Expanded View Figures

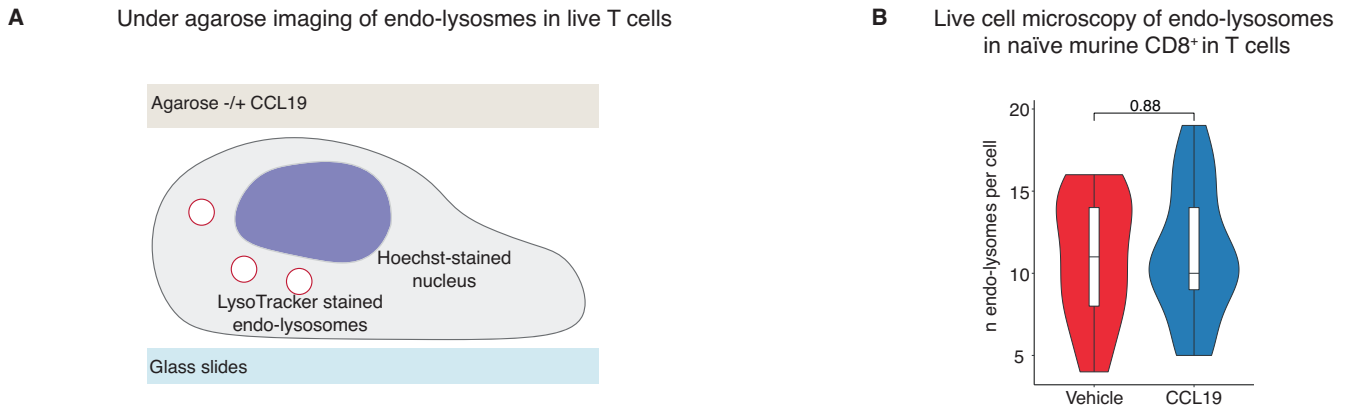


Figure EV1. Endo-lysosomes localize to the uropod of polarized T cells.

(**A**) Schematic of the under-agarose assay used to image endo-lysosomal polarization in live T cells. (**B**) Quantification of endo-lysosome numbers in naive murine CD8$^+$ T cells treated with vehicle ($n = 16$) or CCL19 ($n = 25$). (**C**) Representative fluorescence images of a naive murine CD8$^+$ T cell stained for Lamp1, Rab7 and exposed to CCL19-Dy649P1 (white). Top panels: merge with DAPI (blue; scale bars = 5 µm); box indicates region of interest magnified in bottom panels (scale bar = 500 nm). (**B**) Representative results of three independent experiments. Wilcoxon rank-sum test (**A**, **B**). Box plots indicate the median (central line), first and third quartiles (box bounds; 25th and 75th percentiles), and the minimum and maximum values (whiskers).

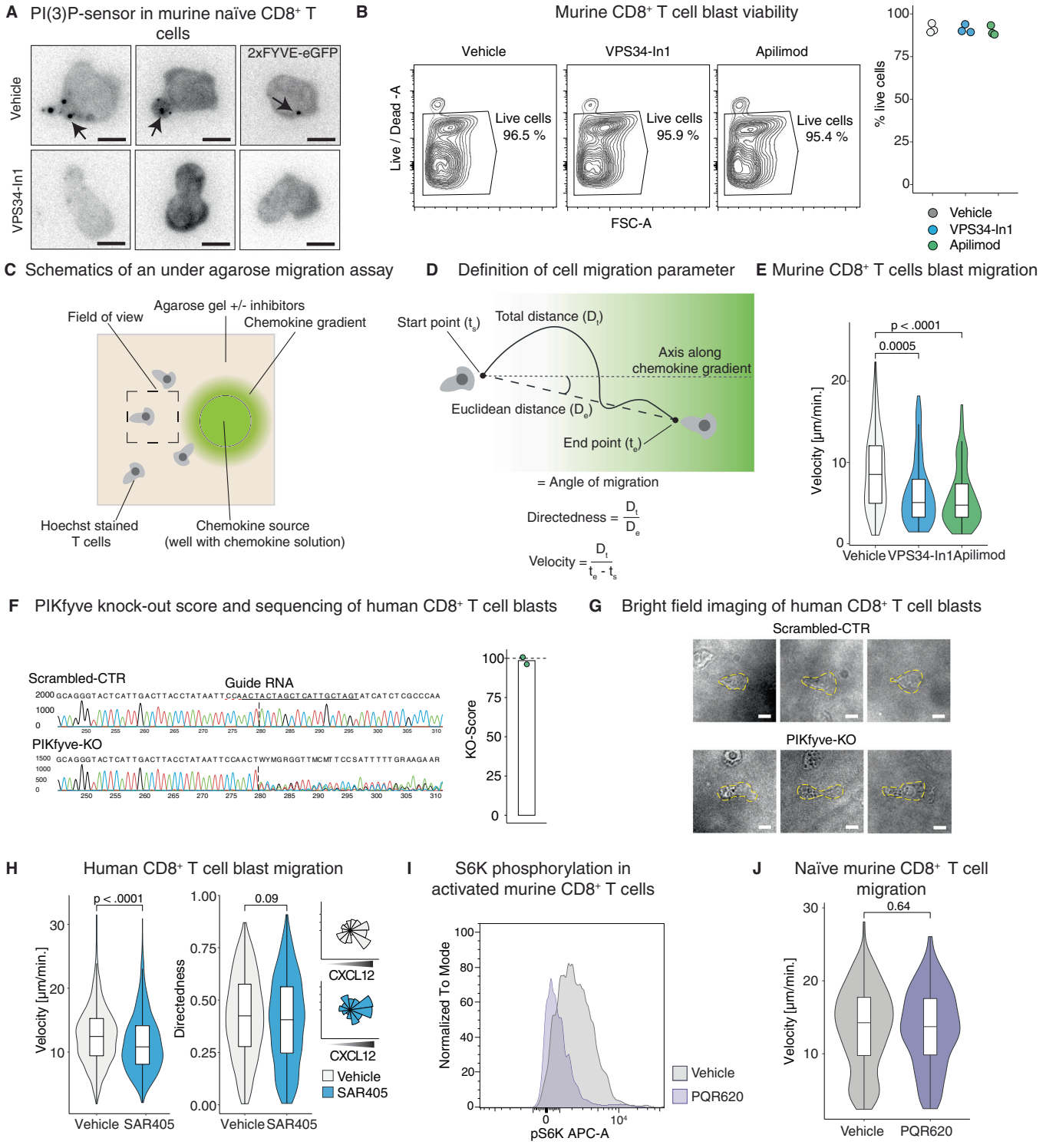

A PI(3)P-sensor in murine naïve CD8+ T cells
Vehicle
VPS34-In1
2xFYVE-eGFP
B Murine CD8+ T cell blast viability
Vehicle
VPS34-In1
Apilimod
Live / Dead -A
Live cells 96.5 %
Live cells 95.9 %
Live cells 95.4 %
FSC-A
% live cells
Vehicle
VPS34-In1
Apilimod
C Schematics of an under agarose migration assay
Field of view
Agarose gel +/- inhibitors
Chemokine gradient
Hoechst stained T cells
Chemokine source (well with chemokine solution)
D Definition of cell migration parameter
Start point (ts)
Total distance (Dt)
Axis along chemokine gradient
Euclidean distance (De)
End point (te)
= Angle of migration
Directedness = Dt / De
Velocity = Dt / te - ts
E Murine CD8+ T cells blast migration
p < .0001
0.0005
Velocity [µm/min.]
Vehicle VPS34-In1 Apilimod
F PIKfyve knock-out score and sequencing of human CD8+ T cell blasts
Scrambled-CTR
Guide RNA
PIKfyve-KO
KO-Score
G Bright field imaging of human CD8+ T cell blasts
Scrambled-CTR
PIKfyve-KO
H Human CD8+ T cell blast migration
p < .0001
0.09
Velocity [µm/min.]
Directedness
CXCL12
CXCL12
Vehicle
SAR405
I S6K phosphorylation in activated murine CD8+ T cells
Normalized To Mode
pS6K APC-A
Vehicle
PQR620
J Naïve murine CD8+ T cell migration
0.64
Velocity [µm/min.]
Vehicle PQR620

Figure EV2. T cell velocity, but not directedness, is controlled by VPS34 and PIKfyve.

(**A**) Naive murine CD8$^+$ T cells transfected with the 2xFYVE–eGFP PI(3)P sensor, placed in a CCL19 gradient, and treated with vehicle or VPS34-In1. Arrows indicate PI(3)P$^+$ vesicles. Scale bar = 5 µm. (**B**) Left panel: representative live/dead staining plots (flow cytometry) of murine CD8$^+$ T cell blasts treated with vehicle, VPS34-In1, or Apilimod for 6 h. Right panel: percentage of live cells from three biological replicates. (**C**) Schematic (top view) of a T cell under-agarose migration assay. (**D**) Definitions of cell migration metrics used in this study. (**E**) Migration speed of murine CD8$^+$ T cell blasts in a CCL19 gradient, treated with vehicle control ($n = 66$), VPS34-In1 ($n = 81$), or Apilimod ($n = 79$). (**F**) Left: representative bulk Sanger sequencing of control *vs.* PIKfyve-KO human CD8$^+$ T cell blasts of the region flanking the first guide RNA (gRNA). The gRNA sequence is underlined, the cutting side indicated by a dashed vertical line. Right: knockout score ($n = 2$). (**G**) Bright-field images of control and PIKfyve-KO human CD8$^+$ T cells placed in a CCL19 gradient. Yellow dashed lines indicate cell outline. Scale bar = 10 µm. (**H**) Velocity and directedness of human CD8$^+$ T cell blasts migrating in a CXCL12 gradient, treated with the VPS34 inhibitor SAR405 (velocity and angles: $n = 1838$, directedness: $n = 694$) or vehicle control (velocity and angles: $n = 1400$, directedness: $n = 594$). (**I**) Representative histogram (flow cytometry) of pS6K expression among murine CD8$^+$ T cells activated for 24 h with CD3 and CD28 targeting antibodies and treated with the mTOR inhibitor PQR620 (purple) *vs.* vehicle control (grey). (**J**) Velocity of naive murine CD8$^+$ T cells in a CCL19 gradient, following treatment with vehicle ($n = 272$) or the mTOR inhibitor PQR602 ($n = 232$). (**A**, **B**, **G**–**J**) Representative of ≥3 independent experiments; (**I**) representative of two independent experiments. Wilcoxon rank-sum test (**E**, **H**, **J**). Box plots indicate the median (central line), first and third quartiles (box bounds; 25th and 75th percentiles), and the minimum and maximum values (whiskers).

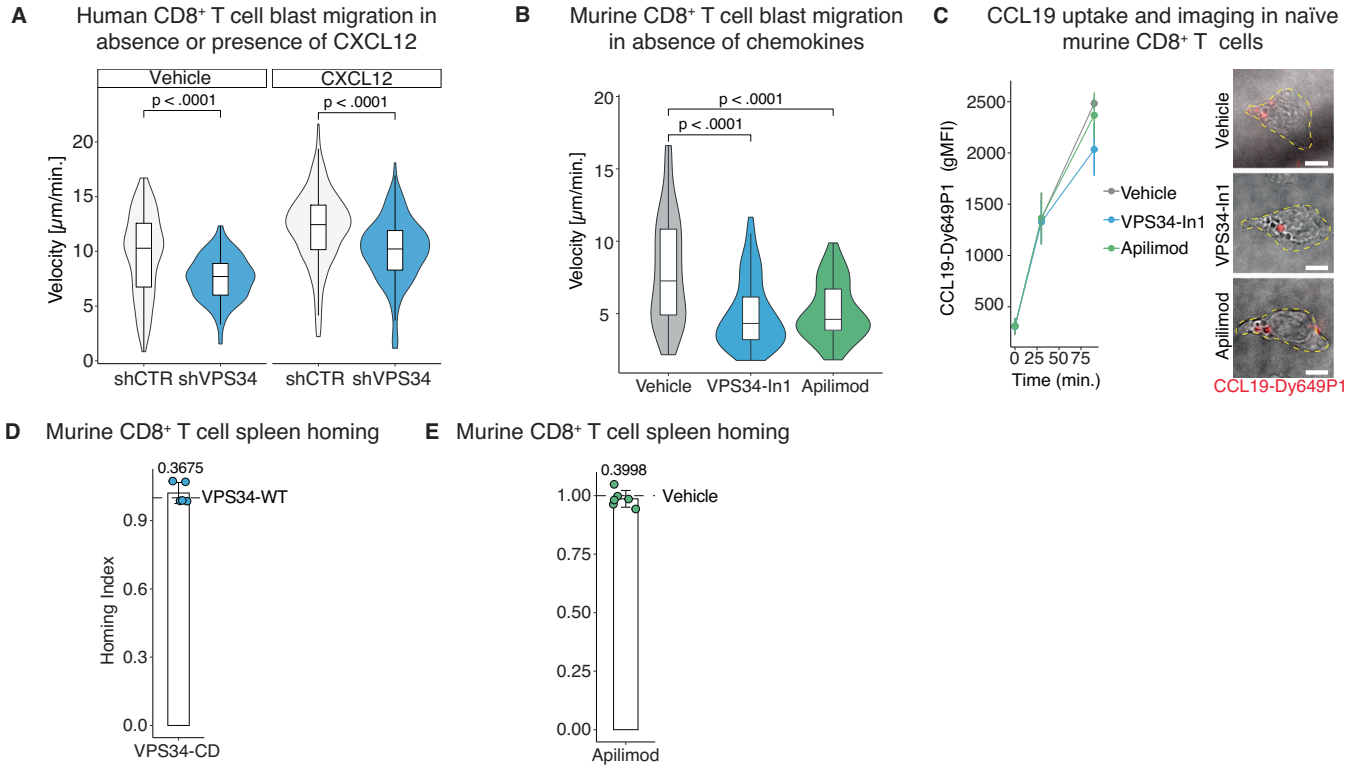


Figure EV3. Chemokine signaling is dispensable for VPS34-PIKfyve-mediated regulation of T cell migration speed.

(**A**) Migration speed in the absence or presence of a CXCL12 gradient, of human CD8$^+$ T cell blasts expressing scrambled control shRNA (shCTR; vehicle: $n = 196$; CXCL12: $n = 326$) or VPS34 knockdown (shVPS34; vehicle: $n = 62$; CXCL12: $n = 210$). (**B**) Migration speed of murine CD8$^+$ T cell blasts treated with vehicle ($n = 90$), VPS34-In1 ($n = 76$), or Apilimod ($n = 46$) in the absence of chemokines. (**C**) Left panel: Time course analysis of CCL19-Dy649P1 uptake in naive murine CD8$^+$ T cells (flow cytometry, geometric mean intensity (gMFI) and standard deviation of three biological replicates). Right panel: Representative bright field images merged with CCL19-Dy649P1 signal (red) of naive murine CD8$^+$ T cells migrating in a fluorescent CCL19 gradient, treated with vehicle control, VPS34-In1, or Apilimod. Yellow dashed lines indicate cell outline. Scale bar = 5 µm. (**D**) Spleen homing index (normalized to each internal control) of murine CD8$^+$ T cells per recipient mice ($n = 5$) expressing the wild-type or catalytic dead (CD) VPS34, and (**E**) spleen homing index (normalized to each internal control, $n = 6$ recipient mice) of vehicle or Apilimod-treated cells. (**A–C**) Representative of three experiments; (**D**, **E**) representative of two experiments. Wilcoxon rank-sum test (**A**, **B**) and paired *t* test (**D**, **E**). (**D**, **E**) Error bars = s.d. Box plots indicate the median (central line), first and third quartiles (box bounds; 25th and 75th percentiles), and the minimum and maximum values (whiskers).

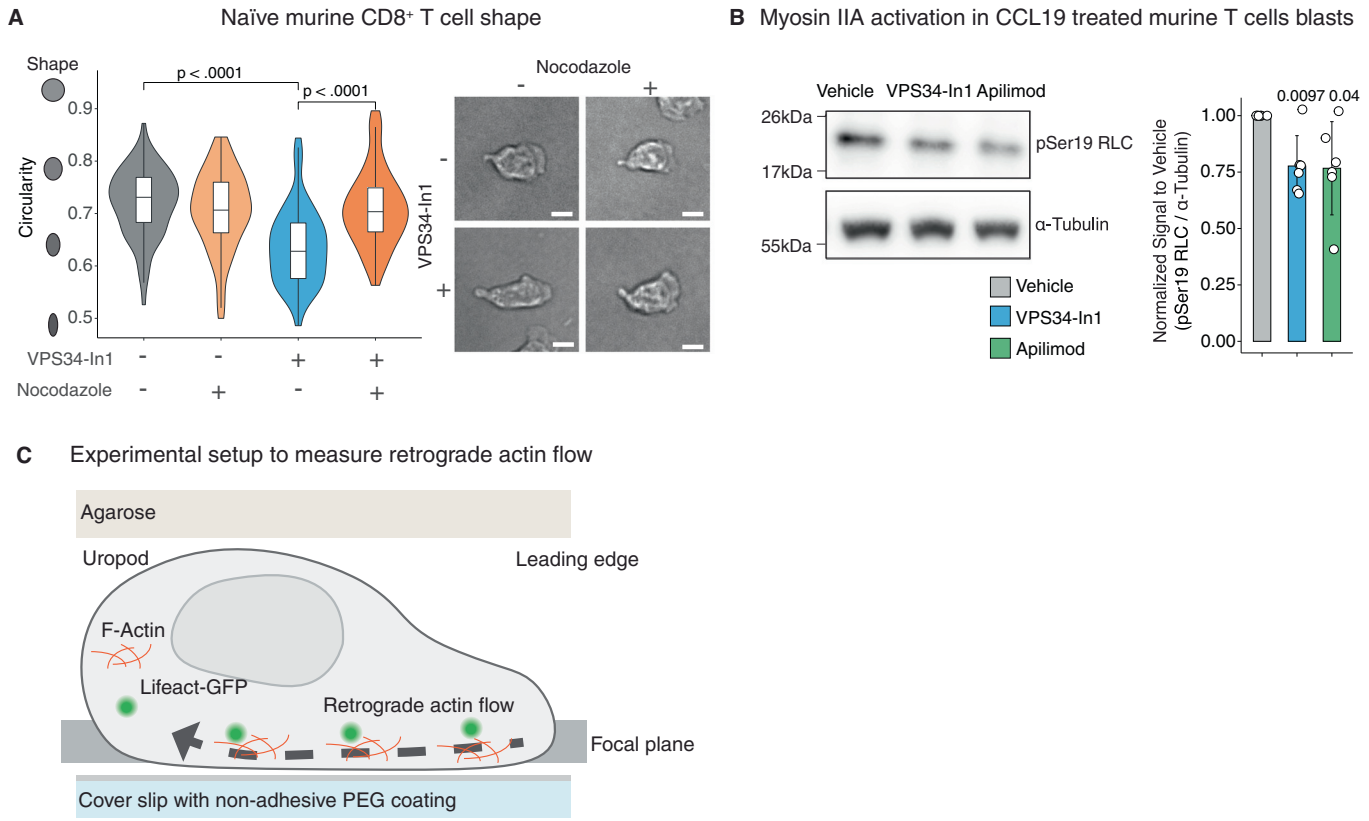


Figure EV4. VPS34 and PIKfyve regulate retrograde actin flow and myosin IIA activation.

(**A**) Left panel: Circularity of naive CD8$^+$ T cells placed in a CCL19 gradient treated with vehicle control ($n = 81$), Nocodazole ($n = 70$), VPS34-In1 ($n = 85$), and VPS34-In1 plus Nocodazole ($n = 56$). Right panels: representative bright field images. Scale bar: 5 µm. (**B**) Left panels: representative Western blots probing for RLC Ser19 phosphorylation and α-tubulin (loading control) in T cell blasts treated with CCL19 and vehicle, VPS34-In1, or Apilimod. Right panel: quantification of six biological replicates, normalized to α-tubulin and vehicle. (**C**) Experimental setup for assessing retrograde actin flow in murine T cell blasts expressing the Lifeact-GFP reporter (to visualize F-actin dynamics). (**A**) Displays representative data of three independent experiments. ART ANOVA for (**A**). Paired *t* test for (**B**). (**B**) Error bars = s.d. Box plots indicate the median (central line), first and third quartiles (box bounds; 25th and 75th percentiles), and the minimum and maximum values (whiskers).

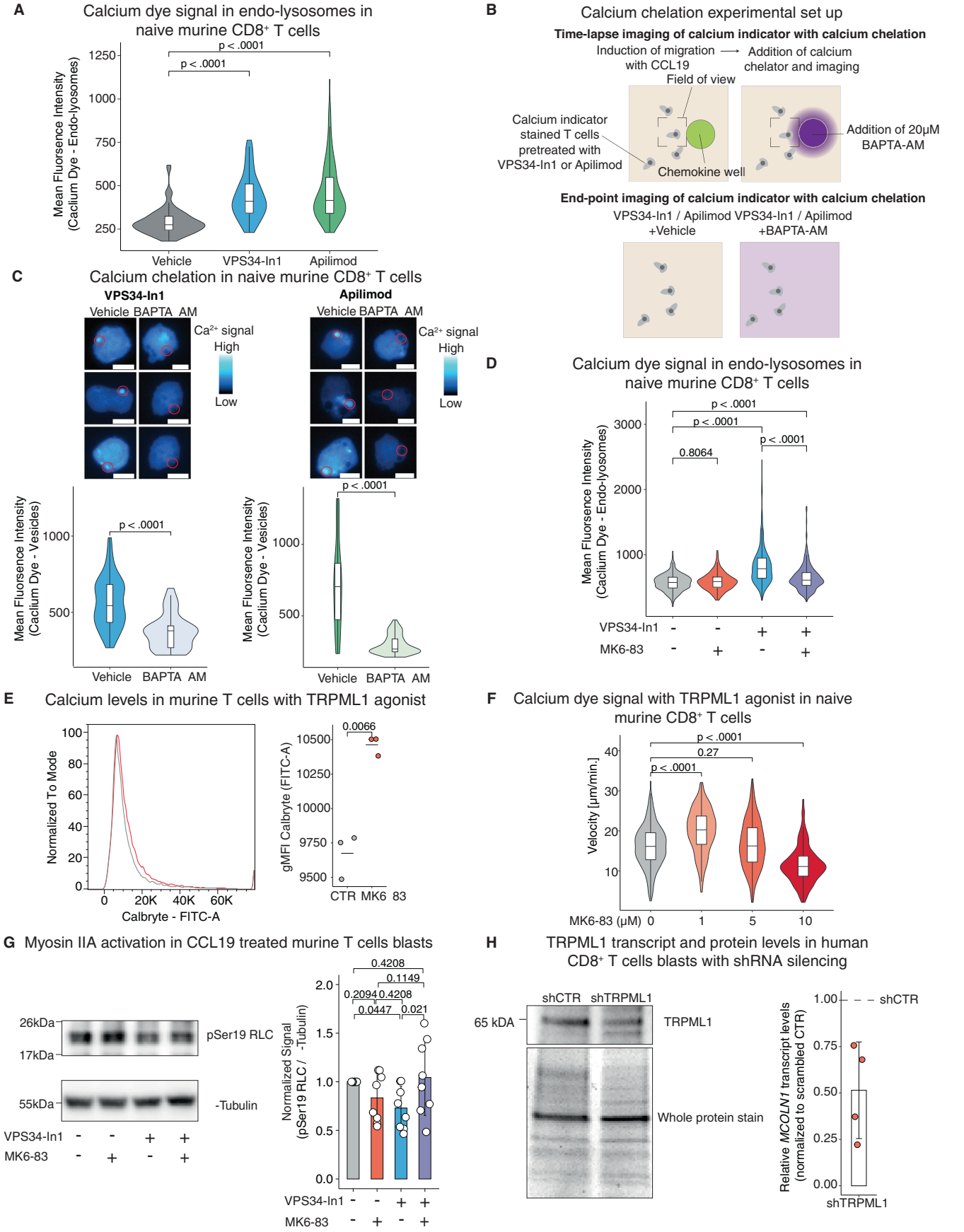
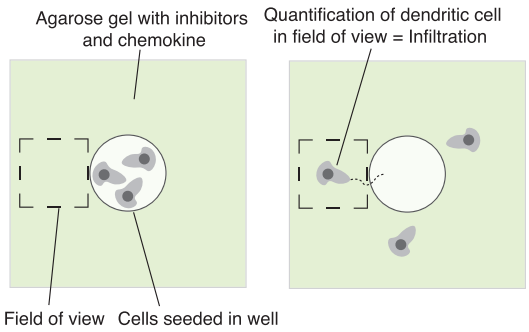

A
Calcium dye signal in endo-lysosomes in naive murine CD8+ T cells
Mean Fluorsence Intensity (Caclium Dye - Endo-lyosomes)
p < .0001
p < .0001
Vehicle
VPS34-In1
Apilimod
B
Calcium chelation experimental set up
Time-lapse imaging of calcium indicator with calcium chelation
Induction of migration with CCL19
Addition of calcium chelator and imaging
Field of view
Calcium indicator stained T cells pretreated with VPS34-In1 or Apilimod
Chemokine well
Addition of 20µM BAPTA-AM
End-point imaging of calcium indicator with calcium chelation
VPS34-In1 / Apilimod +Vehicle
VPS34-In1 / Apilimod +BAPTA-AM
C
Calcium chelation in naive murine CD8+ T cells
VPS34-In1
Apilimod
Vehicle BAPTA AM
Ca2+ signal
High
Low
Mean Fluorsence Intensity (Caclium Dye - Vesicles)
p < .0001
Vehicle
BAPTA AM
D
Calcium dye signal in endo-lysosomes in naive murine CD8+ T cells
Mean Fluorsence Intensity (Caclium Dye - Endo-lyosomes)
p < .0001
p < .0001
p < .0001
0.8064
VPS34-In1
MK6-83
E
Calcium levels in murine T cells with TRPML1 agonist
Normalized To Mode
Calbryte - FITC-A
gMFI Calbryte (FITC-A)
0.0066
CTR MK6 83
F
Calcium dye signal with TRPML1 agonist in naive murine CD8+ T cells
Velocity [µm/min.]
p < .0001
0.27
p < .0001
MK6-83 (µM)
G
Myosin IIA activation in CCL19 treated murine T cells blasts
26kDa
17kDa
pSer19 RLC
55kDa
-Tubulin
Normalized Signal (pSer19 RLC / -Tubulin)
0.4208
0.1149
0.2094
0.4208
0.0447
0.021
VPS34-In1
MK6-83
H
TRPML1 transcript and protein levels in human CD8+ T cells blasts with shRNA silencing
shCTR
shTRPML1
65 kDA
TRPML1
Whole protein stain
Relative MCOLN1 transcript levels (normalized to scrambled CTR)
shCTR
shTRPML1

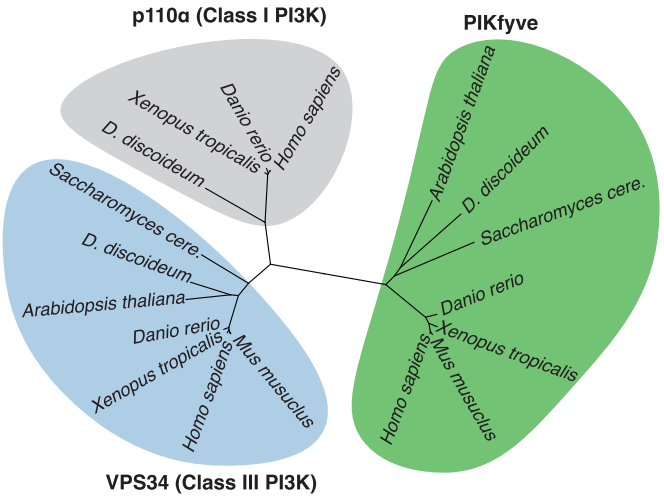
Figure EV5. VPS34 and PIKfyve regulate migration velocity of T cells via lysosomal Ca^{2+}.

(**A**) Quantification of calcium dye MFI per endo-lysosome in naive murine $CD8^+$ T cells migrating in a CCL19 gradient, treated with vehicle ($n = 82$ vesicles in 28 cells), VPS34-In1 ($n = 75$ vesicles in 19 cells), or Apilimod ($n = 152$ vesicles in 39 cells). (**B**) Experimental setup for calcium chelation: naive murine $CD8^+$ T cells stained with Calbryte were treated with VPS34-In1 or Apilimod and placed under agarose. For time-lapse imaging, cells migrated in a CCL19 gradient, then medium was replaced with BAPTA-AM-containing medium. For endpoint imaging, cells were incubated for 30 min at 37 °C with or without VPS34-In1, Apilimod, and/or BAPTA-AM. (**C**) Upper panels: representative images of Calbryte-stained naive murine $CD8^+$ T cells with or without VPS34-In1, Apilimod, and/or BAPTA-AM. Lower panels: quantification of calcium dye MFI per endo-lysosome for VPS34-In1 ($n = 72$ vesicles in 32 cells), VPS34-In1 + BAPTA-AM ($n = 41$ vesicles in 18 cells), Apilimod ($n = 25$ vesicles in 10 cells), or Apilimod plus BAPTA-AM ($n = 64$ vesicles in 23 cells). Representative quantified vesicles are marked by a red circle. Scale bar = 5 µm. (**D**) Quantification of calcium dye MFI per endo-lysosome in naive murine $CD8^+$ T cells migrating in a CCL19 gradient, treated with vehicle ($n = 335$ vesicles in 93 cells), MK6-83 ($n = 194$ vesicles in 65 cells), VPS34-In1 ($n = 518$ vesicles in 108 cells), or VPS34-In1 plus MK6-83 ($n = 450$ vesicles in 121 cells). (**E**) Left panel: Representative histogram of cytosolic calcium levels as captured by flow cytometry in murine T cells stained with Ca^{2+} dye and treated with MK6-83 (5 µM) or vehicle. Right panel: Summary of $n = 3$ technical replicates. (**F**) Migration velocity of naive murine $CD8^+$ T cells placed in a CCL19 gradient, treated with vehicle ($n = 646$), or increasing concentrations of MK6-83 (1 µM: $n = 173$; 5 µM: $n = 487$; 10 µM: $n = 262$). (**G**) Left panel: representative Western blots probing for RLC Ser19 phosphorylation (top) and α-tubulin (bottom; loading control) in T cell blasts treated with CCL19 and vehicle, VPS34-In1, and/or MK6-83. Right panel: quantification of eight biological replicates normalized to α-tubulin and vehicle. (**H**) Left panel: representative Western blots probing for TRPML1 (*top*) and whole protein stain (bottom) in human $CD8^+$ T cells transduced with scrambled control shRNA (shCTR) or TRPML1-targeting shRNA (shTRPML1). Right panel: relative *MCOLN1* transcript levels from four biological replicates, normalized to SDHA and scrambled control. (**A**, **C**, **D**, **F**) Representative of ≥3 independent experiments. Two-way ANOVA with Fisher's least-squares test (**G**), *t* test (**E**), Wilcoxon rank-sum test (**A**, **C**), ART ANOVA (**D**). (**G**, **H**) Error bars = s.d. Box plots indicate the median (central line), first and third quartiles (box bounds; 25th and 75th percentiles), and the minimum and maximum values (whiskers).

A Under-agarose dendrictic cell migration and infiltration assay



B VPS34, PIKfyve and p110α phylogenetic tree of model organisms



C TRPML1 orthologue sequence alignment

```
                41 43   55      62
                 **      * *    *
Mus musculus    RRR....KFRAKGRK
D. discoideum   RRC....KWRKYNRF
```

AA - $PI(3,5)P_2$ binding amino acid

* - Conserved AA

Figure EV6. Regulation of amoeboid migration is a conserved function of the VPS34–PIKfyve axis.

(**A**) Schematics of the migration and infiltration assay of DCs: Cells were added to the well in the agarose (containing inhibitors and CCL19, left panel). DCs crawled under the gel (= infiltration) and the number of cells in the field of view was quantified (right panel). (**B**) Phylogenetic analysis of the amino acid (AA) sequences of VPS34, PIKFYVE, and p110α (as an example for Class I PI3K). (**C**) Alignment of the $PI(3,5)P_2$-binding protein region from *Mus musculus* TRPML1 and the *D. discoideum* orthologue. $PI(3,5)P_2$-binding AAs are highlighted in green, conserved AAs indicated by an asterisk.