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Supporting Information for

MTM1-mediated production of phosphatidylinositol 5-phosphate fuels the formation of podosome-like protrusions regulating myoblast fusion

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Extended Methods
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Legend for Movie S1 and S2
Uncropped Western blot

Other supporting materials for this manuscript include the following:

Movie S1
Movie S2

Extended Methods

Cell culture

C2C12 mouse myoblasts (ECACC 91031101) were maintained at low density in DMEM-glutamax without pyruvate (Gibco) supplemented with 20% fetal bovine serum and differentiated in DMEM-glutamax without pyruvate supplemented with 2% horse serum when cells reached 90-95% confluency. Briefly, cells were seeded at 8,000 cells/cm^2 with or without glass coverslips coated with 0.2% gelatin. Two days after, cells were switched to differentiation medium and culture media was changed every day. CRISPR-Mediated Genome Editing in C2C12 Cells is described in *SI Appendix*, *Extended Methods.* Cells were routinely tested for *Mycoplasma* contamination and all tests were negative.

Plasmids

pMJ915 used for recombinant Cas9 purification was a gift from Jennifer Doudna (*University of California,* Berkeley/HHMI). N174-MCS (puromycin) was a gift from Adam Karpf (Addgene plasmid # 81068; http://n2t.net/addgene:81068; RRID:Addgene_81068), N174-MCS (neomycin) was a gift from Adam Karpf (Addgene plasmid # 81061; http://n2t.net/addgene:81061 ; RRID:Addgene_81061). peGFP-C1 was from Clontech. peGFP-PIP4K2A, peGFP-PIP4K2B and peGFP-PIP4K2C were a gift from Jonathan Clarke (Uni. Of Cambridge). The pBabe-X-SF1-myomaker plasmid was a gift from Douglas Millay (*Cincinnati Children's Hospital Medical Center).* N174(puro)-flag-MTM1, N174(puro)-flag-MTM1-CS, N174(neo)-Flag-PIP4K2A and N174(neo)-Flag-PIP4K2C were constructed using the plasmids and primers described in Supplementary Table 2 using *In-fusion kit (Takara Bio)* according to the manufacturer's instructions. N174(neo)-Flag-PIP4K2A-sh2-resist and N174(neo)-Flag-PIP4K2C-sh2-resist were constructed using the plasmids and primers described in *SI Appendix*, Table S2 and the mutagenesis protocol of the *In-fusion kit (Takara Bio)* according to the manufacturer's instructions. pGEX-2TK-PH (PLCδ1) and pGEX-2TK-FYVE (HRS) were a gift from Mark Lemmon (Yale, U.S.A). PH (PLCδ1) and FYVE (HRS) were

first subcloned into pmCherry-C1 (Clontech) using the restriction enzymes and primers listed in Supplementary Table 2 then subcloned into pGEX-4T-1 (Pharmacia Biotech) using the restriction enzymes and primers listed in *SI Appendix*, Table S2. shRNAs constructs: Mouse *Mtm1* (Stock: TRCN0000080723 (*Mtm1* shRNA #1), TRCN0000080727 (*Mtm1* shRNA #2)), *Pip4k2a* (Stock: TRCN0000415033 (*Pip4k2a* shRNA #1), TRCN0000025576 (*Pip4k2a* shRNA #2), TRCN0000425695 (*Pip4k2a* shRNA #3)), *Pip4k2c* (Stock: TRCN0000024701 (*Pip4k2c* shRNA #1), TRCN0000024702 (*Pip4k2c* shRNA #2)) TRC shRNA were from Sigma-Aldrich (St. Louis, USA), *Tks5* (Stock: TRCN0000105730 (*Tks5* shRNA)), *Dnm2* (Stock: TRCN0000317550 (*Dnm2* shRNA). SHC003 (Sigma-Aldrich) was used as a control.

CRISPR-Mediated Genome Editing in C2C12 Cells

The webtool CRISPOR (1) was used to design gRNAs targeting *MTM1* based on their specificity score. Low-passage C2C12 cells were electroporated with Cas9-sgRNA *RNP targeting MTM1 exon 8 using* the Neon transfection system (*Life Technologies*). 12.5 pmol of recombinant Cas9 purified as described (2) were incubated with 75 pmol of sgRNA synthetized using the EnGen sgRNA Synthesis Kit (*New England Biolabs) from the template oligonucleotide* described in Supplementary Table 2 and purified with the RNA clean & concentrator-25 kit (Zymo research). The complex was incubated 10 minutes at room temperature. 100,000 cells were mixed with the RNP and electroporated using the *following* conditions: 1,650V, 10 ms, 3 pulses and then placed in a well of a twelve-well plate containing 500 µl growth medium without antibiotics. 24 hours after electroporation, cells were detached using accutase (BD biosciences) and half was used to monitor genomic editing efficiency and half was sorted as single cell into 96-well plates using FACS. Briefly, for genomic editing efficiency, *genomic DNA was isolated using the* GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich) and was used as template to amplify by a nested PCR (primers described in Supplementary Table 2), a 638-bp region surrounding the predicted site of Cas9 activity using PrimeSTAR Max DNA Polymerase (Takara Bio). Genomic editing efficiency verification was done using a T7 endonuclease assay according to the manufacturer's instructions (*New*

England Biolabs. After single cell sorting, each clone was amplified and genotyped by amplifying the 638-bp region described previously and analyzed with the TIDE webtool (Tracking of Indels by Decomposition) (3) to identify clones with frameshift mutations *(TIDE results for the isolated clone used in this study presented in SI Appendix*, Fig. S1 *A*). Identified clones were further assessed for their capacity of differentiation. The isolated clone used in this study was further characterized by sequencing predictive off-targets provided by CRISPOR (amplified with primers described in Supplementary Table 2 and results presented in *SI Appendix*, Fig. 1 *C*); and *bacterial* single colony *sequencing of modified alleles (amplified* using the primers listed in *SI Appendix*, Table S2 from the genomic DNA and subcloned in a pcDNA3.1(+) vector using the *In-fusion kit (Takara Bio))*. Frameshift mutations are presented in *SI Appendix*, Fig. S1 *B*.

Western Blotting

Total cellular proteins were extracted with Cell Lysis Buffer (Cell Signaling) and 20 µg proteins were separated by electrophoresis on *4-12% gradient* SDS-*polyacrylamide gel* (Life Technologies) and transferred on Immobilon-P membranes (Millipore). Membranes were then incubated with appropriate antibodies (references, dilutions, buffer and incubation times are available in *SI Appendix*, Table S1) and immunoreactive bands were detected by chemiluminescence using ChemiDoc MP (Bio-Rad Laboratories) with clarity Western ECL substrate detection system (Bio-Rad Laboratories). Full length original western blots are provided in *SI Appendix*.

Lentivirus production and transduction

Vector particles were produced as previously described (4). C2C12 cells were transduced by incubation with lentiviral particles and then washed 48 h later with growth medium in the presence of 2 µg.ml^{-1} puromycin supplemented with 1 mg.ml^{-1} G418 for rescue experiment with shRNA resistant gene. When cells reached 90-95% confluency, differentiations were

started as previously described in the presence of 1 µg.ml^{-1} puromycin supplemented with 500 µg.ml−1 G418 for rescue experiment with shRNA resistant genes.

Protein purification

Recombinant GST-mCherry-PH-domain (PLCδ1) and GST-mCherry-FYVE-domain (HRS) were expressed in BL21(DE3) bacteria overnight at 18°C using 0.5 mM IPTG and purified by affinity chromatography using *Glutathione* Sepharose 4B beads (*GE Healthcare)* according to the manufacturer's instructions. GST-mCherry-PH-domain (PLCδ1) / GST-mCherry-FYVE-domain (HRS) were purified in 50 mM Tris at pH 8.0, 100 mM NaCl, 10% glycerol, snap-frozen and stored at -80°C.

Microscopy and image analysis

For average area of MYH4 positive cells, fusion index, clustered nuclei and relative directionality calculations, cells were fixed with 3.7% formaldehyde for 10 minutes at room temperature, quenched with 50 mM NH_4Cl for 10 minutes and permeabilized by 0.1% Triton X-100 in DPBS during 10 minutes. After 1 hour of saturation with 10% goat serum in DPBS, cells were incubated for 1 hour with antibodies at room temperature. After incubation with fluorescent secondary antibodies (Thermofisher), nuclei were stained with DAPI (Euromedex), and cells were washed and maintained in DPBS. Imaging was performed with the ZOE Fluorescent Cell Imager (Bio-Rad Laboratories). Area, fusion and differentiation indexes were calculated using ImageJ. Relative myotube alignment was quantified using a two-dimensional fast Fourier transform using ImageJ by following the protocol described in (5). *Endomembrane staining protocol*: cells were fixed with 3.7% formaldehyde, quenched with NH_4Cl for 10 minutes and permeabilized with 20 µM Digitonin/PIPES-BS (PIPES 20 mM pH 6.8, NaCl 137 mM, KCl 2.7 mM) for 5 minutes. After 1 hour saturation period in 10% goat serum/PIPES-BS, cells were incubated with 50 µg/ml of the GST-mCherry-FYVE-domain (HRS) probe and/or primary antibodies for 2 hours at room temperature. After 3 washes with PIPES-BS, secondary fluorescent antibodies in 10% goat serum/PIPES-BS were added for 1 hour, washed 3 times

with PIPES-BS. Cells were then fixed a second time with 3.7% formaldehyde, nuclei were stained with DAPI, washed 3 times with DPBS, mounted with FluorSave reagent (Calbiochem). *Plasma membrane staining protocol*: $PI(4,5)P_2$ and proteins staining were done as described in (6) but with minor modifications. Briefly, all steps were performed on ice until the second fixation and with ice cold solutions. Cells were fixed with 3.7% formaldehyde and 0.2% glutaraldehyde for 15 min. After three washes with NH_4Cl, cells were incubated for 1 hour in blocking buffer (PIPES-BS, NH_4Cl 50mM, Goat Serum 10%, Saponin 0.05%), then incubated for two hours with primary antibodies and the probe against $PI(4,5)P_2$ in antibodies buffer (PIPES-BS, Goat Serum 10%, Saponin 0.1%). After three washes with PIPES-BS for 5 min, secondary fluorescent antibodies were added in antibodies buffer for 1 hour. After a wash with PIPES-BS, cells were fixed a second time with 3.7% formaldehyde for 10 min, then 5 min room temperature. After three washes with DPBS, nuclei were stained with DAPI, washed 3 times with DPBS, and mounted with FluorSave reagent (Calbiochem). Imaging was performed with confocal LSM780 Zeiss microscope (Zen software, x63 objective) or LSM900 Zeiss microscope (Zen software, x63 objective). Fluorescence intensities were calculated with ImageJ. To calculate relative cell area to volume ratio of WT and *Mtm1*-KO C2C12 myoblasts, cell areas were determined from 10 images per condition per experiment accounting for 50 to 100 cells divided by forward scatter values measured by FACS (obtained from 10,000 cells per experiment per condition). For time-lapse microscopy, 3 days differentiated myoblasts were imaged in gelatin-coated ibidi 35 mm glass bottom (Ibidi, 81158). Imaged were acquired with a Zeiss Cell Observer.Z1 microscope, objective Plan-Apochromat 40x/1.4 oil, camera CCD Hamamatsu Orca R2 (Model C10600-10B-H). Imaging parameters: 20% LED intensity, 600ms exposure time, every 10 minutes for 2 days. For confocal time-lapse microscopy, 3 days differentiated myoblasts were imaged in gelatin-coated Ibidi µ-Slide 4 well (Ibidi, 80426). Imaged were acquired with a Zeiss LSM900 confocal microscope, objective Plan-Apochromat 20x/0.8 M27). Imaging parameters: 0.4% laser intensity, 600ms exposure time, every 10 minutes for 2 days. For the random cell migration assay, C2C12 cells were initially seeded onto gelatin-coated Ibidi µ-Slide 4 well (Ibidi, 80426). Live cell imaging was then carried out at

37°C with 5% CO2 using a Zeiss Cell Observer.Z1 microscope equipped with a Plan-Apochromat 20x objective and a CCD Hamamatsu Orca R2 camera (Model C10600-10B-H). Brightfield images were acquired at 10-minute intervals over a span of 17 hours. Subsequently, Cellpose 2.0 was used for cellular segmentation (7), and cell tracking was performed using the TrackMate plug-in within the Fiji Image J software (8). Pearson's correlation coefficients were calculated using the Fiji JaCoP Plugin.

Phospholipid extraction and analysis

Metabolic labeling: C2C12 cells were seeded on 10 cm plate and differentiated for the indicated periods. Metabolic labeling with [^{32}P] Phosphate (1 mCi; Perkin Elmer) was performed the day before phospholipids extraction. Phospholipids were extracted according to Bligh and Dyer procedure (9), purified by TLC (thin layer chromatography), and analyzed by high-performance liquid chromatography (HPLC) as described in (4). Mass assay for PI5P were performed exactly as described in (10) from C2C12 cells seeded on 10 cm plate and differentiated for the indicated periods.

PIP_2 measurement by mass spectrometry

PIP_2 levels were measured from C2C12 cells cultured in 6-well plates as described in (11).

Structural analysis

The structure of mouse Myomaker dimer is entry 8T03 in the PDB database. Molecular graphics and analyses were performed with Swiss-PdbViewer (12) and Pov-Ray (Persistence of Vision Pty. Ltd.).

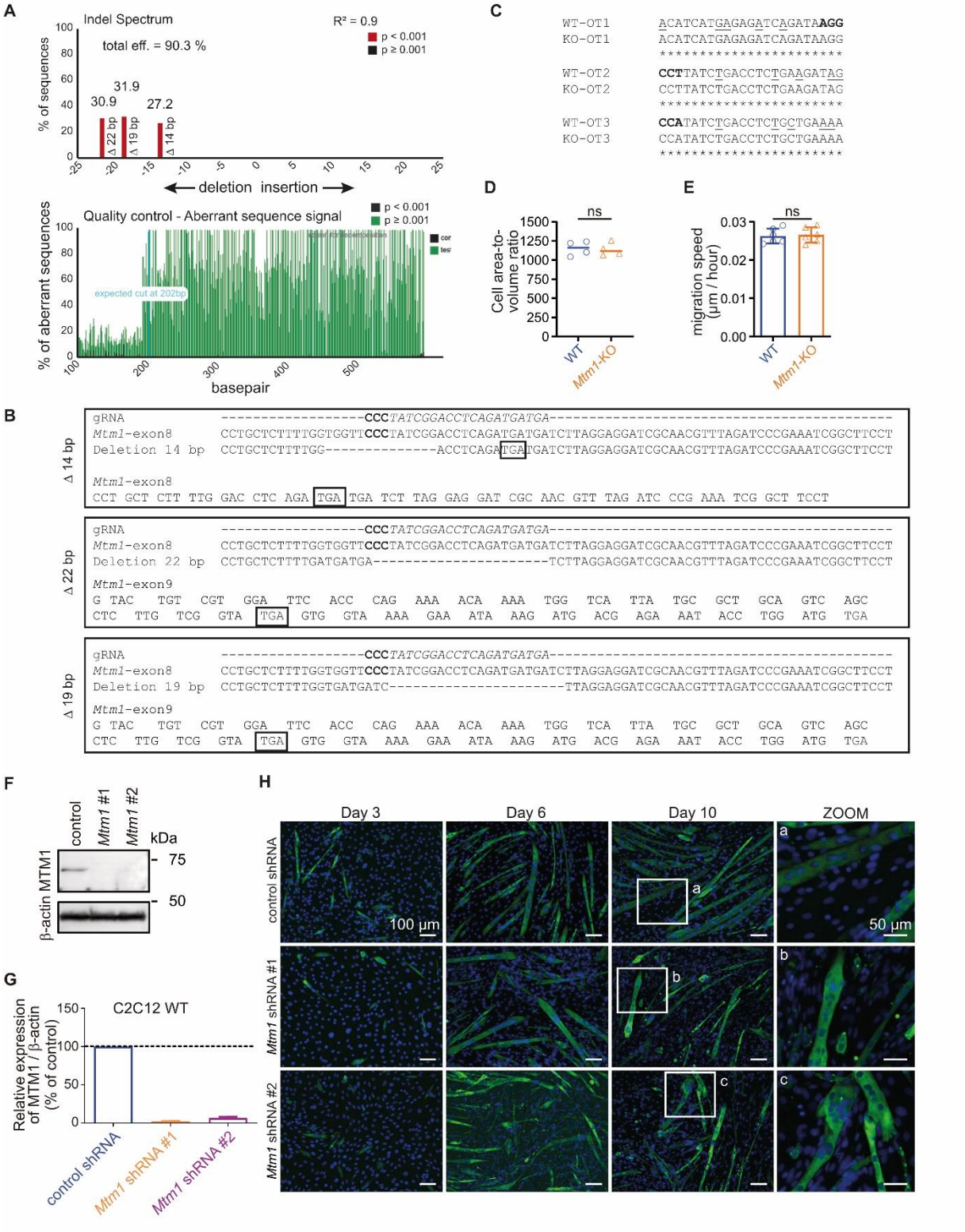


Figure S1: Generation and characterization of the C2C12 *Mtm1* knockout cell line.

(*A*) TIDE analysis of the *Mtm1*-KO isolated clone. (*B*) Alleles sequencing of the isolated clone confirming the TIDE result and identification of the premature stop codons. (*C*) Sequencing of the three main predictive off-targets. PAM sequences are in bold. Mismatches with the gRNA targeting MTM1 are underlined. (*D*) Cell area-to-volume ratio of WT and *Mtm1*-KO C2C12 myoblasts. Each data point represents the average area obtained from 4 independent experiments, with cell area values (μm^2) normalized to the mean forward scatter values obtained through flow cytometric analysis. To measure cell area, 30 cell areas were measured per experiment, and 10,000 cells per experiment were analyzed by FACS to determine forward scatter values. ns=not significant according to Student's t-test. (*E*) Migration speed of WT and *Mtm1*-KO C2C12 myoblasts. For each replicate, 30 to 60 cells were analyzed. Data are represented as mean ± S.E.M, n=6, ns=not significant according to Student's t-test. (*F*) Expression of MTM1 on day 6 of C2C12 differentiation, expressing control shRNA, *Mtm1* shRNA #1 or *Mtm1* shRNA #2 analyzed by Western blot. (*G*) Quantification of the extinction of MTM1 in WT C2C12 expressing control shRNA or *Mtm1* shRNAs on day 6 of differentiation, analyzed by Western blot in (*E*). (*H*) MYH4 and DAPI staining of WT C2C12 expressing control shRNA or *Mtm1* shRNAs at the indicated time points. Scale bar, 100 μm. On the right, higher magnifications for the indicated areas shown as boxes. Scale bar, 50 μm.

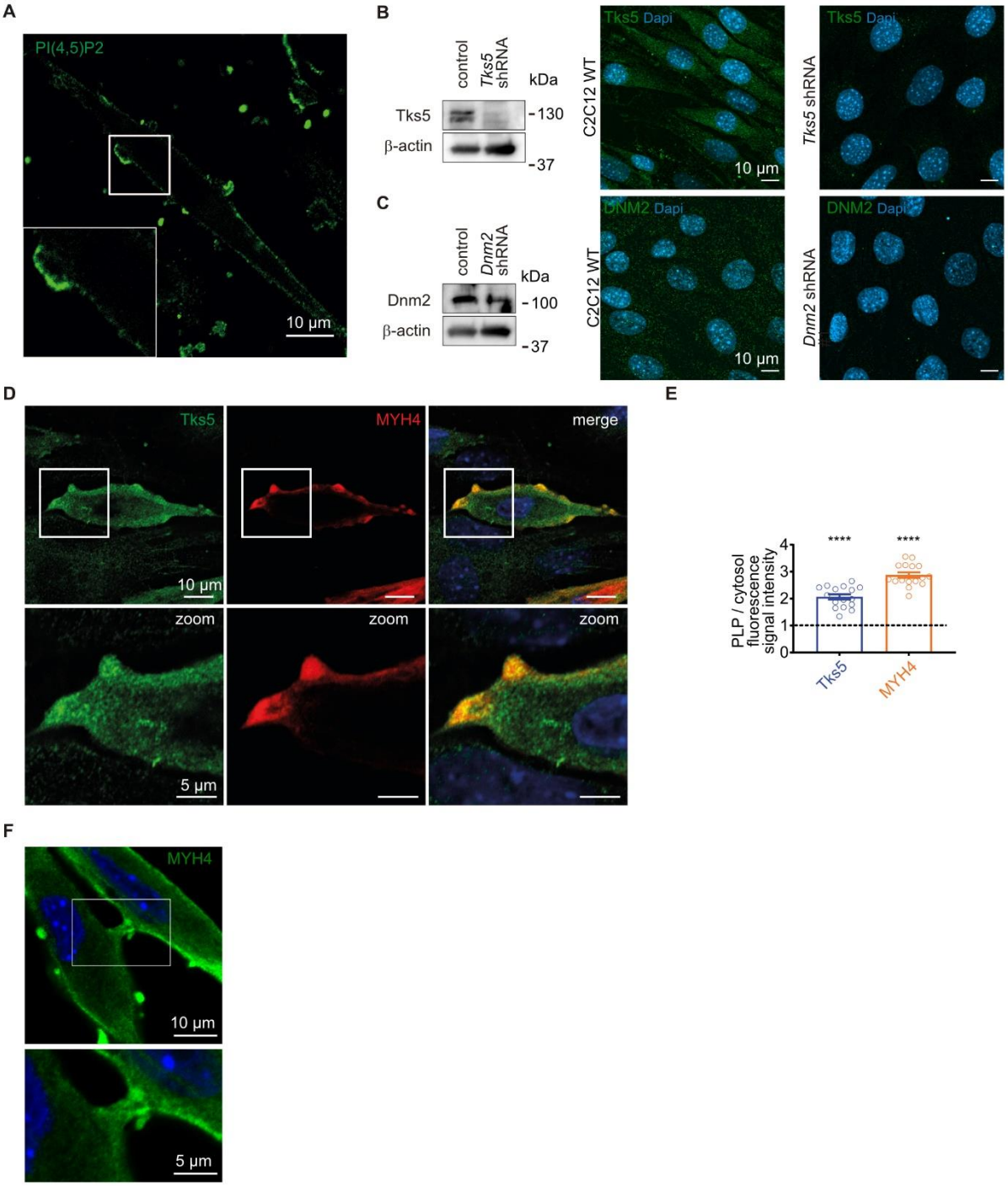


Fig. S2. PI(4,5)P_2 is highly enriched in podosome-like protrusions containing Tks5 and MYH4.

(*A*) Confocal image of C2C12 WT on day 3 of differentiation labeled with a $PI(4,5)P_2$ antibody. Scale bar, 10 µm. (*B*) Tks5 was depleted in C2C12 myoblasts by a lentiviral shRNA and selected with puromycin for 3 days. After 3 days of differentiation, cells were processed for immunoblotting (left) or immunostaining (right). Scale bar, 10 µm. (*C*) Dynamin2 was depleted in C2C12 myoblasts by a lentiviral shRNA and selected with puromycin for 3 days. After 3 days of differentiation, cells were processed for immunoblotting (left) or immunostaining (right). Scale bar, 10 µm. (*D*) Top: representative confocal images of C2C12 WT on day 3 of differentiation and labeled for Tks5, MYH4 and DAPI. Scale bar, 10 µm. Bottom: higher magnification of the areas shown as a box on the top. Scale bar, 5 µm. (*E*) Quantification of the fluorescence ratio of the indicated proteins in podosome-like protrusions (PLPs) versus cytosol. Data are represented as mean ± S.E.M, n=3, each point represents the quantification of one field of view, ****p<0.0001 according to One-sample t-test (two tailed). (*F*) Confocal images of fusing C2C12 WT on day 3 of differentiation labeled for MYH4 and DAPI. Scale bar, 10 µm. A magnification of the boxed area depicting the fusion site is shown on the right. Scale bar, 5 µm.

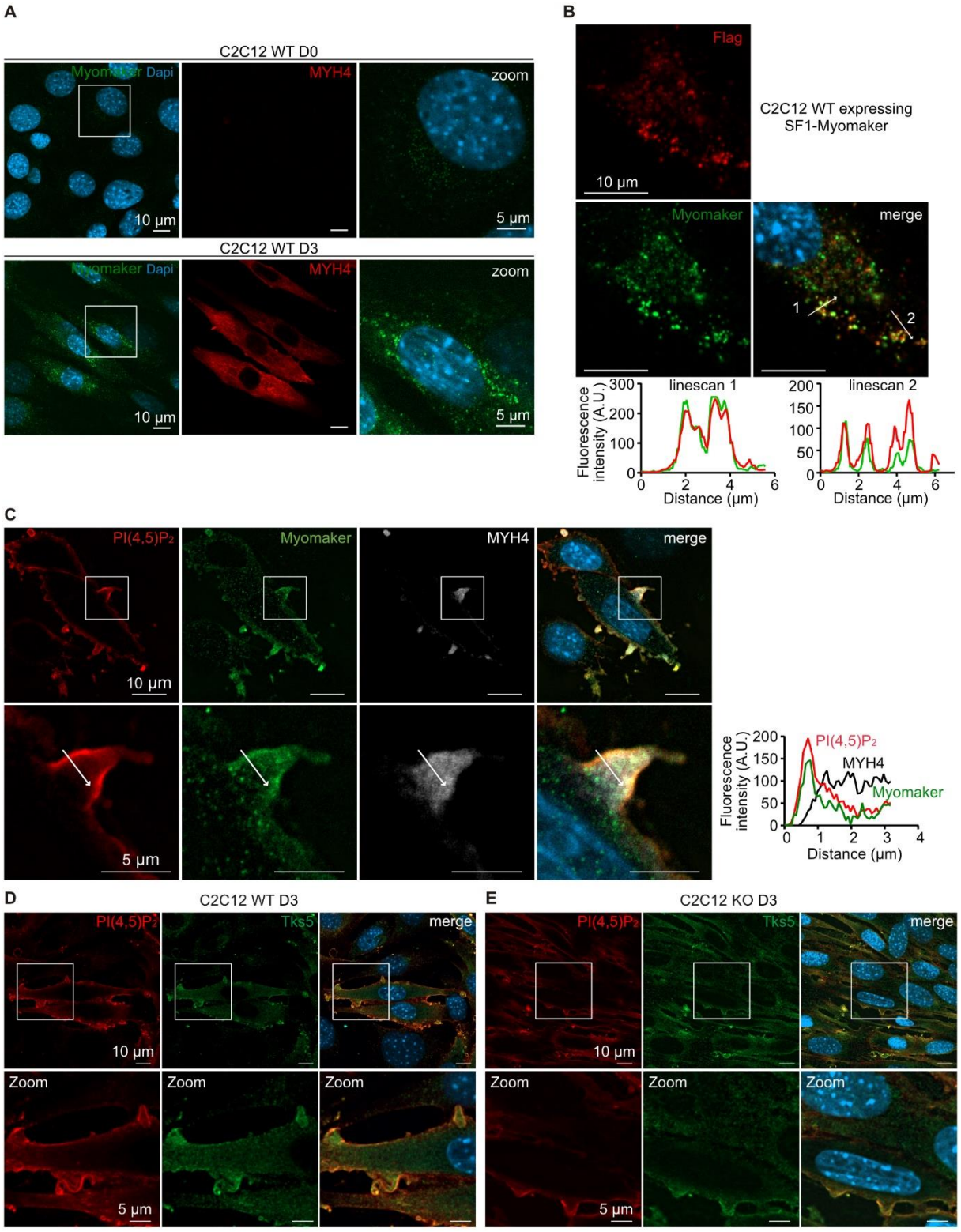


Fig. S3. Myomaker is enriched in the plasma membrane of podosome-like protrusions.

(*A*) Representative confocal images of C2C12 WT on day 0 and day 3 of differentiation and labeled for Myomaker and MYH4. Scale bar, 10 μm. A magnification of the boxed area is shown on the bottom. Myomaker localized primarily to vesicular structures in the perinuclear region. This agrees with the previously reported Golgi localization of Myomaker (13, 14). Scale bar, 5 μm. (*B*) Representative confocal images of SF1-Myomaker-expressing C2C12 WT myoblasts labeled for Myomaker and flag are shown, along with respective line scans are shown on the bottom. Scale bars, 10 μm, Complete colocalization is not observed can be observed, possibly due to the SF1-Myomaker construct, which contained a synthetic cleavable signal sequence upstream of FLAG, followed by full-length Myomaker protein. This may have rendered the flag tag partially inaccessible to the antibody. Importantly, no fluorescence signal was detected in untransfected cells. (*C*) Representative confocal images of C2C12 WT after 3 days of differentiation and labeled with a PI(4,5)P_2 probe, Myomaker, MYH4 and DAPI. Scale bar, 10 μm. Magnification of the boxed areas shown on the bottom. A line scan is shown on the right. Scale bar, 5 μm. (*D*) Representative confocal images of C2C12 WT on day 3 of differentiation labeled with a PI(4,5)P_2 antibody and for Tks5 and DAPI. Scale bar, 10 μm. Magnification of the boxed area shown on the bottom. Scale bar, 5 μm. (*E*) Representative confocal images of *Mtm1*-KO C2C12 on day 3 of differentiation labeled with a PI(4,5)P_2 antibody and for Tks5 and DAPI. Scale bar, 10 μm. Magnification of the boxed area shown on the bottom. Scale bar, 5 μm.

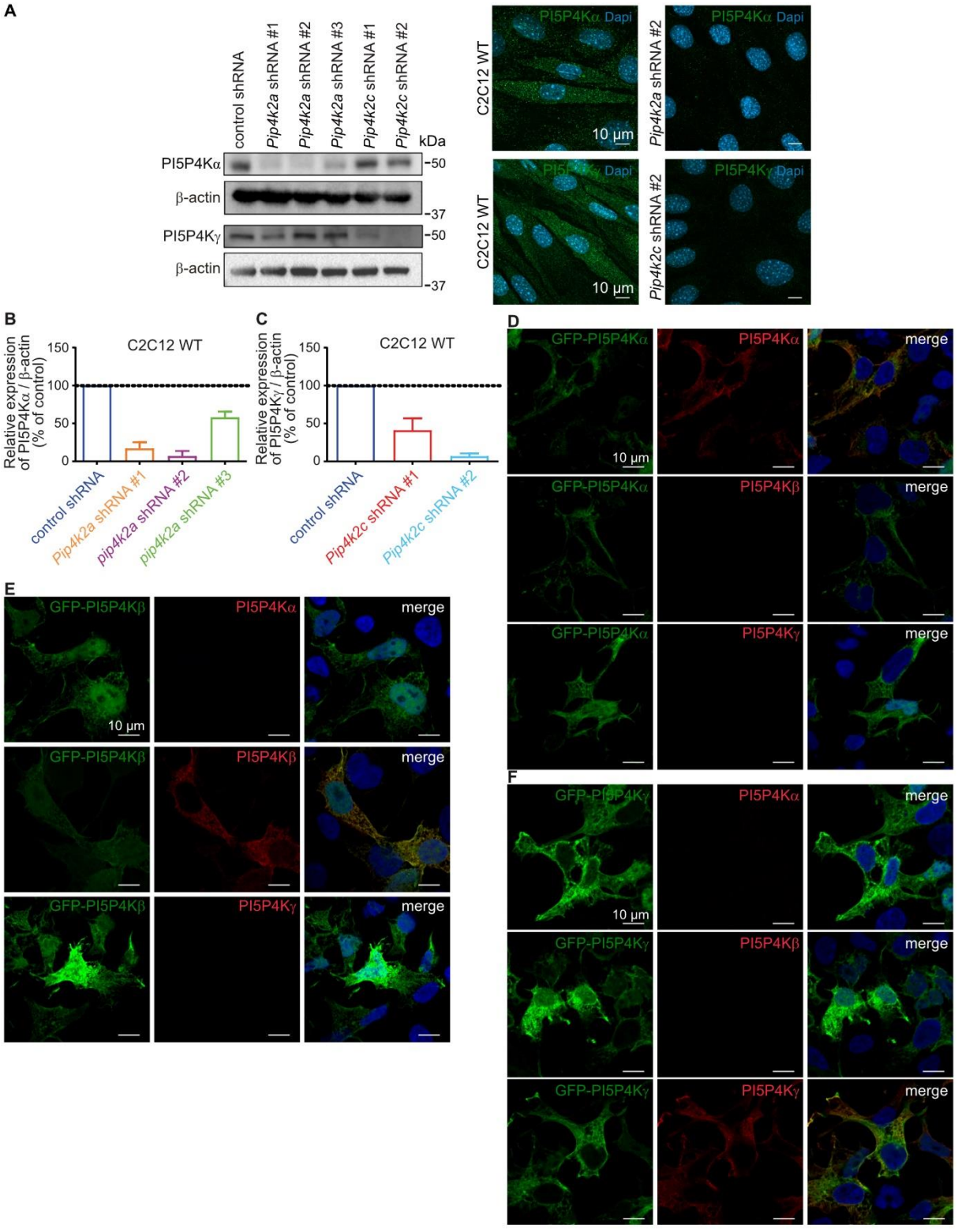


Fig. S4. Validation of PI5P4Kα and PI5P4Kγ antibodies.

(*A*) Left: Expression of PI5P4Kα and PI5P4Kγ on day 3 of differentiation of C2C12 expressing control shRNA, *Pip4k2a* shRNAs or *Pip4k2c* shRNAs analyzed by Western blot. Right: PI5P4Kα and PI5P4Kγ were depleted in C2C12 myoblasts by a lentiviral shRNA and selected with puromycin for 3 days. After 3 days of differentiation, cells were processed for immunostaining. Scale bar, 10 µm. (*B*) Quantification of the extinction of PI5P4Kα in WT C2C12 expressing control shRNA or *Pip4k2a* shRNAs on day 3 of differentiation analyzed by Western blot in (*A*). (*C*) Quantification of the extinction of PI5P4Kγ in WT C2C12 expressing control shRNA or *Pip4k2c* shRNAs on day 3 of differentiation analyzed by Western blot in (*A*). (*D-F*) Representative images of HEK cells expressing GFP-PI5P4Kα (*D*) GFP-PI5P4Kβ (*E*) or GFP-PI5P4Kγ (*F*) and labeled with antibodies directed against PI5P4Kα, PI5P4Kβ, or PI5P4Kγ. Note that although cells transfected with GFP-PI5P4Kbeta display nuclear staining, no staining for PI5P4Kβ is detected within the nucleus because digitonin is ineffective at permeabilizing the nuclear membrane (15). Scale bar, 10 µm.

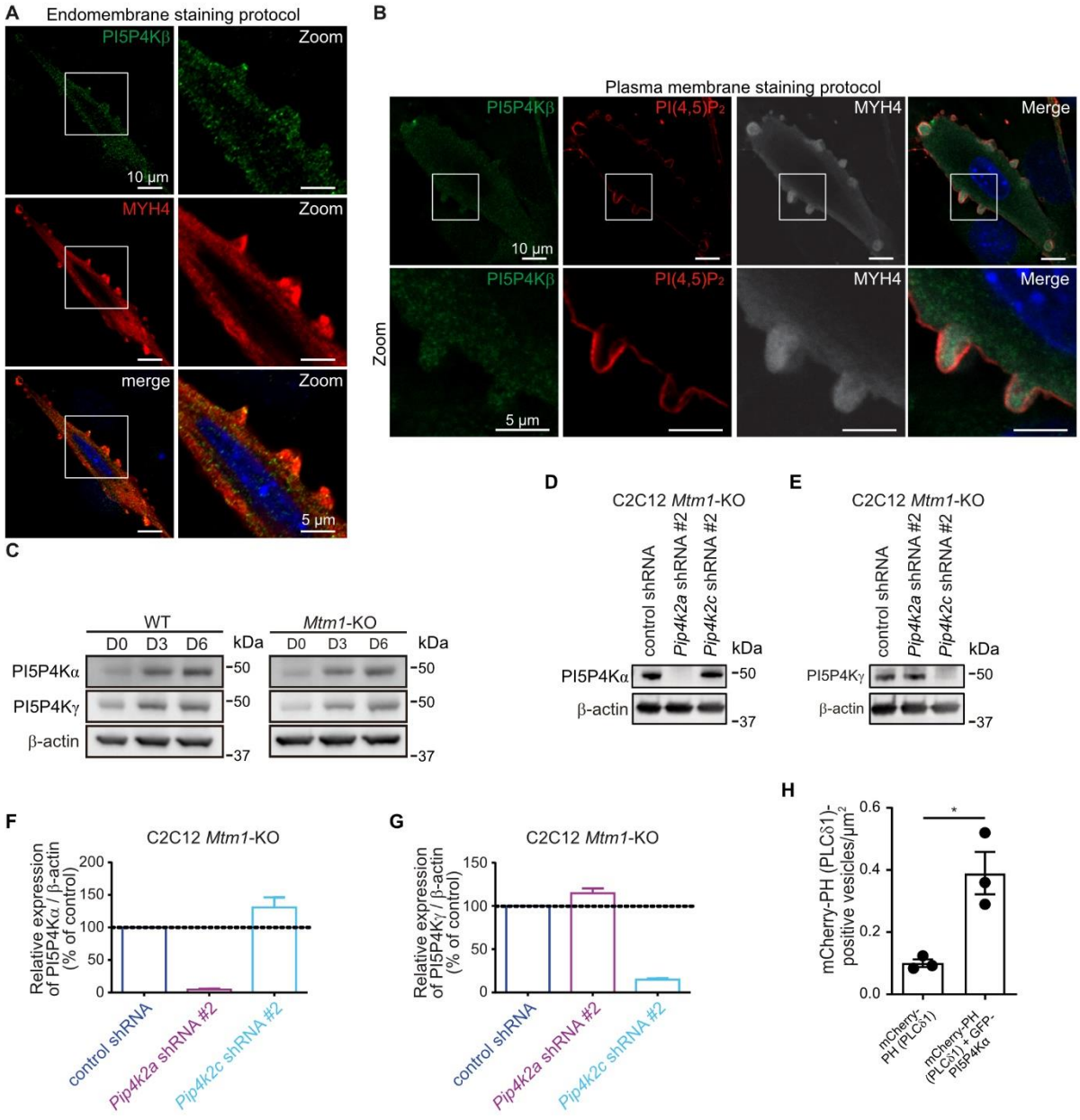


Fig. S5. PI5P4K2β is not enriched in podosome-like protrusions.

(*A*) Left: representative confocal images of C2C12 WT on day 3 of differentiation and labeled for PI5P4K2β, MYH4 and DAPI. Scale bar, 10 µm. Right: higher magnification of the area shown as a box on the left. Scale bar, 5 µm. (*B*) Top: representative confocal images of C2C12 WT on day 3 of differentiation and labeled for PI5P4Kβ, PI(4,5)P2 probe, MYH4 and DAPI. Scale bar, 10 µm. Bottom: higher magnification of the area shown as a box on the top. Scale bar, 5 µm. (*C*) Expression of PI5P4Kα and PI5P4Kγ during WT and *Mtm1*-KO C2C12 differentiation analyzed by Western blot. Images are representative of 3 independent experiments. (*D*) Expression of PI5P4Kα on day 3 of differentiation of C2C12 *Mtm1*-KO expressing control shRNA, *Pip4k2a* shRNA #2 or *Pip4k2c* shRNA #2 analyzed by Western blot. (*E*) Expression of PI5P4Kγ on day 3 of differentiation of C2C12 *Mtm1*-KO expressing control shRNA, *Pip4k2a* shRNA #2 or *Pip4k2c* shRNA #2 analyzed by Western blot. (*F*) Quantification of the extinction of PI5P4K2α in C2C12 *Mtm1*-KO expressing control shRNA, *Pip4k2a* shRNA #2 or *Pip4k2c* shRNA #2 on day 3 of differentiation analyzed by Western blot in (D). (*G*) Quantification of the extinction of PI5P4Kγ in C2C12 *Mtm1*-KO expressing control shRNA, *Pip4k2a* shRNA #2 or *Pip4k2c* shRNA #2 on day 3 of differentiation analyzed by Western blot in (*E*). (*H*) Quantification of mCherry-PH(PLCδ1)-positive vesicles density in C2C12 transfected with mCherry-PH(PLCδ1) alone or co-transfected with GFP-PI5P4Kα, and differentiated for 3 days. Data are represented as mean ± S.E.M, n=3, each point represents the mean of 10 cells per experiment, *p<0.05 according to Student's t-test.

Supplementary Table S1. List of antibodies used for immunoblotting and immunofluorescence.

Immunoblotting

Primary Antibody	**Species**	**Source**	**Catalog Number**	**clone**	**dilution**	**RRID**
β-Actin	mouse	Sigma-Aldrich	A5441	AC-15	1:5000	AB_476744
MTM1	rabbit	home made	n/a	2827	1:5000	n/a
PI5P4Kα	rabbit	Cell Signaling	#5527S	D83C1	1:1000	AB_2722636
PI5P4Kγ	rabbit	Proteintech	17077-1-AP	n/a	1:5000	AB_2715526
Tks5	rabbit	Santa Cruz	sc-30122	M-300	1:1000	AB_2254551
Dynamin-2	rabbit	Invitrogen	PA1-661	n/a	1:1000	AB_2293040
Myogenin	mouse	DSHB	F5D	n/a	1 :500	AB_2146602
Secondary Antibody	**Species**	**Source**	**Catalog Number**	**dilution**	**RRID**	
anti mouse HRP	goat	Promega	W4021	1:5000	AB_430834	
anti rabbit HRP	goat	Promega	W4011	1:5000	AB_430833	

Immunofluorescence

Primary Antibody	Species	Source	Catalog Number	clone	dilution	RRID
Myosin Heavy Chain	mouse	eBioscience	14-6503-82	MF-20	1:500	AB_2572894
PI5P4Kα	rabbit	Atlas Antibodies	HPA065440	n/a	1:300	AB_2685490
PI5P4Kβ	rabbit	gift from J. Clarke	n/a	n/a	1:300	n/a
PI5P4Kγ	rabbit	gift from J. Clarke	n/a	n/a	1:300	n/a
$PI(4,5)P_2$	mouse	Echelon Bio.	Z-P045	2C11	1:500	AB_427225
Tks5	rabbit	Santa Cruz	sc-30122	M-300	1:300	AB_2254551
Dynamin-2	rabbit	Invitrogen	PA1-661	n/a	1:100	AB_2293040
Myomaker	rabbit	Sigma-Aldrich	HPA055785		1:100	AB_2682920
α-actin	mouse	Sigma-Aldrich	A2172		1:300	AB_476695
Pan-Cadherin	mouse	Sigma-Aldrich	C1821	CH-19	0,25	AB_476826
active β1 integrin	mouse	BD Biosciences	553715	9EG7	1:300	AB_395001
Secondary Antibody	**Species**	**Source**	**Catalog Number**	**dilution**	**RRID**	
anti rabbit Alexa 488	goat	Thermo Fisher Scientific	A11008	1:500	AB_143165	
anti mouse Alexa 594	goat	Thermo Fisher Scientific	A11005	1:500	AB_2534073	
anti rabbit Alexa 594	goat	Thermo Fisher Scientific	A11012	1:500	AB_2534079	
anti mouse Alexa 647	goat	Thermo Fisher Scientific	A21235	1:500	AB_2535804	

Supplementary Table S2. List of primers and oligonucleotides used in this study.

Name of the plasmid constructions and/or purpose	Backbone plasmid	Linearized by	insert	Primers and oligonucleotides sequences
N174 (puro)-flag-MTM1	N174-MCS (puromycin) Addgene # 81068	EcoR1/BamH1	Flag-MTM1 amplified from pcDNA3-flag-MTM1	Forward : CGTGAGGATCGAATTCATGGAAGACTACAAAGACGACGACG
				Reverse : CGGTAGAATTGGATCCTCAGAAGTGAGTTTGCACATGGG
N174 (puro)-flag-MTM1-CS	N174-MCS (puromycin) Addgene # 81068	EcoR1/BamH1	Flag-MTM1-CS amplified from pcDNA3-flag-MTM1	Forward : CGTGAGGATCGAATTCATGGAAGACTACAAAGACGACGACG
				Reverse : CGGTAGAATTGGATCCTCAGAAGTGAGTTTGCACATGGG
N174 (Neo)-Flag-PIP4K2A	N174-MCS (puromycin) Addgene # 81061	EcoR1/BamH1	Flag-PIP4K2A amplified from peGFP-PIP4K2A	Forward : GGTGTCGTGAGGATCGAATTCATGGACTACAAAGACGACGACGACAAAGGTGGCGGTGGCTCCATGGCGACCCCCGGCAAC
				Reverse : CTACCCGGTAGAATTGGATCCTTACGTCAAGATGTGGCCAATAAAG
N174(Neo)-Flag-PIP4K2A-sh2-resist	N174 (Neo)-Flag-PIP4K2A			**Mutagenesis** Forward : CAAGTCACTTTAAATTCAAGGAATACTGCCCGATGGT
				Reverse : ATTTAAAGTGACTTGGCATGTTTTCTTTGTTAAAAAGGTGA
N174(Neo)-Flag-PIP4K2C	N174-MCS (Neomycin) Addgene # 81061	EcoR1/BamH1	Flag-PIP4K2C amplified from peGFP-PIP4K2C	Forward : GGTGTCGTGAGGATCGAATTCATGGACTACAAAGACGACGACGACAAAGGTGGCGGTGGCTCCATGGCGTCCTCCTCGGTC
				Reverse : CTACCCGGTAGAATTGGATCCTTAGGCAAAGATGTTGGTAATAAAA

Name of the plasmid constructions and/or purpose	Backbone plasmid	Linearized by	insert	Primers and oligonucleotides sequences
N174(Neo)-Flag-PIP4K2C-sh2-resist	N174(Neo)-Flag-PIP4K2C			**Mutagenesis** Forward : GCAAAATTAAAGTGAATAATCACCTTTTCCACAGGGA
				Reverse : TCACTTTAATTTTGCTGCTGGCCTTAAAGTCATCTGG
pGEX-4T-1-mCherry-PH (PLC□1)	pGEX-4T-1	BamH1/Sal1	mCherry- PH (PLCδ1) amplified from pmCherry- PH (PLCδ1)	Forward : CCGGGATCCATGGTGAGCAAGGGCGAGGAG
				Reverse : CGCGTCGACCTACTTCTGCCGCTGGTCCATG
pmCherry-C1-FYVE (HRS)	pmCherry-C1	Xho1/BamH1	FYVE (HRS) amplified from pGEX-2TK - FYVE (HRS)	Forward : CCGCTCGAGCAGAGAGAGCCCCAGACT
				Reverse : CGCGGATCCTACCTGTTCAGCTGCTCGT
pGEX-4T-1-mCherryFYVE (HRS)	pGEX-4T-1	BamH1/Sal1	mCherry- FYVE (HRS) amplified from pmCherry-FYVE (HRS)	Forward : CCG GGATCCATGGTGAGCAAGGGCGAGGAG
				Reverse : CGCGTCGACCTACCTGTTCAGCTGCTCGT
N174 (puro)-Tks5-GFP	N174-MCS (puromycin) Addgene # 81068	EcoR1/BamH1	gene synthesis	Forward : CGTGAGGATCGAATTCATGCTCGCCTACTGCGTGC
				Reverse : CGGTAGAATTGGATCCCTACTTGTACAGCTCGTCCATGCC

Name of the plasmid constructions and/or purpose	Backbone plasmid	Linearized by	insert	Primers and oligonucleotides sequences
template oligonucleotide for sgRNA synthesis (gRNA in bold)				TAATACGACTCACTATAGG**TCATCATCTGAGGTCCGATA**GTTTTAGAGCTAGAAATAGCAAG
1st PCR of the nested PCR to amplify the region surrounding the of Cas9 cleavage site				Forward : GAAATGAGCCTCCCCAAAAG
				Reverse : TCTTTTCTTTGCTGCCCAGT
2nd PCR of the nested PCR to amplify the region surrounding the site of Cas9 cleavage				Forward : GGTGCATGGGTAGGAGATGG
				Reverse : CCTGGACTGGCAGCTCATAG
First PCR of the nested PCR to amplify the region surrounding OT1				Forward : TGTAACAGTAAGTTTCCGCTTTCAC
				Reverse : AAGATGCCTTGGTTCAATTTAGGTC
Second PCR of the nested PCR to amplify the region surrounding OT1				Forward : CCAGAGTACGAGAGTAACGAATAAG
				Reverse : GGTCCAGAAACTTCAGAAAACTCTC

Name of the plasmid constructions and/or purpose	Backbone plasmid	Linearized by	insert	Primers and oligonucleotides sequences
First PCR of the nested PCR to amplify the region surrounding OT2				Forward : CTGTGACAAATAGTTGGCATTATGG
				Reverse : AGACAAGTGGTAGTAAGTCTGAAGG
Second PCR of the nested PCR to amplify the region surrounding OT2				Forward : TTTATCTTGACTCACAGTTTGAGGG
				Reverse : GCACTTTCTTCAAGCAGCATTTTAG
First PCR of the nested PCR to amplify the region surrounding OT3				Forward : ATAGGCACTCAAGGTCAGAAAGTAG
				Reverse : AGCAGAGGACAGTCAGATGAAAAGG
Second PCR of the nested PCR to amplify the region surrounding OT3				Forward : CCAAGATAATTCAAACAGTTCCCCC
				Reverse : CAAAGACAGCTAACTGGGAATACTG
Alleles sequencing by bacterial single colony sequencing	pcDNA3.1(+)	NheI/XhoI		Forward : GGGAGACCCAAGCTGGCTAGCggtgcatgggtaggagatgg
				Reverse : AACGGGCCCTCTAGACTCGAGcctggactggcagctcatag

Supplementary Table S3. List of main reagents used in this study.

Reagent	Source	Catalog number
Accutase	BD Biosciences	561527
Gelatin from porcine skin (type A)	Sigma-Aldrich	G1890
DMEM glutaMAX (without pyruvate)	Gibco	61965-026
DPBS	Eurobio Scientific	CS1PBS01-01
Fetal Bovine Serum	Gibco	16000-036
Cell Lysis Buffer (10X)	Cell Signaling	#9803
Formaldehyde	Sigma-Aldrich	F8775
Glutaraldehyde	Sigma-Aldrich	G5882
Digitonin	Sigma-Aldrich	D141
Saponin	Sigma-Aldrich	84510
Triton X-100	Sigma-Aldrich	93443
Goat Serum	Sigma-Aldrich	S26
FluorSave	Calbiochem	345789-20
NH4Cl	Sigma-Aldrich	A9434
PIPES	Sigma-Aldrich	P6757
DAPI	Euromedex	1050

Legend for Movie

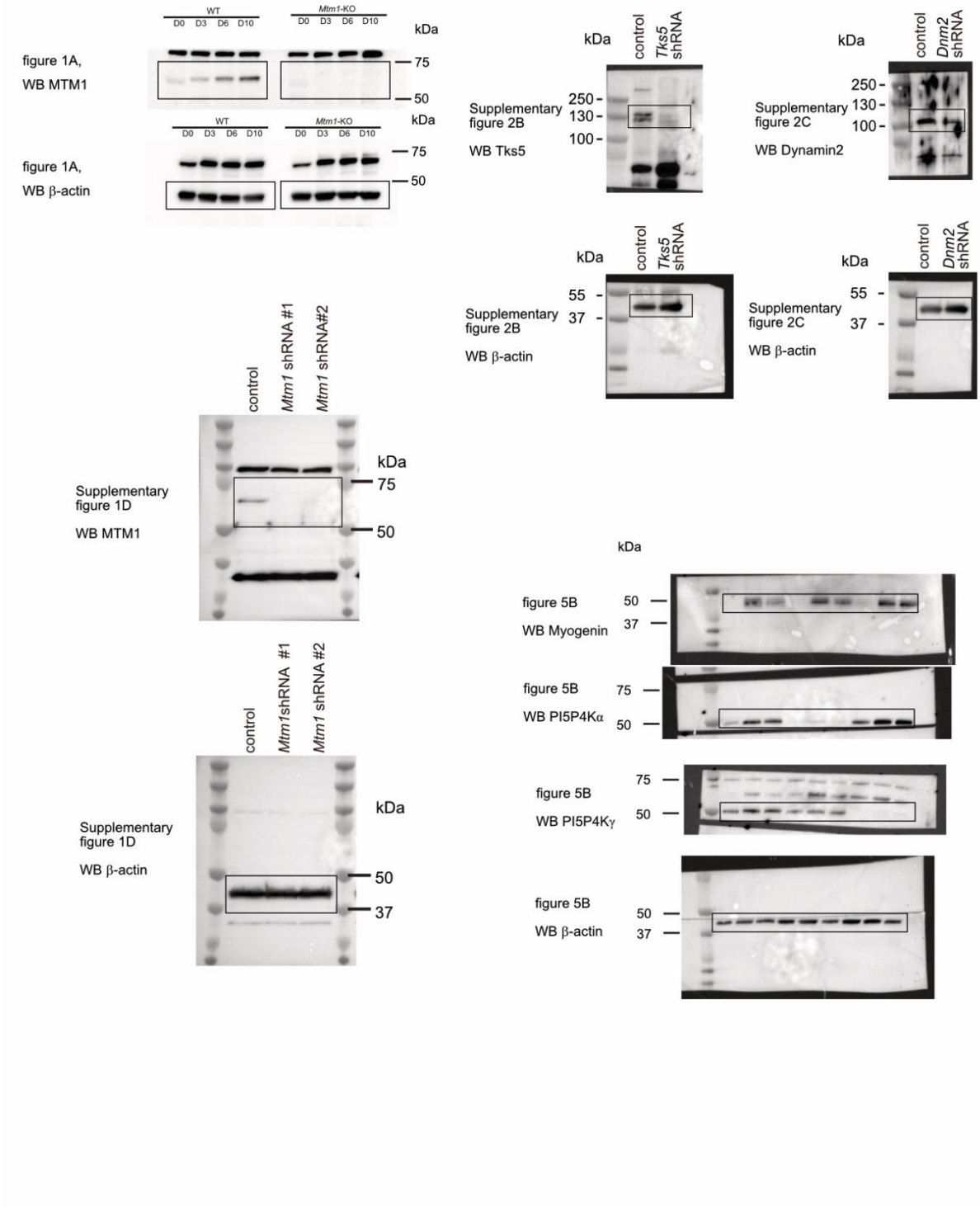
Movie 1 | Time-lapse microscopy of Tks5-GFP in differentiated C2C12 myoblasts.

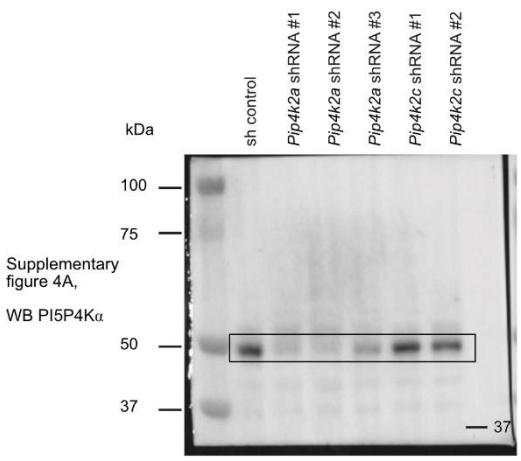
Day 3 differentiated C2C12 myoblasts transduced with lentivirus encoding Tks5-GFP were imaged with videomicroscopy using a 10-min time-interval. Arrows indicate contact-site that look-like podosome-like protrusions enriched in Tks5-GFP.

Movie 2 | Time-lapse confocal microscopy of Tks5-GFP during C2C12 fusion.

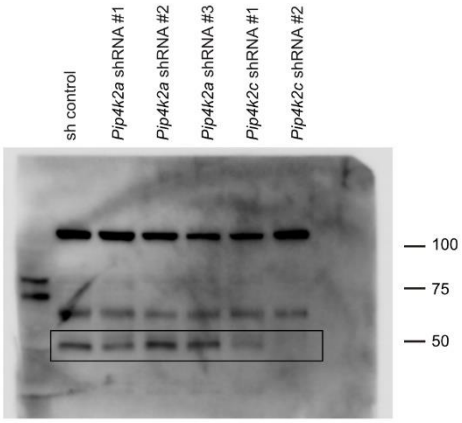
Day 3 differentiated C2C12 myoblasts transfected with a plasmid encoding Tks5-GFP were imaged with an LSM900 Zeiss microscope (Zen software, x20 objective) equipped with a ibidi stage Top incubator, using a 15-min time-interval and a single Z-plane. An arrow indicates the presence of a podosome-like protrusion enriched in Tks5-GFP before fusion.

Uncropped western blot images

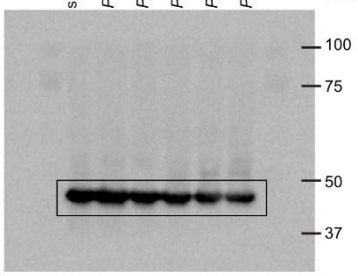




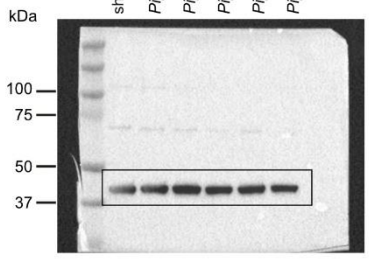
Supplementary figure 4A, WB PI5P4Kα



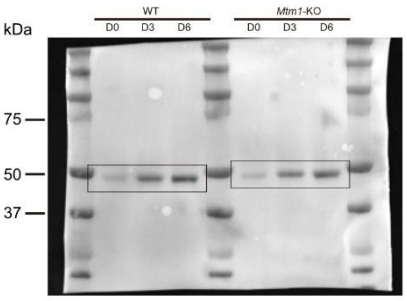
Supplementary figure 4A, WB PI5P4Kγ



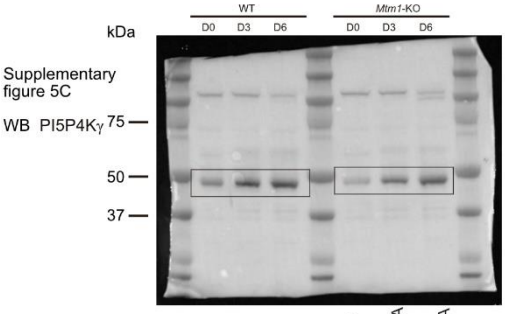
Supplementary figure 4A, WB β-actin



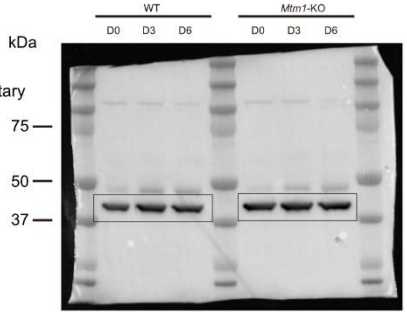
Supplementary figure 4A, WB β-actin



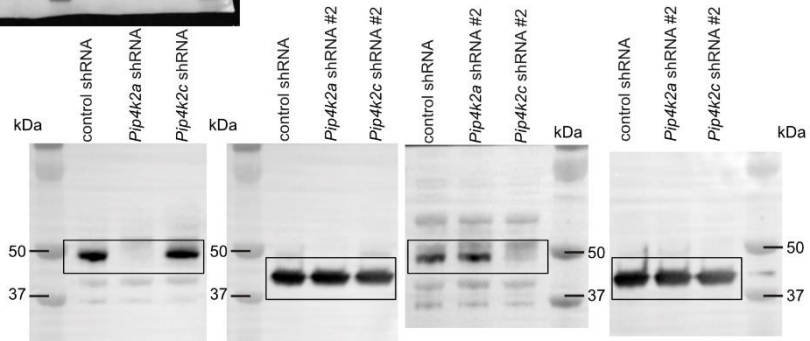
Supplementary figure 5C WB PI5P4Kα



Supplementary figure 5C WB PI5P4Kγ



Supplementary figure 5C WB β-actin



Supplementary figure 5C, WB PI5P4Kα

Supplementary figure 5C, WB β-actin

Supplementary figure 5D, WB PI5P4Kγ

Supplementary figure 5D, WB β-actin

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