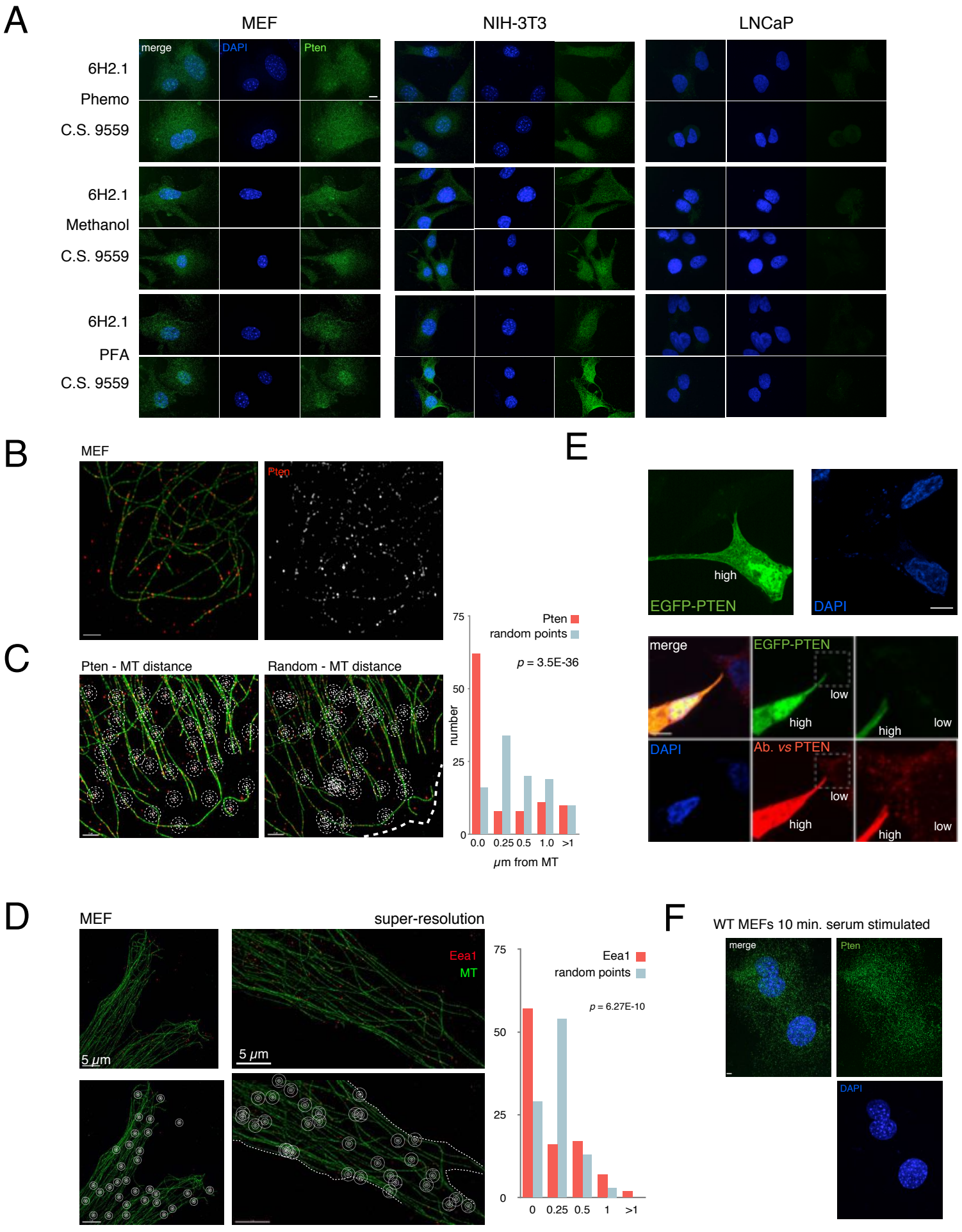
Figure S1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LNCaP
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DAPI
Pten
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Phemo
C.S. 9559
6H2.1
Methanol
C.S. 9559
6H2.1
PFA
C.S. 9559
B
MEF
Pten
C
Pten - MT distance
Random - MT distance
Pten
random points
p = 3.5E-36
number
75
50
25
0
0.0 0.25 0.5 1.0 >1
μm from MT
E
high
EGFP-PTEN
DAPI
merge
EGFP-PTEN
low
high
high
low
DAPI
Ab. vs PTEN
low
high
high
low
D
MEF
super-resolution
5 μm
Eea1
MT
5 μm
Eea1
random points
p = 6.27E-10
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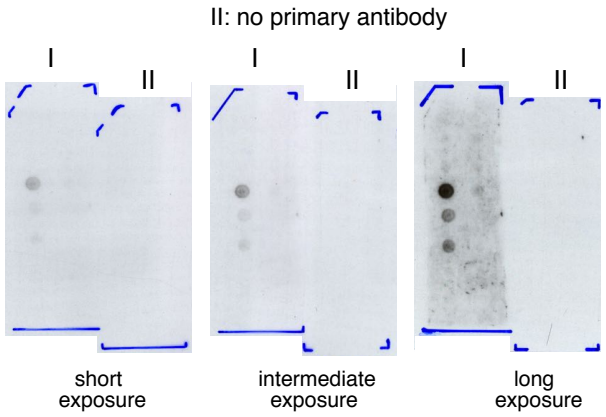
50
25
0
0 0.25 0.5 1 >1
F
WT MEFs 10 min. serum stimulated
merge
Pten
DAPI

Figure S2

A



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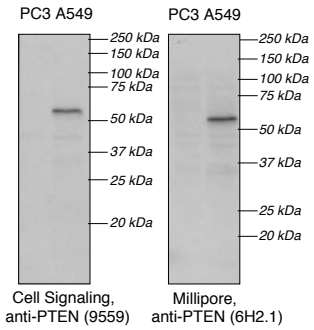

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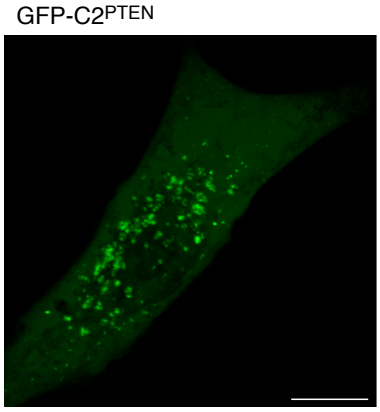
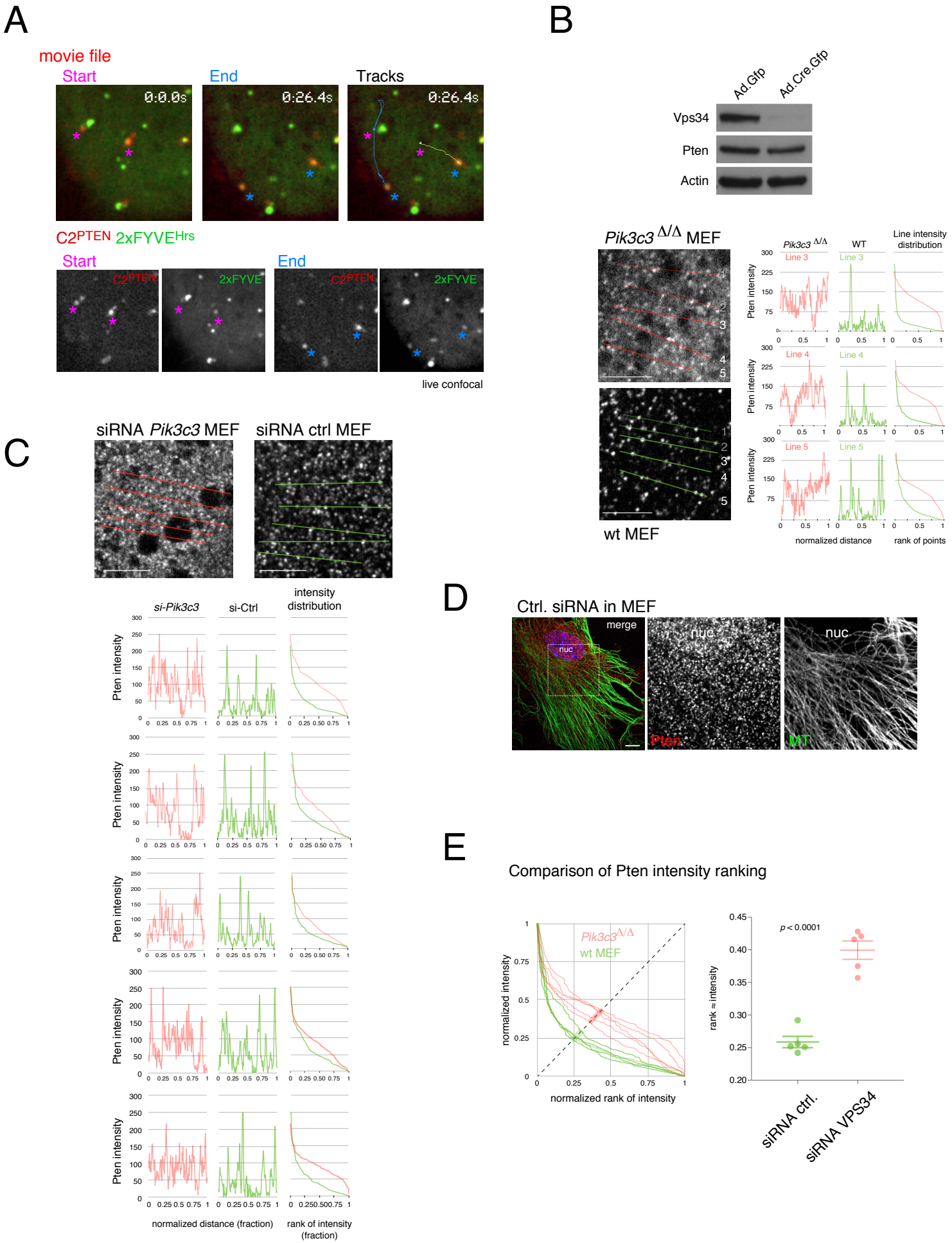


Figure S3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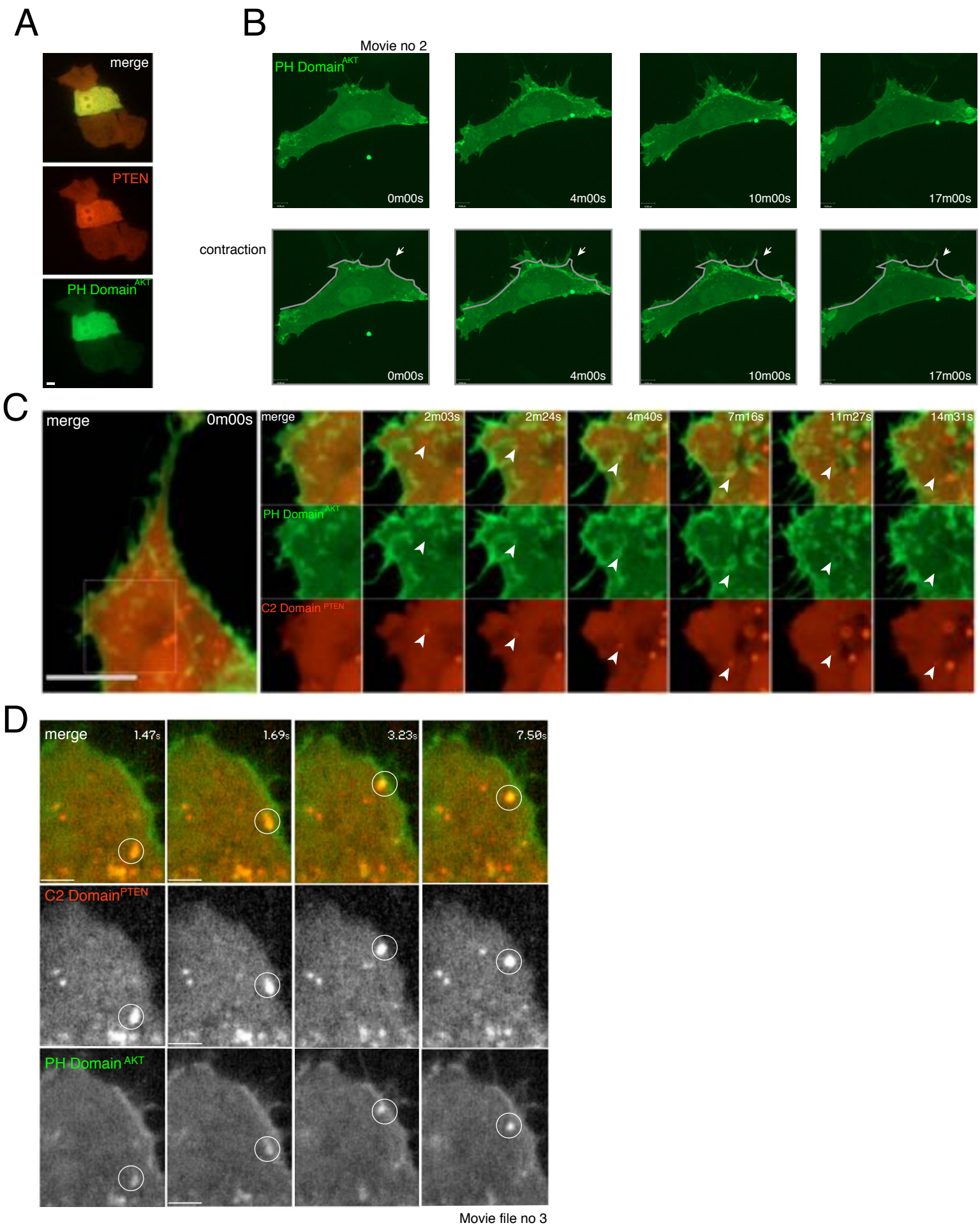
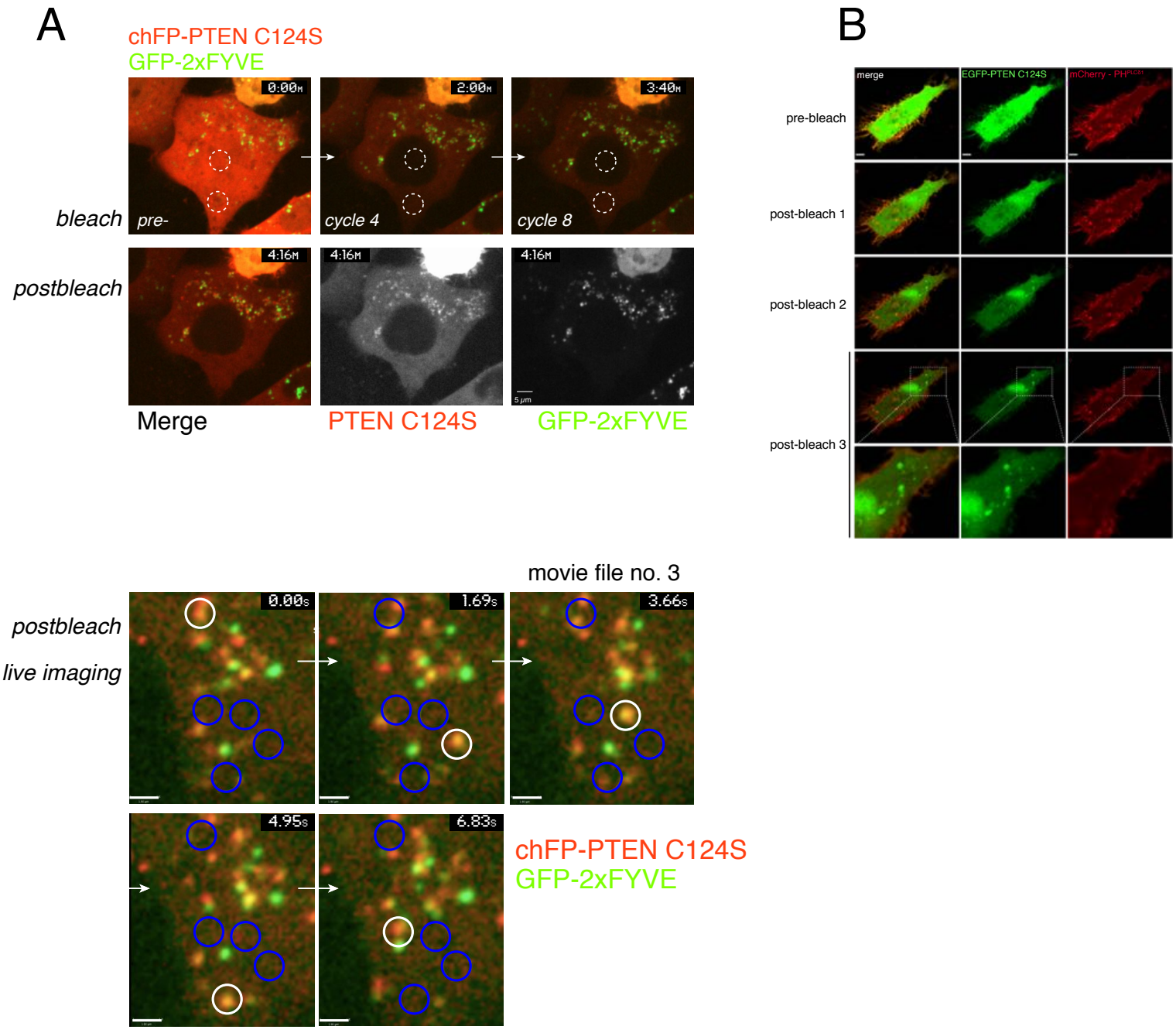
Start
End
C2PTEN
2xFYVE
live confocal
B
Ad.Gfp
Ad.Cre.Gfp
Vps34
Pten
Actin
Pik3c3 Δ/Δ MEF
wt MEF
Line intensity distribution
Pten intensity
normalized distance
rank of points
C
siRNA Pik3c3 MEF
siRNA ctrl MEF
si-Pik3c3
si-Ctrl
intensity distribution
normalized distance (fraction)
rank of intensity (fraction)
D
Ctrl. siRNA in MEF
merge
nuc
Pten
MT
E
Comparison of Pten intensity ranking
normalized intensity
normalized rank of intensity
Pik3c3Δ/Δ
wt MEF
p < 0.0001
rank ≈ intensity
siRNA ctrl.
siRNA VPS34

Figure S4



Movie file no 3

Figure S5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GFP-2xFYVE
bleach
postbleach
pre-
cycle 4
cycle 8
Merge
PTEN C124S
GFP-2xFYVE
B
merge
EGFP-PTEN C124S
pre-bleach
post-bleach 1
post-bleach 2
post-bleach 3
movie file no. 3
postbleach
live imaging
chFP-PTEN C124S
GFP-2xFYVE

Supplemental Figure Legends

Figure S1 related to Figure 1. **Validation and quantification of PTEN localization**. (**A**) The punctate distribution of PTEN is observed across multiple cell lines and was retained using three independent fixation and permeabilization methodologies (phemo-fixation, methanol and paraformaldehyde, see Methods). Note that HEK293 cells showed the same results (not shown). PTEN-null LNCaP cells demonstrate no PTEN signal when processed under identical conditions to PTEN-positive cells and using identical microscopy settings, confirming signal specificity. All images in extended focus, scale bar, 10 µm.

(**B**) Pten/MT dual labeled immunofluorescence, visualized using super-resolution microscopy reveals colocalization along curved MTs. WT MEFs, Z=1, scale bar, 2 µm.

(**C**) Demonstration of quantification of the distance of Pten signal intensity relative to MT position showing distance markers placed (left panel) and comparative randomly selected cytoplasmic positions (right panel; see Materials and Methods). The thick intermittent white line represents the cell boundary. WT MEFs, Z=1, scale bar, 2 µm. Right graph: The distance of Pten signal intensity from MTs is statistically significantly lower than randomly selected locations in the cell cytoplasm (p = 3.5 x 10-36, $\chi 2$). (**D**) Visualization and quantification of Eea1 association with microtubules in wt MEFs, done as in (C). Z=1.

(**E**) Top panels: EGFP-PTEN overexpression in *PTEN*-null PC3 recapitulates endogenous PTEN distribution only in cells with low PTEN levels, which require indirect IF for visualization. Z=1, scale bar, 10 µm. Antibody visualization of exogenous EGFP-PTEN in *PTEN*-null PC3 cells. The higher sensitivity achieved using antibody detection reveals that low expressing cells have a punctate PTEN distribution.

(**F**) Endogenous Pten distribution is not noticeably altered subsequent to PI3-K activation by serum stimulation in WT MEFs. Z=1, scale bar, 4 µm.

Figure S2 related to Figure 2. PTEN - PI(3)P binding *in vitro* and *in vivo*

(**A,B**) Confirmation of the mono-specificity of the two anti-PTEN antibodies used for immunofluorescence and lipid protein overlay assays.

(**C**) Overexpression of EGFP-$C2^{PTEN}$. Z=1, scale bar, 10 µm.

Figure S3 related to Figures 3 & 4. Live co-localization of PTEN C2 and FYVE domains and mislocalization of PTEN

(**A**) Live tracking shows co-migration of ChFP-C2^{PTEN} domain-positive and GFP-2xFYVE^{HRS} positive vesicles. Starting position (purple asterisks), end positions (blue asterisks) and migratory paths (blue and yellow lines) are depicted at indicated time points. See also Movie file 1. Note that color channel separation during motion bursts is due to sequential channel recording. Z=1.

(**B**) Top, immunoblot confirmation of Vps34 loss in *Vps34$^{fl/fl}$* MEFs infected with Cre recombinase-encoding adenovirus at 7 days post Cre infection. Bottom, Pten distribution in *Pik3c3* deleted and wt MEFs. Lines of intensity measurements are indicated. Z=1, scale bar, 10 µm. Quantification of Pten intensity values in both *Pik3c3* null and wt MEFs is shown, 3 distinct line plots are displayed.

(**C**) Top: Pten line intensity plots for MEFs treated with siRNA targeting *Pik3c3* transcripts and scrambled siRNA controls. Z=1, scale bar, 10 µm. Bottom: quantification of Pten intensity values in both cell types are shown for the 5 line plots.

(**D**) Images of Pten distribution in MEFs treated with scrambled siRNA control. Z=1, scale bar, 10 µm.

(**E**) Left panel: quantification of Pten intensity plots. Normalized intensity is plotted vs. normalized rank of intensity on 5 lines shown. Right panel: comparison of a measure for rank : intensity distribution (see methods) shows significant difference between wt and knockout cells ($p<0.0001$), two-tailed Student's T-test. Bars show mean with S.E.M.

Figure S4 related to Figure 6. The C2 domain of PTEN colocalizes with the PH domain of AKT after serum stimulation

(**A**) In PC3 cells (*PTEN*-null) expression of mCherry-PTEN in the presence of EGFP-PH^{AKT} leads to broad distribution of EGFP signal throughout the cell. Z=1, scale bars: 10 µm. (**B**) In the absence of active PTEN (*Trp53$^{\Delta/\Delta}$*, *Pten$^{\Delta/\Delta}$* MEFs), expression of EGFP-PH^{AKT} leads to accumulation of EGFP signal at the cell periphery. Serum stimulation leads to internalization of the PIP_3-binding EGFP-PH^{AKT} probe and a reduction in cell surface area. Time points indicate time-post serum addition to cells, with the first image representing a serum-starved state. Z=1, scale bar, 10 µm.

(**C**) In the absence of active PTEN (PC3 cells), expression of EGFP-PH^{AKT} leads to accumulation of EGFP signal at the cell periphery. Serum stimulation leads to internalization of the PIP_3-binding EGFP-PH^{AKT} probe and subsequent colocalization

with ChFP-C2^{PTEN}. Time points indicate time-post serum addition to cells, with the first image representing a serum-starved state. Z=1, scale bar, 10 µm.

(**B**) Movie 2 still images after serum stimulation of Pten/ p53-null MEF cells shows partial membrane association of the EGFP-PH domain of AKT and contraction of cell surface as indicated in the lower panels using the time 0 cell outline. Projection of entire Z-stack, Scale bar, 10 μm.

(**C**) Movie 3 still images after serum stimulation of PC3 (*PTEN*-null) cells shows ChFP-C2^{PTEN} domain colocalization and co-migration with the EGFP-PH domain of AKT. Z=1, scale bar, 5 µm.

Figure S5 related to Figure 6. Photobleaching of excess exogenous protein reveals enrichment of PTEN in a PI(3)P-labeled compartment

(**A**) Top: Photobleaching of mobile catalytically-inactive mCherry-PTEN(C124S) after overexpression reveals a pool of vesicle associated PTEN. Z=1, scale bars: 10 µm. Bottom: time lapse images showing co-migration of the mCherry-PTEN with GFP-2xFYVE^{HRS} subsequent to bleaching (see also Movie 2). Z=1, scale bars: 5 µm. Position of a dual-labelled vesicle is highlighted (white circles) throughout its trafficking through multiple subsequent positions (blue circles). Z=1, scale bars: 1.9 µm.

(**B**) Photobleaching of the excess mobile pool of catalytically-inactive EGFP-PTEN(C124S) in cells expressing the PH domain of PLCδ1 fused to mCherry, a PI45P_2-binding probe. Bleaching reveals a pool of vesicle-associated PTEN but with no enrichment at the cell periphery.

Table 1: *Antibody manufacturer, uses and concentrations*

Antigen	*Manufacturer*	*Application*	*Working Dilution*	*Secondary Antibody*	*Secondary antibody dilution*
PCNA (Hu)	Santa Cruz (PC10)	Immunoblot	1:6,000, 4°c, 12 hours, 5% BSA• TBS-T	anti-mouse HRP conjugated (GE Healthcare)	1:5000, 23°c, 60 minutes, 5% BSA• TBS-T
PTEN (Hu), Pten (Mu)	Millipore (6H2.1)	immunoblot	1:10,000, 4°c, 12 hours, 5% BSA• TBS-T	anti-mouse HRP conjugated (GE Healthcare)	1:5000, 23°c, 60 minutes, 5% BSA• TBS-T
PTEN (Hu), Pten (Mu)	Millipore (6H2.1)	Immunofluorescence	1:2000, 4°c, 60 minutes, 10% GS	anti-mouse Alexa Fluor (Invitrogen)	1:1000, 23°c, 15 minutes, 10% GS
PTEN (Hu), Pten (Mu)	Millipore (6H2.1)	PIP strip binding assay	1:2000, 23°c, 60 minutes, 3% milk• PBS-T	anti-mouse HRP conjugated (GE Healthcare)	1:1000, 23°c, 60 minutes, 3% milk• PBS-T
PTEN (Hu), Pten (Mu)	Cell Signaling (9559)	immunoblot	1:5000, 4°c, 12 hours, 5% BSA• TBS-T	anti-Rabbit HRP conjugated (GE Healthcare)	1:5000, 23°c, 60 minutes, 5% BSA• TBS-T
PTEN (Hu), Pten (Mu)	Cell Signaling (9559)	Immunofluorescence	1:1000, 4°c, 60 minutes, 10% GS	anti-rabbit Alexa Fluor (Invitrogen)	1:1000, 23°c, 15 minutes, 10% GS
PTEN (Hu), Pten (Mu)	Cell Signaling (9559)	PIP strip binding assay	1:1000, 23°c, 60 minutes, 3% milk PBS-T	anti-Rabbit HRP conjugated (GE Healthcare)	1:1000, 23°c, 60 minutes, 3% milk• PBS-T
pS6 (Hu), pS6 (Mu)	Cell Signaling (2211)	immunoblot	1:5000, 4°c, 12 hours, 5% BSA• TBS-T	anti-rabbit HRP conjugated (GE Healthcare)	1:5000, 23°c, 60 minutes, 5% BSA• TBS-T
α Tubulin (Hu), α Tubulin (Mu)	Sigma (T6199)	Immunofluorescence	1:400, 4°c, 60 minutes, 10% (GS)	anti-mouse Alexa Fluor (Invitrogen)	1:1000, 23°c, 15 minutes, 10% GS
EEA1 (Hu), EEA1 (Mu)	Cell Signaling (2411)	Immunofluorescence	1:100, 4°c, 60 minutes, 10% (GS)	anti-rabbit Alexa Fluor (Invitrogen)	1:1000, 23°c, 15 minutes, 10% GS
EEA1 (Mu)	Santa Cruz (33585)	Immunoblot	1:1000, 4°c, 12 hours, 5% BSA• TBS-T	anti-Rabbit HRP conjugated (GE Healthcare)	1:5000, 23°c, 60 minutes, 5% BSA• TBS-T
$pAKT^{ser473}$ (Hu), $pAKT^{ser473}$ (Mu)	Cell Signaling (4058)	immunoblot	1:5000, 4°c, 12 hours, 5% BSA• TBS-T	anti-Rabbit HRP conjugated (GE Healthcare)	1:5000, 23°c, 60 minutes, 5% BSA• TBS-T
VPS34 (Hu), Vps34 (Mu)	Cell Signaling (4263)	immunoblot	1:1000, 4°c, 12 hours, 5% BSA• TBS-T	anti-Rabbit HRP conjugated (GE Healthcare)	1:5000, 23°c, 60 minutes, 5% BSA• TBS-T
GST	Cell Signaling (2625)	PIP strip binding assay	1:100, 23°c, 60 minutes, 3% milk• PBS-T	anti-Rabbit HRP conjugated (GE Healthcare)	1:1000, 23°c, 60 minutes, 3% milk• PBS-T

Hu: human, *Mu*: mouse. * Provided as a kind gift by Professor Seong-Seng Tan, Howard Florey Institute, Melbourne, Australia. *BSA•TBS-T*: bovine serum albumin in trizma buffered saline, 0.1% Tween-20. *GS*: goat serum in phosphate buffered saline. *milk•PBS-T*: non-fat milk in phosphate buffered saline, 0.1% Tween-20.

Supplemental Experimental Procedures

Immunofluorescence microscopy

Cells were plated onto coverslips of 0.13-0.17 mm thickness (Electron Microscopy Sciences, PA, USA) and incubated in DMEM (Mediatech inc., VA, USA), 10% FBS, penicillin 50 units/ml, (Sigma, MO, USA) streptomycin 100 µg/ml (Sigma) at 37°c, 5% CO_2, 100% humidity overnight. Growth medium was then removed and coverslips were washed once with phosphate buffered saline (PBS). Cells were then fixed and permeabilized by one of three methods: (1) methanol fixation and permeabilization by addition of ice-cold methanol for 5 minutes. (2) Paraformaldehyde (PFA) fixation by addition of PFA for 5 minutes, then a subsequent PBS wash, followed by addition of 25 mM NH_4Cl for five minutes as a fixative quencher. Another PBS wash preceded permeabilization with 0.5% Triton X-100 in PBS for 5 minutes. (3) Phemo fixation and permeablization by addition of a solution of 3.7% formaldehyde, 0.05% gluteraldehyde, 0.5% Triton X-100 in phemo buffer (0.068 M PIPES, 0.025 M HEPES, 0.015 M EGTA•Na_2, 0.003 M $MgCl_2$•H_2O, 10% DMSO, pH 6.8) for 5 minutes. Following fixation and permeabilization, coverslips were washed three times with PBS and non-specific antibody binding blocked by addition of 10% goat serum in PBS for 30 minutes. Coverslips were then incubated at 4°c for 60 minutes in primary antibody diluted in 10% goat serum (see antibody concentration table for working dilutions). Coverslips were then washed three times in PBS and incubated with secondary, fluorescent-conjugated antibodies (see antibody table) for 15 minutes at room temperature. Primary and secondary incubations were performed in a light proof, humidified chamber. Subsequently, coverslips were washed 3 times in PBS and mounted using Prolong Gold anti-fade reagent containing DAPI (Invitrogen, OR, USA) and allowed to cure overnight. Coverslips were finally sealed using clear nail polish. Slides were imaged using a Perkin Elmer Ultra*VIEW* VoX spinning disk confocal microscope (Perkin Elmer, MA, USA) using Volocity v.6.3 software (Perkin Elmer). For visualization of transferrin-containing vesicles, cells were incubated for 60 minutes prior to fixation with fluorescent-conjugated transferrin (Invitrogen), 5 µg/ml in complete growth medium.

Structured Illumination for Super-Resolution Imaging (OMX microscopy)

Cell fixation and preparation was as described above. High resolution images were acquired using an OMX 3D Structured Illumination Microscope (Applied Precision, WA, USA). Solid state lasers were used for excitation at 405, 488 or 593 nm. Excitation light was coupled to a fiber optic cabling, scrambled and passed through a diffraction grating

prior to sample illumination. Diffraction grating was mounted on a piezoelectric and a rotational stage to allow for control of lateral shift and angular orientation (+/- 60 degree: required for 3D structured illumination imaging). Subsequent to beam diffraction, the innermost 3 beam orders (orders 0 and +/- 1) were used for image reconstruction with additional orders obstructed by a beam blocker and not utilized. The interference pattern of the beams in the focal plane of the objective (UPlanS Apochromat 100x 1.4NA: Olympus, PA, USA) was used to generated 3D sinusoidal pattern. The fluorescent light emitted by the sample was gathered by the same objective, passed through different dichroic mirrors and filters and measured by Cascade II EMCCD back-illuminated cameras (Photometrics, AZ, USA). Exposure times of each frame were typically between 100 and 200 ms, and the power of each laser was adjusted to achieve optimal intensities in the raw image of a 16-bit dynamic range. Multi-channel images were achieved through sequential acquisition. The original z-stacks were saved and processed using SoftWoRx 4.5.0 (Applied Precision) to reconstruct the high resolution information. The dataset was further processed to achieve a 3D reconstruction or maximum intensity projections using the same software.

PTEN/ microtubule association quantification

Quantification of Pten position relative to microtubules was achieved by assessment of Pten staining distances from microtubule stain. For 3 individual OMX images of wildtype MEFs, the Pten (red) channel was visualized in the absence of the α-tubulin (microtubule: green) channel. Thirty-three Pten intensities were chosen at random per image. The distribution of randomly selected intensities was throughout the image. A scale marker, as determined by the digitally assigned marker added by the SoftWoRx software, was placed over the centre pixel of the Pten intensity. Subsequently, the green (α-tubulin) channel was overlaid onto the red channel/scale marker image. Each Pten intensity was then assigned a relative distance to the closest green pixel (microtubule stain). Intensities were assigned as either 0 μM from the closest microtubule (i.e. red and green channels demonstrated direct colocalization, with pixels both red and green in the corresponding channels), 0.5 μm, 1 μm or 2 μm. Scoring was ‘outward’, such that any intensity greater than 1 μM but less than 2 μM from the closest microtubule was assigned as ‘2 μm’. As a control comparison, random pixels in the same images were selected and assessed in a similar manner. In order to select random pixels, a random number generator (www.randomizer.org) was used to provide co-ordinates of pixels within each image by providing *X* and *Y* values. Random pixels were only used if they

were within the cell periphery in the image (co-ordinates corresponding to regions of the image outside of the cell were discarded). Thirty-three random pixels were assessed in each image. The relative distances were then compared using the non-parametric chi-squared (χ^2) test, as not all observed data demonstrated a Gaussian distribution. Pten/random pixels were categorized as either overlapping (0 μm from microtubules) or other (> 0 μm) for statistical testing.

PTEN mutagenesis

Mutation of PTEN was performed using the Quikchange II site directed mutagenesis kit (Agilent Technologies, CA, USA) according to the manufacturer's instructions. Generation of the 5KE PTEN mutant was achieved by converting the following lysine residues of PTEN to glutamic acid: 260, 263, 266, 267 and 269. Residues 264 and 265 were converted to alanine and glycine respectively. Insertion to the C-terminus of EGFP and N-terminus of PTEN of $FYVE^{Hrs}$ domains was performed either in tandem or triple to generate EGFP-FYVE-PTEN constructs.

cDNA overexpression

cDNA constructs were transfected using Lipofectamine reagent (Invitrogen) according to the manufacturer's instructions and cells were processed further between 12-24 hours after transfection. MTM1 plasmids were a kind gift by G.S. Taylor (Univ. of California San Diego).

Drug treatments of cells

For nocodazole treatment cells were treated with nocodazole (Sigma) at a final concentration of 10 μg/ml (33 μM) for 30 minutes in DMEM, 10% FBS, penicillin 50 units/ml, streptomycin 100 μg/ml at 37°c, 5% CO_2, 100% humidity prior to fixation for microscopy. Stock solutions were prepared in DMSO (Mallinckrodt, NJ, USA). For Vps34 inhibition, cells were treated with KU-55933 (Abcam, MA, USA) at a final concentration of 2 μM for 4 hours in DMEM, 10% FBS, penicillin 50 units/ml, streptomycin 100 μg/ml at 37°c, 5% CO_2, 100% humidity prior to fixation for microscopy. Stock solutions were prepared in DMSO (Mallinckrodt, NJ, USA).

Phosphatidylinositol (PIP) strip binding assay

GST-tagged human recombinant PTEN protein (Echelon, UT, USA) was resuspended in nuclease free water (Qiagen, MD, USA) to a concentration of 50 μg/ml. PIP strips

(membrane blotted with immobilized lipids-Echelon) were blocked in 3% non-fat milk, PBS•0.1% Tween-20 (PBS-T) for 60 minutes at room temperature. Recombinant human PTEN protein was then added to the strip at 0.5 µg/ml in 3% non-fat milk, PBS-T for 120 minutes at room temperature. Subsequently, three five minute washes of the strip in PBS-T were followed by incubation of primary anti-PTEN antibody. Strips were then washed for 5 minutes three times and incubated with corresponding secondary antibody (see antibody list) and visualized using ECL detection reagent (GE Healthcare, Buckinghamshire, UK). GST-tagged lipid binding protein domains were purchased (Echelon) and used as comparative binding controls

Recombinant PTEN protein production

Untagged PTEN protein was expressed in insect cells using baculovirus and purified from Hi5 cell lysate by ion exchange chromatography on SP sepharose. For screening of PTEN mutants, PURExpress *In Vitro* Protein Synthesis kits (New England Biolabs, MA, USA) were used according to the manufacturer's instructions.

Liposome Binding Assays

Liposomes were prepared by mixing 200 µl of the stock solution of phosphatidylcholine (5 mg/ml) with either 500 µl of PI(3)P solution (100 µg/ml) or 500 µl of PI(3,4,5)P_3 solution (100 µg/ml). All lipids were obtained from Avanti Polar Lipids (AZ, USA). The final concentration of the individual PI's in the liposome was 5% of the total lipid. The mixtures were diluted with chloroform, homogenized, and the solvent was removed completely in vacuum overnight. The lipids were resuspended in distilled water and homogenized with 1 minute of sonication. Protein (1 µg) was added to the liposome solution (100 µl) and incubated with agitation for 30 minutes at room temperature. Mixtures were then centrifuged at 20,000 RPM in a TLA 100.3 rotor (Beckman Coulter, CA, USA) and the supernatant removed. 100 µl of wash buffer (100 mM tris,HCl, 100 mM NaCl, pH 7.5) was added to the lipid pellets and the samples were spun again with the supernatant removed. Washing was performed 3 times in total. Samples were analyzed by SDS PAGE. The supernatant was analyzed as a loading control, the third wash was analyzed to confirm that all soluble protein had been removed and the lipid pellet was analyzed to ascertain protein-liposome binding.

Vps34 (*Pik3c3*) knockdown and knockout

Four independent siRNAs targeting *Vps34* were pooled for use and compared to a scramble control (all siRNAs from Qiagen) and transfected into NIH3T3 cells using Dharmafect1 reagent (Thermo Scientific) according to the manufacturer's instructions. After 72 hours cells were analyzed for VPS34 knockdown effects. $Vps34^{fl/fl}$ MEFs were treated with Adenovirus-Cre-GFP or Adenovirus-GFP control as described previously (Jaber et al., 2012, PMID 3277541). Cell lysates were collected at the indicated times for immunoblot analysis with concurrent fixation of cells for immunofluorescence microscopy. For quantification of Pten intensity, lines through Pten foci (and vacuoles) were drawn suing the Volocity software (see Confocal microscopy and image analysis). Pten intensity was plotted as a function of normalized distance along the line and intensity ranking was calculated both on absolute intensity values, as well as on normalized intensity. Normalized ranking was used to define and plot the last ranked value where normalized rank > normalized intensity, as a measure for intensity distribution.

Confocal microscopy and image analysis

All confocal microscopy was analyzed using Volocity software (v.6.3). Vesicle counting was done using the Find Spots function (see Volocity User Guide, version 23.0, page 240 ff.) on at least 20 cells per condition. For vesicle live tracking the Find Spots, Clip To ROIs, Track and Measure Position functions were used (p. 215 ff.) and movie files were exported at 8x real time speed. Note that color channel separation during motion bursts is due to sequential channel recording. 3D rendering was done using the 3D Opacity function in Fluorescence mode (page 306 ff.) and images with varying density settings of one channel were included to show signal overlap. For line graph quantification, the Measure Line Profiles function was used (page 248 ff.) on a 14 μm line across the cytoplasm. Representative results from at least 2 biological replicates are shown. For FLIP bleaching of excess fluorescence tagged PTEN, photobleaching was done as previously(Howitt et al., 2012), but through 8 cycles in ten second intervals on the indicated nuclear and cytoplasmic regions followed by live imaging for up to ten minutes. Note that the procedure showed no cell toxicity after several hours.

PTEN enzymatic activity assays

PTEN activity against soluble (non-membrane) substrates was performed using malachite green and DiC8 PIP_3 (both Echelon). Soluble lipid concentration was 20 µM

in 10 mM PIPES, pH 7.5. For assessment of PTEN activity on membranes, synthetic liposomes were generated (see above). The enzyme reaction was stopped after 5 minutes by freezing in liquid nitrogen, and the samples dried by lyophilization. The pellets were dissolved in 50% chloroform:methanol containing 0.1% NH_4OH, and 5 µl was injected with the injection port of a Thermo TSQ Vantage Mass-spectrometer, using a Thermo Accela Pump at 350 µl/min flow for the injection. A heated electrospray ionization (HESI-II) probe ion source was used for the ionization in negative ion mode, and the mass-spectrometer was used in selected reaction monitoring mode (SRM). Argon gas for collision-induced dissociation (CID) was used at 1.5 Torr. The major fragments of the PIs and the optimal collision energy of each fragment were determined by method development prior to the enzyme reaction. Tune parameters were as follows: spray voltage 2500 V, vaporizer temperature 250ºC, sheath gas pressure 35, ion sweep gas pressure 8.0, aux gas pressure 10, capillary temperature 350ºC. S-lens values were optimized in the instrument calibration process. The peak area of each injection was calculated with the Qual Browser software of the Thermo Xcalibur 2.1 software.
We monitored the fragment ions of the double charged ion form of the PI(3)P (470.18 m/z) and PI(3,4,5)P3 (550.17 m/z) because their abundance was comparable with the single ions' abundance in the standard compounds under the previously described conditions.

Cellular EGF internalization and assessment

Cells were cultured and washed three times with cold PBS before addition of cold DMEM, 0.1% FBS, penicillin 50 units/ml, streptomycin 100 µg/ml containing Cy5-tagged EGF (Invitrogen) at 2 µg/ml followed by incubation at 4°c for 30 minutes. Cells were then washed with cold PBS three times followed by addition of warm DMEM, 10% FBS, penicillin 50 units/ml, streptomycin 100 µg/ml and incubated at 37°c. Cells were fixed at designated time points subsequent to addition of warm medium.

PCNA assessment

Immunoblot identification of PCNA was quantified using ImageJ software, using the gel analyzer function.

Supplementary Movie Files

Movie 1.mov: A 2.7 Mb QuickTime Movie file of cell shown in Figure S3A.
Co-migration of ChFPC2^{PTEN} (red) and GFP2xFYVE^{HRS} (green) in PC3 cells.
Note that color channel separation during motion bursts is due to sequential channel recording.

Movie 2.mov: A 2.2 Mb QuickTime Movie file of the Pten-null cell shown in Figure S4B expressing the GFP-PH domain of AKT

Movie 3.**mov**: A 3.4 Mb QuickTime Movie file of the images shown in Figure S4D post-bleach.
Co-migration of ChFP-PTEN (C124S), (red) and GFP2xFYVE^{HRS} (green) in PC3 cells.
Note that color channel separation during motion bursts is due to sequential channel recording.

Abbreviations

Gene/ Protein species nomenclature convention was used. Example: *PTEN* (human gene), PTEN (human protein), *Pten* (murine gene), Pten (murine protein).

EEA1:	Early endosomal antigen 1 (human protein), Eea1, mouse homolog
FLIP:	Fluorescence Loss In Photobleaching
MEFs:	Mouse Embryonic Fibroblasts
NDFIP1:	Nedd4-interacting protein 1
PI 3K:	PI 3-Kinase
Pik3c3:	mouse gene for the catalytic subunit of the Pik3c3 protein, a.k.a,Vps34
PIK3C3:	human gene for the catalytic subunit of the PIK3C3 protein, a.k.a. VPS34
PI:	Phosphatidylinositol
PIP:	Phosphatidylinositol phosphate
PI(3)P:	Phosphatidylinositol 3-phosphate
PI(4)P:	Phosphatidylinositol 4-phosphate
PI(5)P:	Phosphatidylinositol 5-phosphate
$PI(4,5)P_2$:	Phosphatidylinositol 4,5-bisphosphate
$PI(3,4,5)P_3$:	Phosphatidylinositol 3,4,5-trisphosphate
PIP_3:	Phosphatidylinositol 3,4,5-trisphosphate
PTEN:	Phosphatase and TENsin homolog deleted on chromosome TEN
VPS34:	Vacuolar Protein Sorting 34, human protein.
Vps34:	murine protein homolog of VPS34, produced by the *Pik3c3* gene.
wt	wild-type