicated by the arrow), followed by a movie using phase contrast for 10 hours, with 6 frames per hour. The green line indicates the travelled distance of the retransfected cell, and the other coloured lines represent the tracks of MTMR3 knockdown cells without retransfection.