Supplemental Methods

Cell culture, virus production and transduction

Primary HUVEC cells were purchased from Lifeline Cell Technology (Cat: FC-006). 293T cells were cultured in DMEM (ThermoFisher) supplemented with 10% FBS (Gibco). In some cases, 293T cells were maintained in DMEM supplemented with 10% SEV-depleted FBS (Gibco). Primary HUVEC cells were cultured in vasculife medium (Lifeline) containing 5 ng/mL rh EGF (PeproTech), 5 ng/mL rh FGF basic (PeproTech), 15 ng/mL rh IGF-1 (PeproTech), 50 μg/mL Ascorbic Acid (Sigma), 1 μg/mL Hydrocortisone Hemisuccinate (Sigma), 0.75 U/mL Heparin Sulfate (Sigma), 10 mM L-Glutamine (ThermoFisher) and supplemented with 2% SEV-free FBS. LILRB2 chimeric reporter cells were cultured in 1640 medium (ThermoFisher) supplemented with 10% FBS.

To transform normal hematopoietic stem and progenitor cells into AML cells, a MSCV-MLL-AF9-IRES-YFP (or GFP) plasmid together with pKat and pVSVG packaging plasmids was transfected into 293T cells using Lipofectamine 2000 (ThermoFisher) (1). After 48 and 72 h of culture, the supernatant containing retroviruses was harvested and concentrated using an Amicon filter (Millipore). Fetal liver cells or BM cells were enriched using lineage cell depletion beads (Miltenyi) and transduced with MLL-AF9 retroviruses in the presence of 4 μg/ml polybrene (Sigma). The cells were then incubated in IMDM (Gibco) with 15% FBS, 50 ng/ml mouse SCF (Peprotech), 10 ng/ml mouse IL-3 (Peprotech) and mouse IL-6 (Peprotech) for 2 days.

To construct 293T cells expressing ANGPTL2-mCherry, a lentiviral plasmid expressing the ANGPTL2–mCherry fusion protein (PLVX-ANGPTL2-mCherry) was mixed with pSPAX2 and pMD2.G packaging plasmids, and then transfected into 293T cells. To construct VPS33B knockdown 293T cells, a lentiviral vector expressing shRNAs targeting human VPS33B was mixed with the pSPAX2 and pMD2.G packaging plasmids and transfected into 293T cells. Lentiviruses were harvested 48 h and 72 h after transfection. To construct 293T cells overexpressing ANGPTLs, ANGPTL1, 2, 3, 4, 6 or 7 vectors were individually transfected into 293T cells. In some cases, ANGPTL2-mCherry-N1 and CD63-EGFP-N1 plasmids were co-transfected into 293T cells for live-cell imaging.

Generation of Akt1 BM endothelial (Akt1-BM EC) cells

Femurs and tibias from adult mice (8-10 weeks) were dissected out and crushed using a mortar

and pestle. The cells were then digested with 3 mg/ml collagenase I (Worthington) and Dispase II (Roche) in HBSS plus Ca^{2+} and Mg^{2+} (ThermoFisher) at 37°C for 15 min. After digestion, the cells were filtered through a 100 μm nylon mesh (BD Falcon) and washed once with staining buffer (PBS + 2 mM EDTA + 2% FBS). The digested cells were enriched using Dynabeads (ThermoFisher) pre-coated with anti-CD31 antibody (BD Biosciences). Enriched $CD31^+$ cells were seeded into RetroNectin (Takara)-coated 24-well plates, and transduced with an Akt1 lentivirus and cultured in BM EC complete medium, containing 200 ml F-12 medium (Corning) and 200 ml DMEM low-glucose medium (Corning), 100 ml FBS (Gibco), 5 ml nonessential amino acid (Corning), 5 ml of penicillin/streptomycin (Gibco), 10 ml of 1M HEPES (Corning), 2 ml of 25 mg/ml heparin stock (Corning), 5 ml of 7.5 mg/ml EC growth supplement (Alfa Aesar) or 5 ml of 6 mg/ml EC growth supplement (Merck).

SEV isolation and cryo-immunogold electron microscopy

To obtain ANGPTL-abundant SEVs, 1.5×10^7 293T cells that overexpressed ANGPTLs were cultured in 30 ml DMEM supplemented with 10% SEV-free FBS for 3 days and the supernatants were collected for SEV isolation. To isolate SEVs from the primary HUVEC cells, 1×10^6 HUVEC cells were cultured in 30 ml vasculife VEGF medium (Lifeline) supplemented with SEV-depleted FBS for 7 days before the supernatants were collected for SEV isolation.

To isolate SEVs from the BM EC culture supernatants, the BM EC medium was first centrifuged for 11 h at 110,000 xg at 4°C to obtain SEV-deleted medium. Akt1–BM EC cell lines were cultured in 24-well plates and transduced with shScramble or shVps33b lentivirus when the cells reached >80% confluence. After 3 days, the ECs were washed three times with pre-warmed PBS after removing the old supernatant. Subsequently, the cells were cultured with 1 ml SEVs-deleted BM EC medium per well for 3 days, and then the supernatant was collected for SEV isolation.

To purify SEVs from the mouse BM niche, 6-8-week-old C57BL/6J, *Cdh5-Cre;Vps33b*$^{fl/fl}$ mice or *Vps33b*$^{fl/fl}$ mice were sacrificed and the long bones were isolated. The BM cells were flushed out using 1 ml serum-free PBS (per mouse). Then, the BM fluid samples or cell culture supernatants were subjected to differential centrifugation as detailed: the samples were sequentially centrifuged at 600 xg for 10 min, 2,000 xg for 10 min and 12,000 xg for 30 min to remove the cells and cellular

debris. The purified BM fluid samples were further filtered using a 0.22 μm filter, followed by ultracentrifugation (Beckman) at 110,000 xg for 80 min. The isolated SEVs were washed with serum-free PBS and pelleted again by ultracentrifugation at 110,000 xg for 80 min. All ultracentrifugation steps were performed at 4°C and the isolated SEVs were re-suspended in 100-200 μl serum-free PBS. The protein concentration was further determined by BCA quantification (ThermoFisher).

For immunoelectron microscopy, isolated SEVs were fixed with 2% paraformaldehyde for 1 h, and then transferred to the Electron Microscopy Core Facility at Shanghai Jiao Tong University for analyses. Immunogold staining of ANGPTL2 SEVs was conducted by incubating with mouse-anti-Flag antibodies (Prozyme PJ255) for 12 h at 4°C, followed by incubation with anti-mouse 10 nm IgG gold (Sigma 041M1453).

Flow cytometry

To analyze MLL-AF9-tranduced leukemia development and differentiation, PB or BM cells were incubated with anti-Mac-1-APC (eBioscience), anti-Gr-1-PE (eBioscience), anti-CD3-APC (eBioscience) and anti-B220-PE (eBioscience) for 30 min at 4°C. To examine L-GMP cells, BM cells collected from leukemia mice were stained with biotinylated anti-CD3, anti-CD8, anti-B220, anti-Gr-1, anti-Ter119 and anti-CD127 antibodies, followed by staining with streptavidin-PE/Cy5.5, anti-Sca-1-PE/Cy7, anti-c-Kit-APC, anti-CD16/32- eFluo450 and anti-CD34-PE (all from eBioscience) for 30 min at 4°C. For human leukemia cell staining, human primary leukemia cells were incubated with anti-human-CD34-PE for 30 min at 4°C. To analyze the chimerism of human AML cells in NOD-SCID mice, PB was collected and stained with anti-human-CD45-APC for 30 min at 4°C. To detect the frequency of HSCs and hematopoietic progenitor cells (HPCs) in Cdh5-CreER;Vps33b$^{fl/fl}$ mice, the BM cells were flushed and stained with the following antibodies for 30 min at 4°C: anti-lineage cocktail-APC/Cy7 (including CD3, CD4, CD8, B220, Mac-1, Gr-1 and Ter119, all from Biolegend), anti-Sca-1-BV786 or PE/Cy7 (BD Biosciences), anti-c-Kit-BV605 or APC (BD Biosciences), anti-CD150-PE/Cy7 (Biolegend), anti-CD48-BV421 or PerCP/Cy5.5 (BD Biosciences), anti-CD41-BV510 or FITC (BD Biosciences), anti-Flk2-PE (eBioscience), anti-IL7Rα-APC (eBioscience), anti-CD34-FITC (eBioscience) and anti-CD16/32-PerCP/Cy5.5 (eBioscience). During the staining, a BD Horizon™ Brilliant Stain Buffer was used (BD Biosciences).

For megakaryocytes isolation, the BM cells were stained with anti-CD41-FITC and Hoechst 33342 for 30 min at 4°C. To test the purity of the enriched ECs, anti-CD31-APC (eBioscience) and anti-VE-Cadherin-BV421 (BD Biosciences) were used.

To isolate BM niche cells, the marrow was gently flushed out and transferred into 1 ml pre-warmed digestion solution (3 mg/ml collagenase I (Worthington), 3mg/ml collagenase IV (Worthington) and 0.01% DNase I (Roche) in HBSS plus Ca^{2+} and Mg^{2+}). After flushing, the bones were crushed with a mortar and pestle using a digestion solution. The marrow or crushed bones were incubated at 37°C for 30 min with gentle shaking. After digestion, the supernatant was collected and filtered through a 100 μm nylon mesh (BD Falcon) and washed once with staining buffer (2). To isolate splenic stromal cells, the spleen capsule was cut into ~1 mm^3 fragments using scissors and then digested in digestion solution for 10 min. After a brief vortex, the spleen fragments were allowed to sediment for 3 min and the supernatant was transferred to another tube on ice. The sedimented spleen fragments were subjected to a second round of digestion (10 min). The two fractions of digested cells were pooled and filtered through a 100 μm nylon mesh (3). Anti-LepR (R&D), anti-CD45 (Biolegend) and anti-Ter119 (Biolegend) antibodies were used to isolate BM MSCs. For osteoblast and osteo-progenitor cells, 6-8-week-old Col2.3-GFP and Osx-CreER;TdTomato mice were used. For EC isolation, mice were injected intravenously with 10 μg Alexa Fluor 647-conjugated anti-VE-cadherin antibody (BV13, Biolegend) 10 min before being killed (4). The samples were analyzed using a FACSCanto II or FACSAria III flow cytometer (BD Biosciences). All data were analyzed using FACSDiVa (BD Biosciences) and FlowJo v10 (BD) software.

For spectral cytometry analysis, BM cells were stained with following antibodies: anti-B220-BUV496 (BD Bioscience), anti-CD48-BV421 (BD Bioscience), anti-Gr-1-PacificBlue (Biolegend), anti-CD41-BV510 (BD Bioscience), anti-c-Kit-BV605 (BD Bioscience), anti-CD8-BV650 (BD Bioscience), anti-Mac-1-BV711 (BD Bioscience), anti-Sca-1-BV785 (BD Bioscience), anti-CD34-FITC (Biolegend), anti-CD3-SparkBlue550 (Biolegend), anti-CD16/32-PerCP/Cy5.5 (Biolegend), anti-CD45 (Biolegend), anti-Flk2-PE (Biolegend), anti-CD150-PE/Cy7 (Biolegend), anti-IL7Ra-APC (Biolegend), anti-CD4-AF700 (Biolegend) and anti-Ter119-APC/Cy7 (Biolegend).For dead cell exclusion, cells were washed with PBS without serum and stained with Zombie NIR fixable viability kit (Biolegend) for 15 min at room temperature.

Cells were resuspended in staining buffer and filtered through 70 μM nylon mesh before analysis. Data were collected on a 5 Laser (UV [355 nm], Violet [405 nm], Blue [488 nm], Yellow-Green [561 nm], Red [640 nm]) Cytek Aurora spectral flow cytometer and analyzed with SpectroFlo software (Cytek Biosciences, Fremont, CA).

Binding of SEVs to leukemia cells

SEVs (100 μg) were labeled with CFSE (ThermoFisher) for 40 min at 37°C. CFSE-labeled SEVs were washed with serum-free PBS and pelleted again by ultracentrifugation at 110,000 xg for 80 min, then incubated with 2×10^5 AML cells, Mac-1^+c-Kit^+ LSCs or L-GMP cells for 2 h at 37°C. After incubation, the cells were washed with PBS to remove unbound SEVs followed and the CFSE signal was analyzed by flow cytometry. To directly observe the binding of SEVs to leukemia cells, AML cells were labeled with c-Kit and Hoechst 33342 to indicate the cell membrane and nuclei, respectively, and then plated onto a 35 mm Poly-D-lysine hydrobromide-coated glass-bottom dish (Cellvis) for confocal imaging (Nikon A1). To test the binding of ANGPTL2-SEV to LSCs, CFSE labeled ANGPTL2-SEVs were incubated with 2×10^5 L-GMP cells or $CD34^+$ human primary leukemia cells for 2 h at 37°C, followed by washing with PBS to remove the unbound SEVs. The c-Kit or CD34 antibody staining indicated the cell membrane. Before imaging, the cells were stained with Hoechst 33342 to visualize the nuclei. In another case, purified L-GMP cells were co-cultured with ANGPTL2-mCherry SEVs for 24 h, and then the mCherry signal in L-GMP cells was detected by flow cytometry.

***In vitro* SEV treatment and *in vivo* SEV administration**

For *in vitro* SEV treatment, 1×10^5 AML cells were cultured in 12-well plates with 600 μL StemSpan serum-free medium (STEMCELL Technologies) containing 10 ng/mL mouse SCF (Peprotech), 10 ng/mL mouse IL-3 (Peprotech) and 10 ng/mL mouse IL-6 (Peprotech), followed by supplementing with 3 μg of each ANGPTL1, 2, 3, 4, 6, 7-SEVs or control SEVs for 3 days before transplantation. For human primary AML cells, 1×10^6 cells were incubated with 10 μg ANGPTL2-SEVs or control-SEVs and maintained in 5 mL StemSpan serum-free medium containing 10 ng/mL human SCF (Peprotech), 10 ng/mL human IL-3 (Peprotech) and 10 ng/mL human IL-6 (Peprotech). The co-cultured cells were collected for transplantation or colony-forming unit assays 3 days after treatment.

For *in vivo* SEV treatments, 20 μg ANGPTL2-SEVs or control-SEVs were injected into *Cdh5-Cre; Vps33b*$^{fl/fl}$ or *Vps33b*$^{fl/fl}$ mice by intra-tibia injection. SEVs were administrated every 5 days for a total 20 days. In another scenario, 40 μg EC-derived SEVs or PBS were injected into AML mice by intravenous injection every 5 days for a total 40 days.

BM transplantation

To establish the AML model, MLL-AF9 transduced lineage$^-$ cells were injected into lethally irradiated (9.5 Gy) C57BL/6J recipient mice to induce AML development (1). To investigate the effects of specific deletion of *Vps33b* in ECs, MSCs, Mks or OPCs on leukemia progression, 5,000 AML cells plus 2×10^5 BM cells were injected into the lethally irradiated 6-8-week-old *Cre*$^+$ *Vps33b*$^{fl/fl}$ mice and *Cre*$^-$ *Vps33b*$^{fl/fl}$ littermate control mice. Then, 10,000 SEV co-cultured AML cells plus 2×10^5 BM cells were injected intravenously into lethally irradiated C57BL/6 recipient mice or the indicated recipients.

For transplantation of *Pirb*-knockout leukemia cells, 2×10^5 MLL-AF9 transduced lineage$^-$ cells from *Pirb*-WT or *Pirb*-KO mice were injected intravenously into lethally irradiated C57BL/6 recipient mice. Then, 5,000 *Pirb*-WT or *Pirb*-KO AML cells were further sorted from primary recipient mice and co-cultured with ANGPTL2-SEVs or control-SEVs. After co-culture, the cells were transplanted with 2×10^5 BM into lethally irradiated C57BL/6 recipient mice.

For normal hematopoietic cell transplantation, 1x10^6 BM cells from 10-week old *Cdh5*-CreER;Vps33b$^{fl/fl}$ mice or Vps33b$^{fl/fl}$ mice were transplanted with 1x10^6 CD45.1$^+$ competitive BM cells into lethally irradiated CD45.1$^+$ recipients. For reciprocal transplantation, 2x10^6 CD45.1$^+$ BM cells were transplanted into CD45.2$^+$ *Cdh5*-CreER;*Vps33b*$^{fl/fl}$ mice or *Vps33b*$^{fl/fl}$ littermate control mice. PB reconstitution was monitored every 4 weeks. In another case, 2×10^6 CD45.1$^+$ BM cells were injected into lethally irradiated CD45.2$^+$ *Tie2*-Cre *Vps33b*$^{fl/fl}$ mice and *Vps33b*$^{fl/fl}$ littermate control mice. After 16 weeks, 1×10^6 CD45.1$^+$ BM cells were purified from primary recipients and mixed with 1×10^6 CD45.2$^+$ competitor BM cells, followed by transplanting into lethally irradiated CD45.2$^+$ mice to perform secondary transplantation.

For human primary leukemia cell transplantation, 2.5×10^6 human primary AML cells were transplanted into 6-8-week-old NOD-SCID mice. PB cells were stained with anti-human-CD45-PE antibodies for the detection of leukemia progression.

Homing assay

Cdh5-Cre Vps33b$^{fl/fl}$ mice or *Vps33b*$^{fl/fl}$ littermate control mice were lethally irradiated and received 2.5×10^6 AML GFP$^+$ cells. The frequency of the homed cells was measured in the BM and spleen 16 h after transplantation by detecting the GFP fluorescence.

ANGPTL2 SEVs activate LILRB2 reporter cells

Purified 10 μg ANGPTL2-SEVs or control SEVs (SEVs isolated from ANGPTL2 overexpressing or control 293T cells) were pre-coated on a flat-bottomed 96-well plate (BD Falcon) for 4 h at 37°C and then incubated with 50,000 LILRB2 chimeric reporter (5) cells in 200 μL medium. The GFP frequency reporting the cells was analyzed by flow cytometry 48 h after incubation.

Half bone whole-mount preparation for imaging

Freshly dissected femurs from 8-10-week old mice were fixed in cold 4% PFA in PBS (Affymetrix) overnight at 4 °C. The bones were washed with PBS to remove the PFA and cryoprotected in 30% sucrose PBS solution overnight at 4 °C with shaking. The bones were embedded in O.C.T. (Sakura) and flash frozen in liquid nitrogen. A Leica cryostat was used to longitudinally bisect the bones. The intact half bone was washed in PBS to remove O.C.T. and then processed for staining. Half bones were stained with DAPI (Sigma) for 1 h at room temperature (6). The images were acquired using a PerkinElmer UltraVIEW VoX Spinning Disk Confocal Microscope or Olympus FV1200MPE and analyzed by Volocity v6.0.

Colony forming unit assay

For mouse AML cells, 3,000 AML cells were seeded in methylcellulose (M3534, STEMCELL Technologies) according to the manufacturer's instructions. For human primary AML cells, 5,000 cells were seeded into methylcellulose (H4436, STEMCELL Technologies) for colony growth. The colonies were imaged and counted 7 days after plating.

Western blotting

Cell extracts or SEV extracts were prepared using SDS buffer and then separated on 10% SDS polyacrylamide gels and transferred onto PVDF membranes (Millipore). The membranes were blocked in 6% non-fat milk and then incubated with antibodies against GM130 (Proteintech), TSG101 (Proteintech), FLOT1 (Abways), VPS33B (Santa Cruz), FLAG (Sigma), ANGPTL2 (R&D system), CD63 (Abcam), CD9 (Abcam), Calnexin (Abcam), pCREB (Abways), CREB (Abways), p-SHP2 (CST), SHP2 (CST) and β-actin (Cell Signaling), followed by incubation with horseradish peroxidase-conjugated secondary antibody.

Quantitative RT-PCR

Total RNA was isolated using a Qiagen RNeasy Mini Kit. cDNA was synthesized using Improm-II™ reverse transcriptase (Promega). qRT-PCR was performed on a 7500 or StepOne real-time PCR system (Applied Biosystems). The primers used in this study were:

mouse *β-actin*: GGCTGTATTCCCCTCCATCG and CCAGTTGGTAACAATGCCATGT;

mouse *Angptl1*: GGAATGCTGGGGACTCTATG and CCATCTTGGTGCTTGCTTCT;

mouse *Angptl2*: CCACCTCGGGTCTACCAAC and CTTGCAGGCAGTCTCTCCAT;

mouse *Angptl3*: ACAGAGCAAAGGGACAGCTC and TGGAGCATCATTTTGGATGA;

mouse *Angptl4*: GCACAGCATCACAGGGAAC and TAGAGAAGGGCAGGGAAAGG;

mouse *Angptl6*: CAGAGCACCAGAGAGAGCAG and ACCACACGGCTACTACACGA;

mouse *Angptl7*: GCCTCATAAACGCAAGACAC and CTGACCCAGTCGCTCTCCT.

mouse *Vps33b*: AGCTTCCCGACTTCTCTATGC and CAATTCGATCCAAAGGGCTCAT

Isolation of SEV proteins and mass spectrometry

Protein extraction and TMT proteomics analysis was supported by HangZhou Jingjie PTM BioLabs. SEV samples were sonicated three times on ice using an ultrasonic processor (Scientz) in lysis buffer (8 M urea, 1% Protease Inhibitor Cocktail). After centrifugation at 18,000 xg, the supernatant was collected and the protein concentration was determined using a BCA kit. For digestion, the protein solution was reduced with 10 mM DTT for 1 h and alkylated with 20 mM IAA for 45 min at room temperature in darkness. After diluting the urea below 2M with TMT label buffer, trypsin was added at a 1:50 trypsin-to-protein (m/m) ratio for the first digestion overnight and at a 1:100 trypsin-to-protein (m/m) ratio for the second 4 h-digestion. After trypsin digestion, the peptide was desalted on a Strata X C18 SPE column (Phenomenex) and vacuum-dried. The peptides were then

TMT labeled according to the manufacturer's instructions.

The peptides were subjected to NSI source followed by tandem mass spectrometry (MS/MS) in a Q Exactive HF-X (Thermo) coupled with an EASY-nLC 1200 UPLC system. The gradient comprised an increase from 6% to 22% solvent B (0.1% formic acid in 90% acetonitrile) over 47 min, 22% to 32% in 20 min and climbing to 80% in 4 min then holding at 80% for the last 4 min. The electrospray voltage applied was 2.0 kV. The intact peptides were detected in the Orbitrap at a resolution of 120,000. The peptides were then selected for MS/MS with a NCE setting of 28 and the fragments were detected in the Orbitrap at a resolution of 15,000. A data-dependent procedure that alternated between one MS scan followed by 30 MS/MS scans with 30.0s dynamic exclusion was used. The automatic gain control (AGC) was set at 5E4. The fixed first mass was set as 100 m/z.

The resulting MS/MS data were processed using the MaxQuant search engine (v.1.5.2.8). Tandem mass spectra were searched against the Mus musculus database concatenated with the reverse decoy database. Trypsin/P was specified as a cleavage enzyme allowing up to two missing cleavages. The mass tolerance for the precursor ions was set as 20 ppm in the first search and as 5 ppm in the main search, and the mass tolerance for the fragment ions was set as 0.02 Da. Carbamidomethyl on Cys was specified as a fixed modification, and oxidation on Met and acetylation on protein N-term were specified as variable modifications. The FDR was adjusted to <1% and the minimum score for modified peptides was set as >40. The minimum peptide length was set at 7. For the quantification method, the TMT 6-plex was selected. All the other parameters in MaxQuant were set to the default values.

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Supplemental Figure Legends

Figure S1. Morphology of BM fluid SEVs and their binding to AML cells

(A) Electron microscopy of BM fluid SEVs. (B) Images of Mac-1$^+$c-Kit$^+$ AML cells after co-culture with CFSE-labelled SEVs (left). The statistics of membrane bound and internalized SEVs is shown (right). In total, 174 AML cells were counted after staining (Scale bars, 5 μm). (C) After 4-12 h co-culture, the statistics of membrane bound and internalized SEVs is shown. Experiments were conducted 2 to 3 times for validation.

Figure S2. Vps33b is required for SEVs release by BM MSCs and ECs. (A) qRT-PCR analysis of *Vps33b* expression in shRNA transduced MSCs (n = 3). (B) Nanoparticle tracking analysis (NTA) showing knockdown of *Vps33b* in MSCs decreased the number of secreted SEVs (n = 3). (C) BCA analysis showing knockdown of *Vps33b* in MSCs decreased the protein levels of secreted SEVs (n = 3). (D) qRT-PCR analysis of *Vps33b* expression in shRNA transduced ECs (n = 3). (E) NTA showing knockdown of *Vps33b* in ECs decreased the number of secreted SEVs (n = 3). (F) BCA analysis showing knockdown of *Vps33b* in ECs decreased the protein levels of secreted SEVs (n = 3). The data in **A-F** represent the means ± SD, *** $p < 0.001$, Student's t test. Experiments were conducted 2 times for validation.

Figure S3. The deletion efficiency of Vps33b in the different models

FACS sorting strategy and qRT-PCR analyses of *Vps33b* gene expression in different mouse models. (A) BM ECs, (B) BM OPCs, (C) BM MSCs, (D) BM Mks (n = 3). The data in **A-D** represent the means ± SD, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Student's t test. Experiments were conducted 2 times for validation.

Figure S4. Reduced EC derived SEVs impairs the leukemia progression

(A) Flow cytometry (left) and histogram (right) analysis of the frequency of homed AML cells in the BM and spleen of *Vps33b*$^{fl/fl}$ and *Cdh5*-Cre;*Vps33b*$^{fl/fl}$ recipients 16 h after transplantation (n = 5). (B) The frequency of Mac-1$^+$Gr-1$^-$ and Mac-1$^+$Gr-1$^+$ cells in the BM of *Vps33b*$^{fl/fl}$ and *Cdh5*-Cre;*Vps33b*$^{fl/fl}$ recipients (n = 4; the data represent the means ± SD, * $p < 0.05$, ** $p < 0.01$, Student's t test). (C) Survival analysis of *Tie2*-Cre;*Vps33*b$^{fl/fl}$ mice and *Vps33*b$^{fl/fl}$ control mice after AML cell injection (n = 5; *** $p < 0.001$, log-rank test). (D) The percentages of YFP$^+$ leukemia cells in the PB of the recipients 10 days after transplantation (n = 3; the data represent the means ± SD, *** $p < 0.001$, Student's t test). (E) The frequency of Mac-1$^+$Gr-1$^-$ and Mac-1$^+$Gr-1$^+$ cells in the BM of *Vps33b*$^{fl/fl}$ and *Tie2*-Cre;*Vps33b*$^{fl/fl}$ recipients (n = 3; the data represent the means ± SD, * $p < 0.05$, ** $p < 0.01$, Student's t test). (F) The recipient spleen and liver size (left) and weight (right) 13 days

after transplantation (n = 3; the data represent the means ± SD, *** $p < 0.001$, Student's t test). (G) Flow cytometry analysis showing the overlap of TdTomato signal with CD31 and VE-Cadherin antibody staining of BM and spleen cells from *Cdh5*-CreER;TdTomato mice. (H) Images showing the overlap of TdTomato expression with anti-VE-Cadherin antibody staining on a BM section from Cdh5-CreER;TdTomato mice (Scale bar, 50 μm). (I) NTA of EC-SEVs. (J) WB analysis of Calnexin, TSG101, CD9 and β-actin protein levels in cultured BM ECs and EC-SEVs. (K) Electron microscopy of EC-SEVs. Scale bar, 100 nm. Experiments were conducted 2 to 3 times for validation.

Figure S5. Reduced EC derived SEVs has no effect on normal hematopoiesis

(A) Flow cytometry analysis of the HSC, MPP1-4, CMP, GMP, MEP, MkP and CLP frequencies in *Vps33b*$^{fl/fl}$ and *Cdh5*-CreER;*Vps33b*$^{fl/fl}$ mice. MPP, multipotent progenitors; CMP, common myeloid progenitors; GMP, granulocyte-macrophage progenitor; MEP, megakaryocytic-erythroid progenitor; CLP, common lymphoid progenitor; MkP, megakaryocyte progenitor. (B) Representative figures of spectral cytometry analysis of lineage cells, HSCs and HPCs. (C) The frequencies of lineage cells, HPCs and frequencies of HSCs/MPPs are shown (n = 4). The data in **C** and **E** represent the means ± SD, Student's t test. Experiments were conducted 2 to 3 times for validation.

Figure S6. Reduced EC derived SEVs no effect on HSC function *in vivo*

(A) The experimental procedure to test HSC function in *Tie2*-Cre;*Vps33b*$^{fl/fl}$ mice. CD45.1$^+$ BM cells (2×10^6) were injected into lethally irradiated CD45.2$^+$ *Tie2*-Cre*;Vps33b*$^{fl/fl}$ mice and *Vps33b*$^{fl/fl}$ littermate control mice. After 16 weeks, 1×10^6 CD45.1$^+$ BM cells were purified from the primary recipients and mixed with 1×10^6 CD45.2$^+$ competitor BM cells. Then, the cells were transplanted into lethally irradiated CD45.2$^+$ mice for secondary transplantation. (B) The reconstitution of CD45.1$^+$ cells from *Tie2*-Cre*;Vps33b*$^{fl/fl}$ mice and *Vps33b*$^{fl/fl}$ mice in the PB of secondary recipients (n = 5). (C) The long-term multi-lineage reconstitution capacities of CD45.1$^+$ cells in the PB of secondary recipients (n = 5). (D) The percentage of CD45.1$^+$ donor derived HSCs in the BM of secondary recipients (n = 3). The data in **B-D** represent the means ± SD. Experiments were conducted 2 times for validation.

Figure S7. ECs express high level of *Angptl2*

(A) qRT-PCR analysis of Vps33b expression in shRNA transduced BM ECs (n = 3; the data represent the means ± SD, *** $p < 0.001$, Student's t test). The data represent the means ± SD. (B-I) The gating strategy for isolating CD45$^+$ cells (B), HSCs (C), AML cells (D), MKs (E), BM MSCs (F), OBs (G), OPCs (H) and ECs (I). Images for OBs, OPCs and ECs are shown. (J) qRT-PCR analysis

of *Angptl1,3,4,6,7* mRNA levels in the indicated cells (n = 3). β-actin was used as an internal control. n.d., not detected. The data represent the means ± SD. (K) Isolation of ANGPTL-abundant SEVs and downstream analyses. Vectors containing a flag-tagged *Angptl* gene were transduced into 293T cells. The supernatants were collected for SEV isolation. AML cells were co-cultured with SEVs for 3 days, and then collected for colony assay or transplantation. (L) Western blot analysis of Flag, TSG101 and FLOT1 protein levels in the indicated *Angptl* gene-transduced 293T cells and transduced cell-derived SEVs. Experiments were conducted 3 to 4 times for validation.

Figure S8. VPS33B regulates ANGPTL2-SEV maturation

(A) CD63 and ANGPTL2 co-localization in WT and VPS33B-knockdown 293T cells (Scale bars, 5 μm). (B) Experimental design. 293T cells were transduced with shScramble (#1) or shVPS33B (#2) and then transduced with vectors containing the *ANGPTL2* gene. The SEVs from indicated supernatant was collected and incubated with AML cells. After culture, the AML cells were collected for transplantation. For experiment #3, ANGPTL2-SEVs collected from the culture medium of *ANGPTL2*-overexpressed 293T cells were added to the SEVs collected form experiment #2, and co-cultured with AML cells for 3 days. (C) Flow cytometry (left) and histogram (right) analysis of the percentage of YFP^+ leukemia cells in the PB of recipients injected with AML cells (collected from Figure S8B, n = 5; the data represent the means ± SD, * $p < 0.05$, ** $p < 0.01$, one-way ANOVA with Tukey's multiple comparison test). (D) Survival analysis of recipients injected with AML cells collected from Figure S8B (n = 5 ** $p < 0.01$, log-rank test). Experiments were conducted 2 times for validation.

Figure S9. The effect of ANGPTL2 on AML progression

(A) qRT-PCR analysis of *Angptl2* expression in shRNA transduced BM ECs (n = 3; the data represent the means ± SD, *** $p < 0.001$, one-way ANOVA followed by Dunnett's test). (B) WB analysis of ANGPTL2, TSG101 and CD9 protein levels in EC-SEVs after *Angptl2* shRNA knockdown. (C) Cell numbers of AML cells per well after 3 days co-culture (n = 5; the data represent the means ± SD, *** $p < 0.001$, one-way ANOVA followed by Dunnett's test). (D) Flow cytometry analysis of the percentages of co-cultured AML cells in the PB of the recipients 14-21 days after transplantation (n = 5; ** $p < 0.01$, one-way ANOVA followed by Dunnett's test). (E) Survival analysis of the recipients injected with AML cells co-cultured with the indicated ECs (n = 5; ** $p < 0.01$, log-rank test). Experiments were conducted 2 times for validation.

Figure S10. ANGPTL2-SEVs bind to LSCs through LILRB2

A. Flow cytometry analysis of ANGPTL2-SEVs binding to AML cells. 293T cells were transduced

with lentiviruses containing the ANGPTL2–mCherry fusion gene. The supernatant was collected to obtain ANGPTL2-mCherry-SEVs. ANGPTL2-mCherry-SEVs were then co-cultured with LSCs and the mCherry signal was detected by flow cytometry (n = 3; the data represent the means ± SD, *** p < 0.001, Student's t test). (B) Images of L-GMP cells after co-culture with CFSE-labelled ANGPTL2-SEVs (100 μg per 1 x 10^5 AML cells). Scale bars, 50 μm. (C-D) AML recipient spleen size (C) and weight (D) after transplantation with the indicated AML cells (n = 3; the data represent the means ± SD, * p < 0.05, ** p < 0.01, one-way ANOVA followed by Dunnett's test). (E) Western blots analysis of p-SHP2/p-CREB in WT and $Pirb^{-/-}$ AML cells with or without ANGPTL2-SEV treatment. Experiments were conducted 2 to 3 times for validation.

Figure S11. HUVEC-SEVs support human AML development

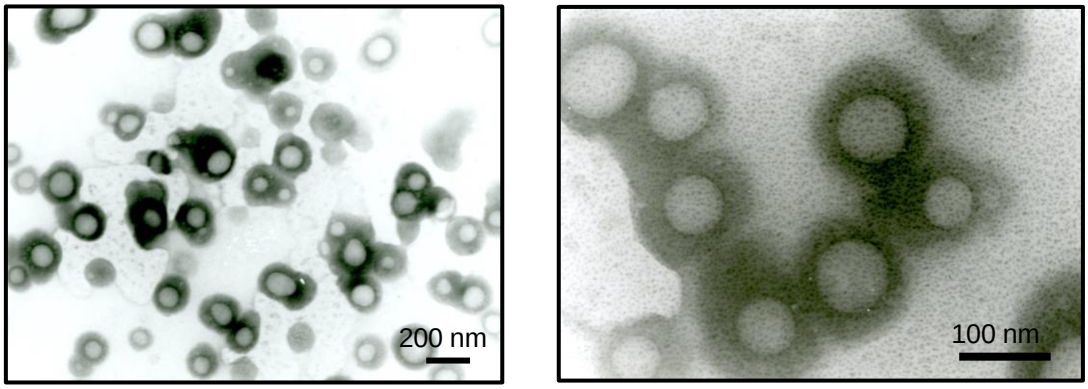
(A) Flow cytometry (left) and histogram (right) analysis of the percentage of $CD34^+$ human AML cells after co-culture with or without HUVEC-SEVs (n = 3; the data represent the means ± SD, * p < 0.05, ** p < 0.01, Student's t test). (B) Western blot analysis of ANGPTL2, TSG101, FLOT1 and VPS33B protein levels in HUVEC cells and HUVEC-SEVs. (C-D) Representative images (C) and histogram (D) analysis of the colony forming ability of human AML cells after co-culture with or without HUVEC-SEVs (n = 3; the data represent the means ± SD, * p < 0.05, ** p < 0.01, *** p < 0.001, Student's t test). (E) Flow cytometry (left) and histogram (right) analysis of the percentage of human $CD45^+$ AML cells in the PB of recipients injected with AML cells after co-culture with or without HUVEC-SEVs (n = 4-5; the data represent the means ± SD, * p < 0.05, Student's t test) mice per group. (F) Survival analysis of recipients injected with AML cells after co-cultured with or without HUVEC-SEVs (n = 5; * p < 0.05, log-rank test). Experiments were conducted 2 to 3 times for validation.

Figure S12. Expression of Vps33b, Angptl2, Angptl3 and LILRB3.

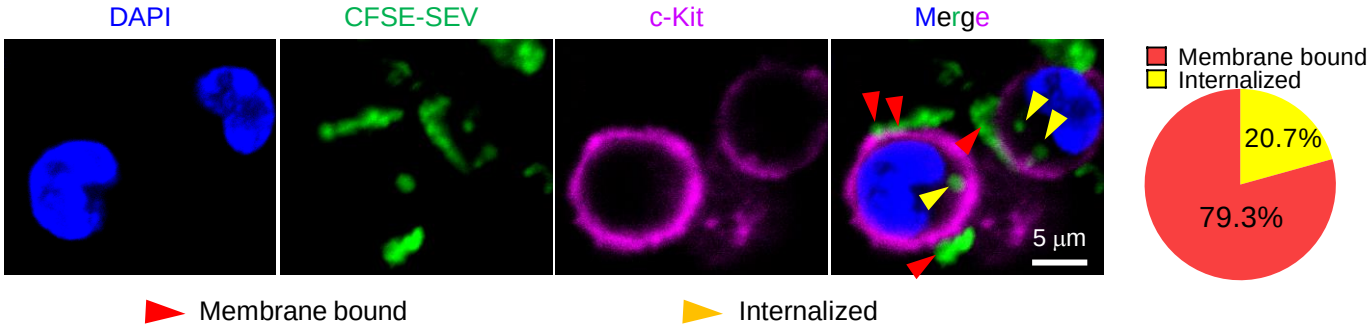
(A) Expression of *Vps33b*, *Angptl2* and *Angptl3* in BM ECs isolated from MLL-AF9-driven AML and WT mice (n = 3; the data represent the means ± SD,* p < 0.05, ** p < 0.01, Student's t test). The data represent the means ± SD, * p < 0.05, ** p < 0.01. n.d., not detected. Experiments were conducted 2 times for validation. (B) Expression of *LILRB3* in AML cells and normal hematopoietic cells. Data is obtained from Bloodspot.

Figure S1

A.



B.



C.

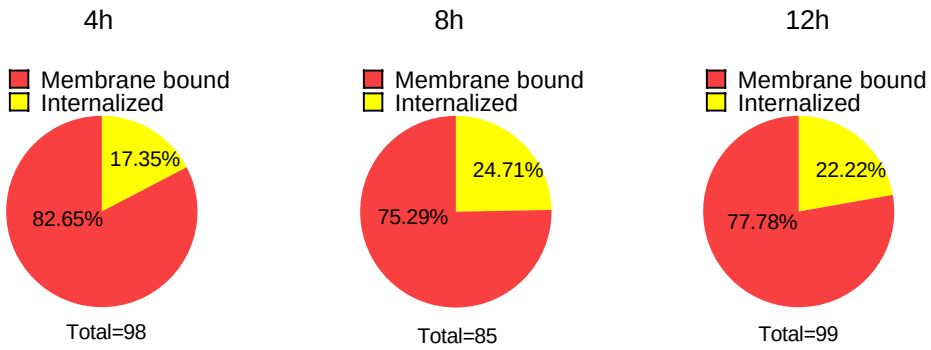


Figure S2

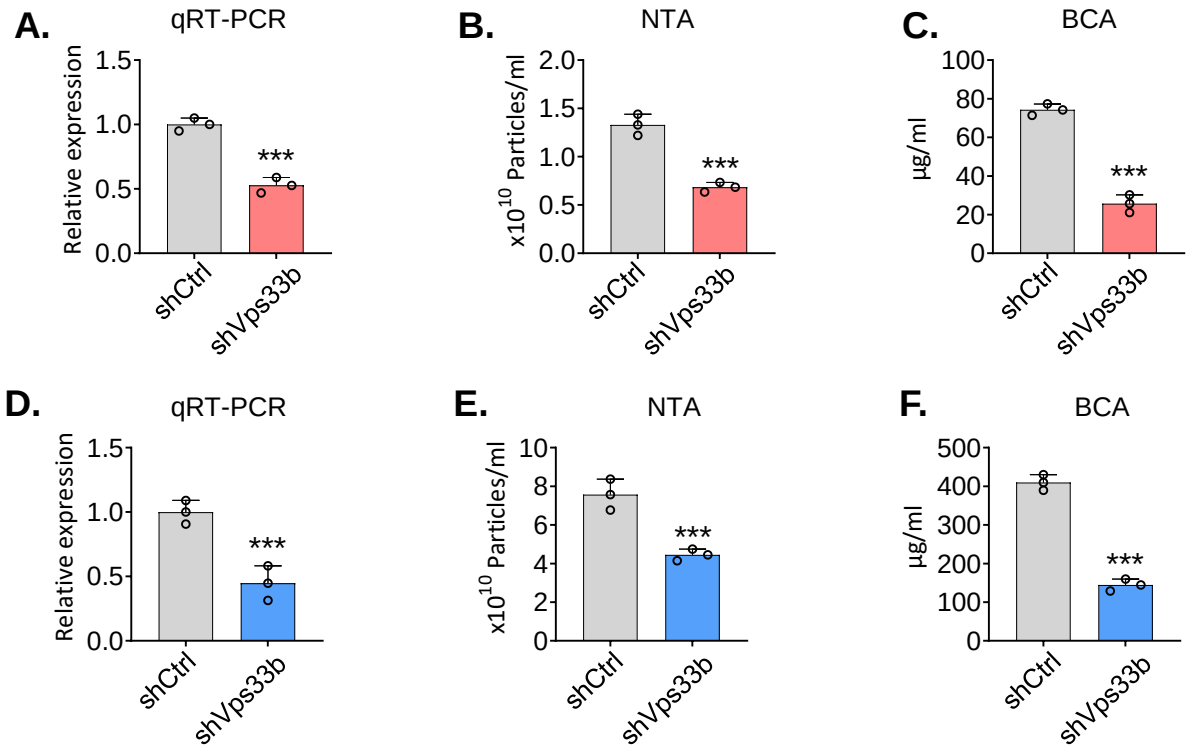

A.
qRT-PCR
Relative expression
1.5
1.0
0.5
0.0
***
shCtrl
shVps33b
B.
NTA
x10^10 Particles/ml
2.0
1.5
1.0
0.5
0.0
***
shCtrl
shVps33b
C.
BCA
µg/ml
100
80
60
40
20
0
***
shCtrl
shVps33b
D.
qRT-PCR
Relative expression
1.5
1.0
0.5
0.0
***
shCtrl
shVps33b
E.
NTA
x10^10 Particles/ml
10
8
6
4
2
0
***
shCtrl
shVps33b
F.
BCA
µg/ml
500
400
300
200
100
0
***
shCtrl
shVps33b

Figure S3

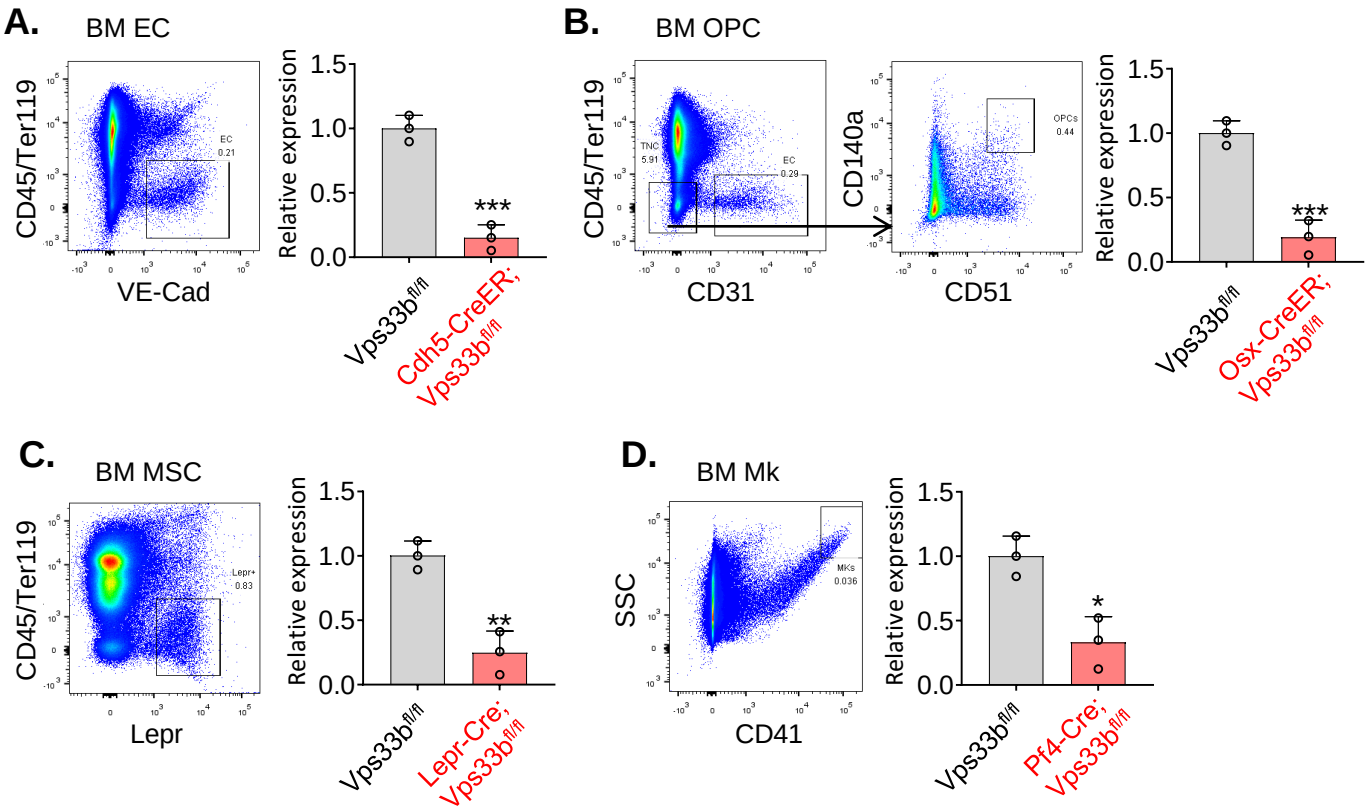

A.
BM EC
CD45/Ter119
VE-Cad
EC
0.21
Relative expression
1.5
1.0
0.5
0.0
***
Vps33b^fl/fl
Cdh5-CreER; Vps33b^fl/fl
B.
BM OPC
CD45/Ter119
CD31
TNC
5.91
EC
0.29
CD140a
CD51
OPCs
0.44
Relative expression
1.5
1.0
0.5
0.0
***
Vps33b^fl/fl
Osx-CreER; Vps33b^fl/fl
C.
BM MSC
CD45/Ter119
Lepr
Lepr+
0.83
Relative expression
1.5
1.0
0.5
0.0
**
Vps33b^fl/fl
Lepr-Cre; Vps33b^fl/fl
D.
BM Mk
SSC
CD41
MKs
0.036
Relative expression
1.5
1.0
0.5
0.0
*
Vps33b^fl/fl
Pf4-Cre; Vps33b^fl/fl

Figure S4

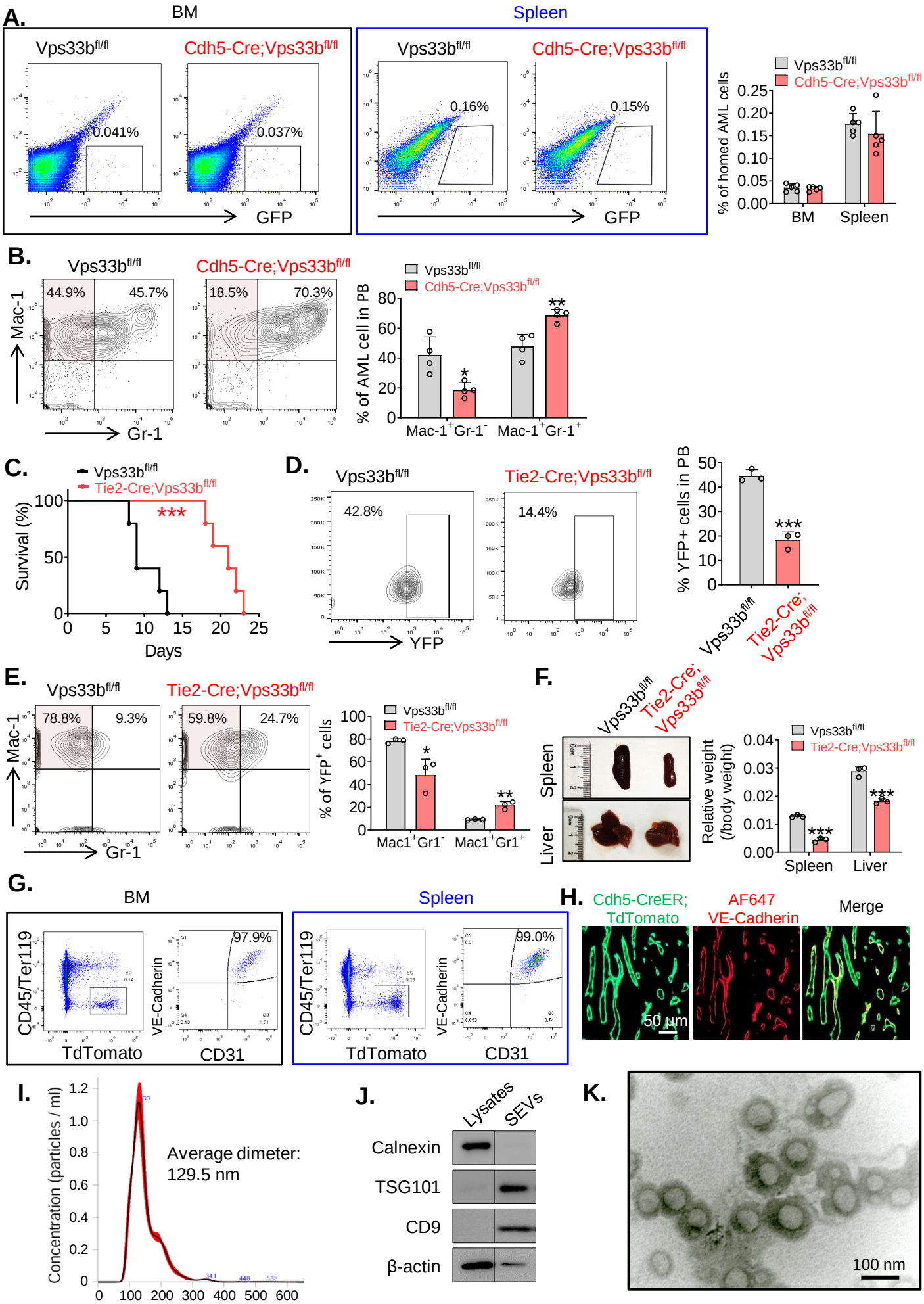

A.
BM
Spleen
Vps33b fl/fl
Cdh5-Cre;Vps33b fl/fl
0.041%
0.037%
0.16%
0.15%
GFP
% of homed AML cells
BM
Spleen
B.
44.9%
45.7%
18.5%
70.3%
Mac-1
Gr-1
% of AML cell in PB
Mac-1+Gr-1-
Mac-1+Gr-1+
C.
Tie2-Cre;Vps33b fl/fl
Survival (%)
Days
***
D.
42.8%
14.4%
YFP
% YFP+ cells in PB
E.
78.8%
9.3%
59.8%
24.7%
% of YFP+ cells
Mac1+Gr1-
Mac1+Gr1+
F.
Spleen
Liver
Relative weight (/body weight)
G.
CD45/Ter119
TdTomato
VE-Cadherin
CD31
97.9%
99.0%
H.
Cdh5-CreER; TdTomato
AF647 VE-Cadherin
Merge
50 μm
I.
Concentration (particles / ml)
Average dimeter: 129.5 nm
J.
Lysates
SEVs
Calnexin
TSG101
CD9
β-actin
K.
100 nm

Figure S5

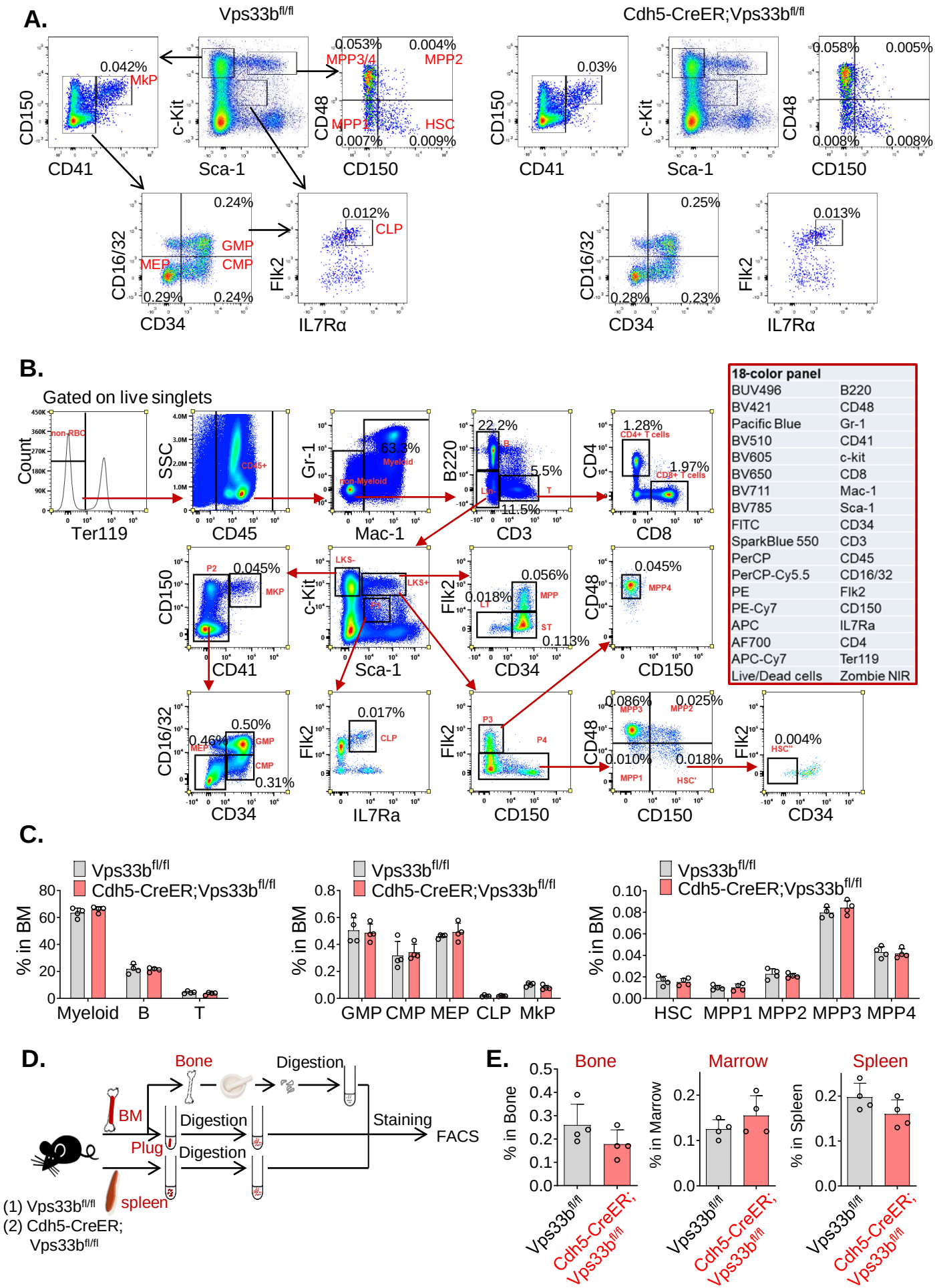
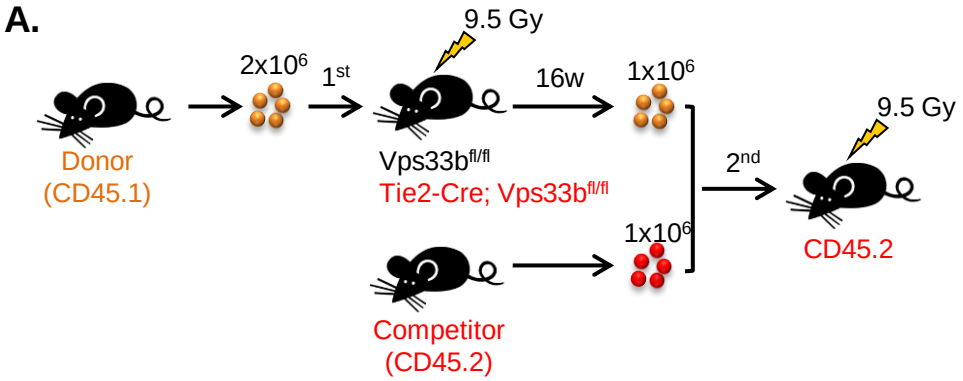
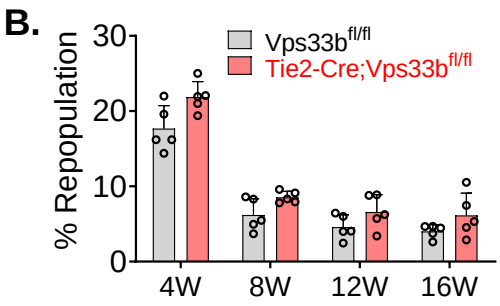
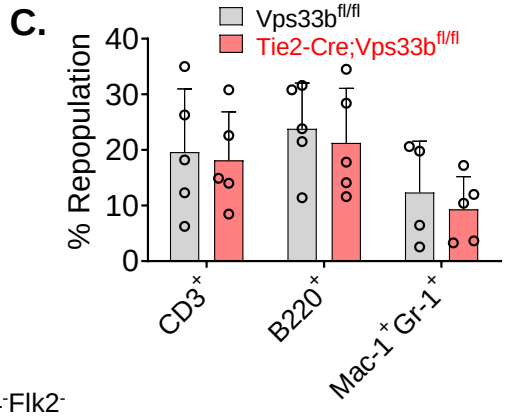

A.
Vps33b fl/fl
Cdh5-CreER;Vps33b fl/fl
0.042%
MkP
CD150
CD41
c-Kit
Sca-1
0.053%
0.004%
MPP3/4
MPP2
MPP1
HSC
0.007%
0.009%
CD48
CD150
0.03%
0.058%
0.005%
0.008%
0.008%
0.24%
GMP
MEP
CMP
0.29%
0.24%
CD16/32
CD34
0.012%
CLP
Flk2
IL7Rα
0.25%
0.28%
0.23%
0.013%
B.
Gated on live singlets
Count
non-RBC
Ter119
SSC
CD45+
CD45
Gr-1
63.3%
Myeloid
non-Myeloid
Mac-1
B220
22.2%
B
5.5%
T
11.5%
CD3
CD4
1.28%
CD4+ T cells
1.97%
CD8+ T cells
CD8
18-color panel
BUV496 B220
BV421 CD48
Pacific Blue Gr-1
BV510 CD41
BV605 c-kit
BV650 CD8
BV711 Mac-1
BV785 Sca-1
FITC CD34
SparkBlue 550 CD3
PerCP CD45
PerCP-Cy5.5 CD16/32
PE Flk2
PE-Cy7 CD150
APC IL7Ra
AF700 CD4
APC-Cy7 Ter119
Live/Dead cells Zombie NIR
P2
0.045%
MKP
CD150
CD41
LKS-
LKS+
P1
c-Kit
Sca-1
0.056%
0.018%
MPP
LT
ST
0.113%
Flk2
CD34
0.045%
MPP4
CD48
CD150
0.50%
0.46%
MEP
GMP
CMP
0.31%
CD16/32
CD34
0.017%
CLP
Flk2
IL7Ra
P3
P4
Flk2
CD150
0.086%
0.025%
MPP3
MPP2
0.010%
0.018%
MPP1
HSC'
CD48
CD150
0.004%
HSC''
Flk2
CD34
C.
Vps33b fl/fl
Cdh5-CreER;Vps33b fl/fl
% in BM
Myeloid
B
T
GMP
CMP
MEP
CLP
MkP
HSC
MPP1
MPP2
MPP3
MPP4
D.
Bone
Digestion
BM
Digestion
Staining
FACS
Plug
Digestion
spleen
(1) Vps33b fl/fl
(2) Cdh5-CreER;
Vps33b fl/fl
E.
Bone
Marrow
Spleen
% in Bone
% in Marrow
% in Spleen
Vps33b fl/fl
Cdh5-CreER;
Vps33b fl/fl

Figure S6


A.
Donor
(CD45.1)
2x10^6
1st
9.5 Gy
Vps33b^fl/fl
Tie2-Cre; Vps33b^fl/fl
16w
1x10^6
Competitor
(CD45.2)
1x10^6
2nd
9.5 Gy
CD45.2


B.
% Repopulation
Vps33b^fl/fl
Tie2-Cre;Vps33b^fl/fl
4W
8W
12W
16W


C.
% Repopulation
Vps33b^fl/fl
Tie2-Cre;Vps33b^fl/fl
CD3+
B220+
Mac-1+Gr-1+

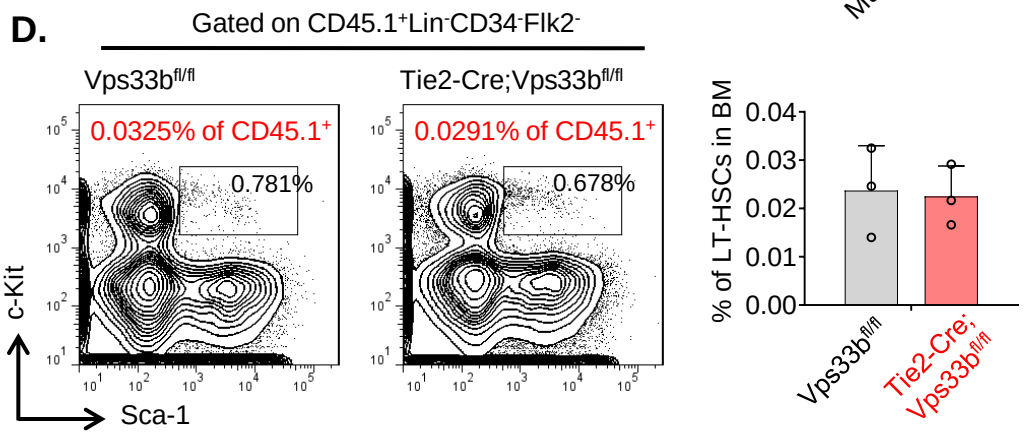

D.
Gated on CD45.1+Lin-CD34-Flk2-
Vps33b^fl/fl
Tie2-Cre;Vps33b^fl/fl
0.0325% of CD45.1+
0.781%
0.0291% of CD45.1+
0.678%
c-Kit
Sca-1
% of LT-HSCs in BM
Vps33b^fl/fl
Tie2-Cre; Vps33b^fl/fl

Figure S7

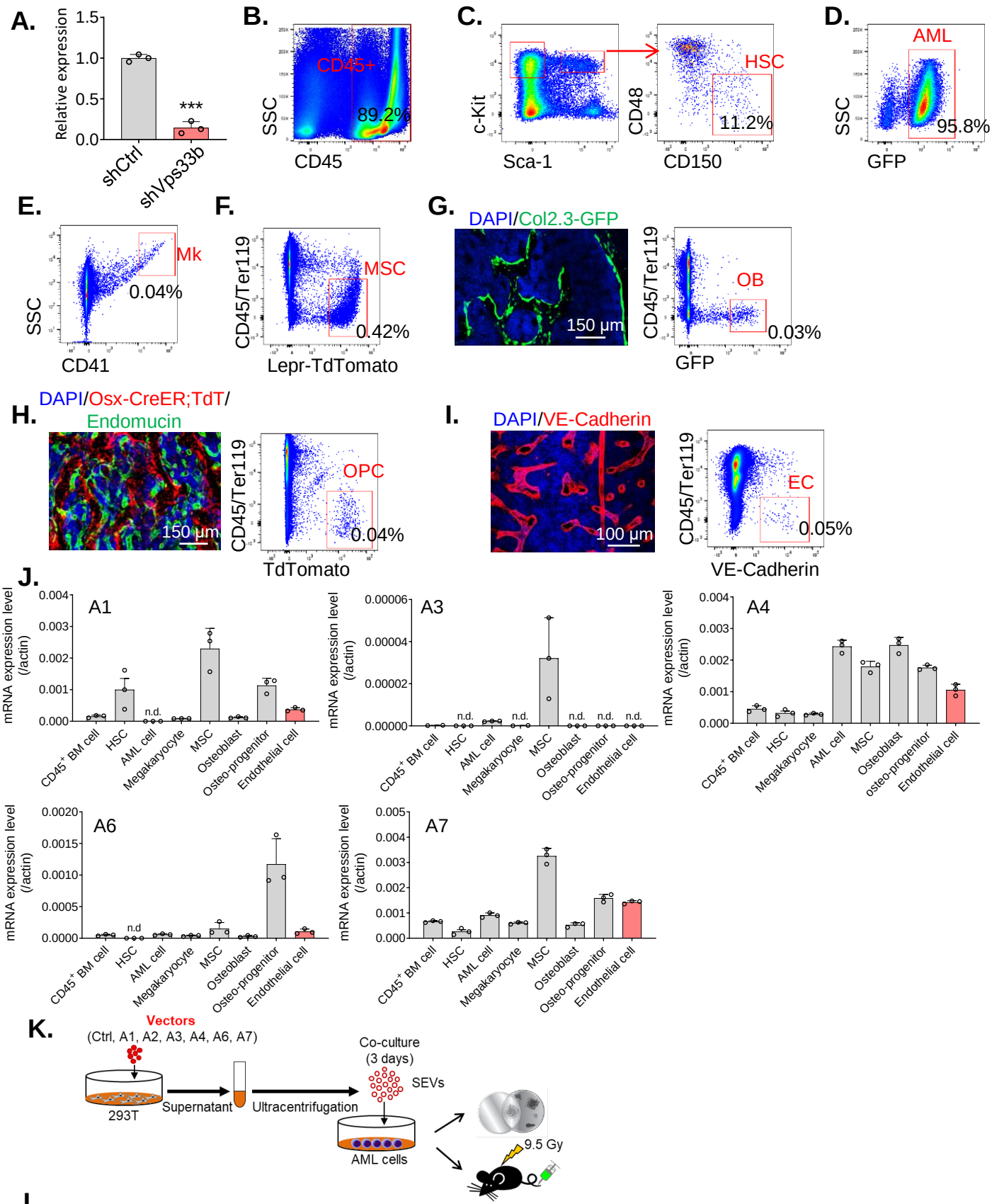

A.
Relative expression
1.5
1.0
0.5
0.0
***
shCtrl
shVps33b
B.
CD45+
89.2%
SSC
CD45
C.
c-Kit
Sca-1
CD48
CD150
HSC
11.2%
D.
AML
95.8%
SSC
GFP
E.
Mk
0.04%
SSC
CD41
F.
MSC
0.42%
CD45/Ter119
Lepr-TdTomato
G.
DAPI/Col2.3-GFP
150 μm
OB
0.03%
CD45/Ter119
GFP
H.
DAPI/Osx-CreER;TdT/
Endomucin
150 μm
OPC
0.04%
CD45/Ter119
TdTomato
I.
DAPI/VE-Cadherin
100 μm
EC
0.05%
CD45/Ter119
VE-Cadherin
J.
A1
A3
A4
A6
A7
mRNA expression level
(/actin)
n.d.
n.d
CD45+ BM cell
HSC
AML cell
Megakaryocyte
MSC
Osteoblast
Osteo-progenitor
Endothelial cell
K.
Vectors
(Ctrl, A1, A2, A3, A4, A6, A7)
293T
Supernatant
Ultracentrifugation
Co-culture
(3 days)
SEVs
AML cells
9.5 Gy
L.
A1
A2
A3
A4
A6
A7
Cells SEV
Flag
TSG101
FLOT1

Figure S8

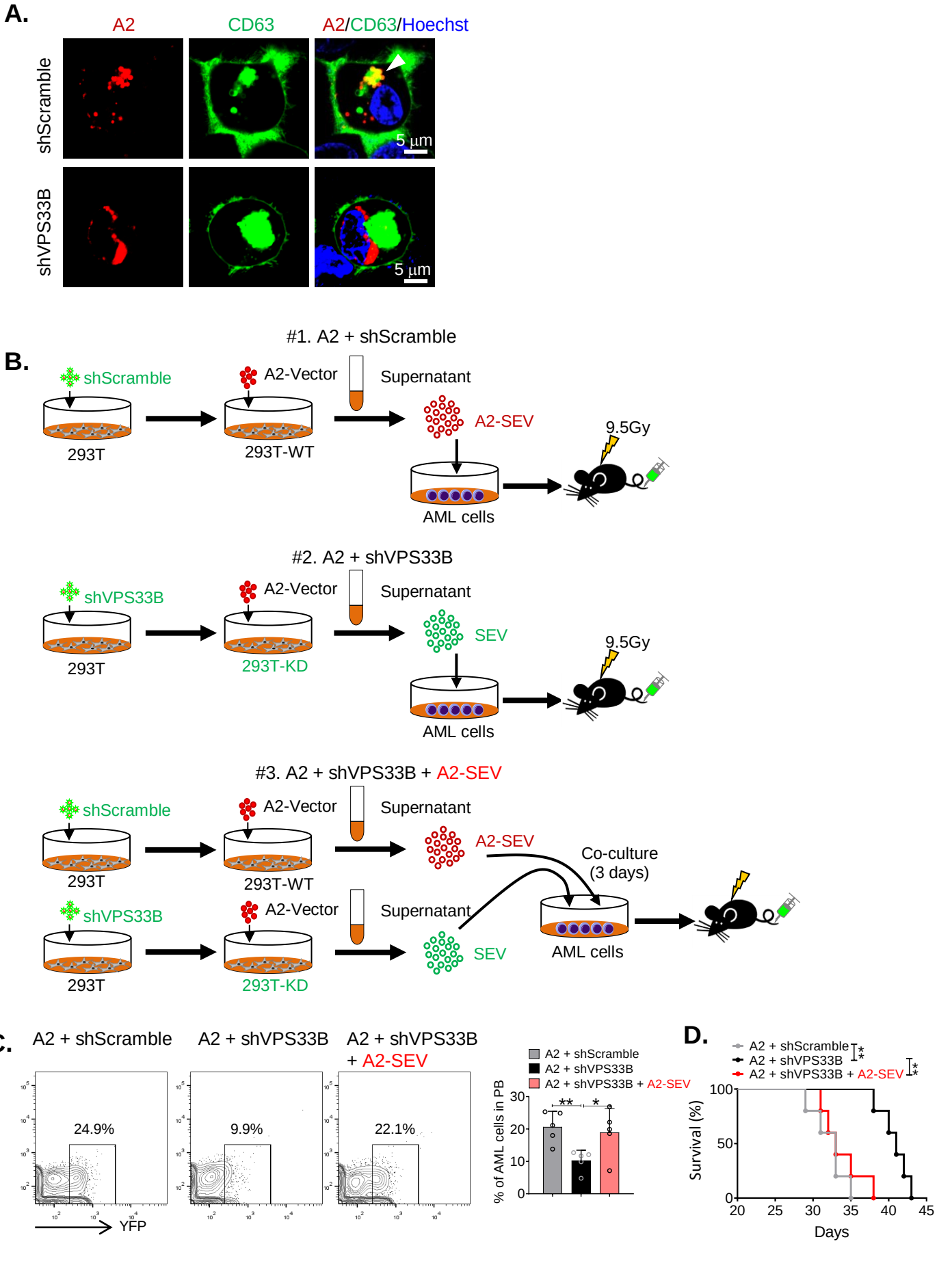

A.
A2
CD63
A2/CD63/Hoechst
shScramble
shVPS33B
5 μm
5 μm
B.
#1. A2 + shScramble
shScramble
293T
A2-Vector
293T-WT
Supernatant
A2-SEV
AML cells
9.5Gy
#2. A2 + shVPS33B
shVPS33B
293T
A2-Vector
293T-KD
Supernatant
SEV
AML cells
9.5Gy
#3. A2 + shVPS33B + A2-SEV
shScramble
293T
A2-Vector
293T-WT
Supernatant
A2-SEV
shVPS33B
293T
A2-Vector
293T-KD
Supernatant
SEV
Co-culture
(3 days)
AML cells
C.
A2 + shScramble
A2 + shVPS33B
A2 + shVPS33B
+ A2-SEV
24.9%
9.9%
22.1%
YFP
A2 + shScramble
A2 + shVPS33B
A2 + shVPS33B + A2-SEV
% of AML cells in PB
30
20
10
0
**
*
D.
A2 + shScramble
A2 + shVPS33B
A2 + shVPS33B + A2-SEV
Survival (%)
100
50
0
20
25
30
35
40
45
Days

Figure S9

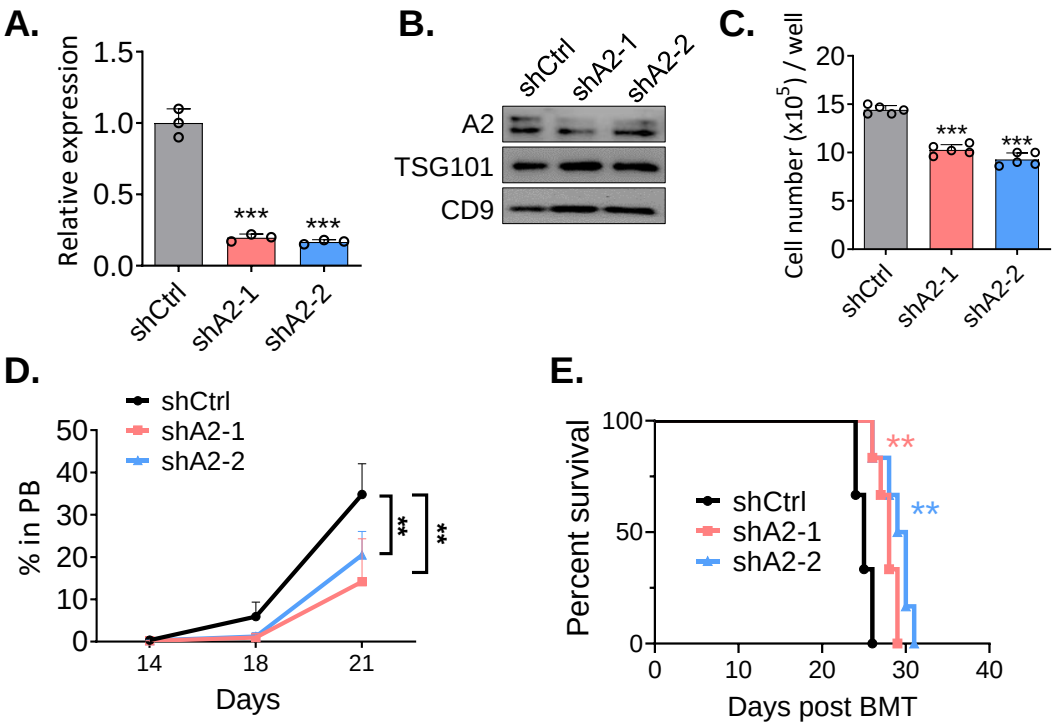

A.
Relative expression
1.5
1.0
0.5
0.0
***
***
shCtrl
shA2-1
shA2-2
B.
shCtrl
shA2-1
shA2-2
A2
TSG101
CD9
C.
Cell number (x10^5) / well
20
15
10
5
0
***
***
shCtrl
shA2-1
shA2-2
D.
shCtrl
shA2-1
shA2-2
% in PB
50
40
30
20
10
0
**
**
14
18
21
Days
E.
Percent survival
100
50
0
shCtrl
shA2-1
shA2-2
**
**
0
10
20
30
40
Days post BMT

Figure S10

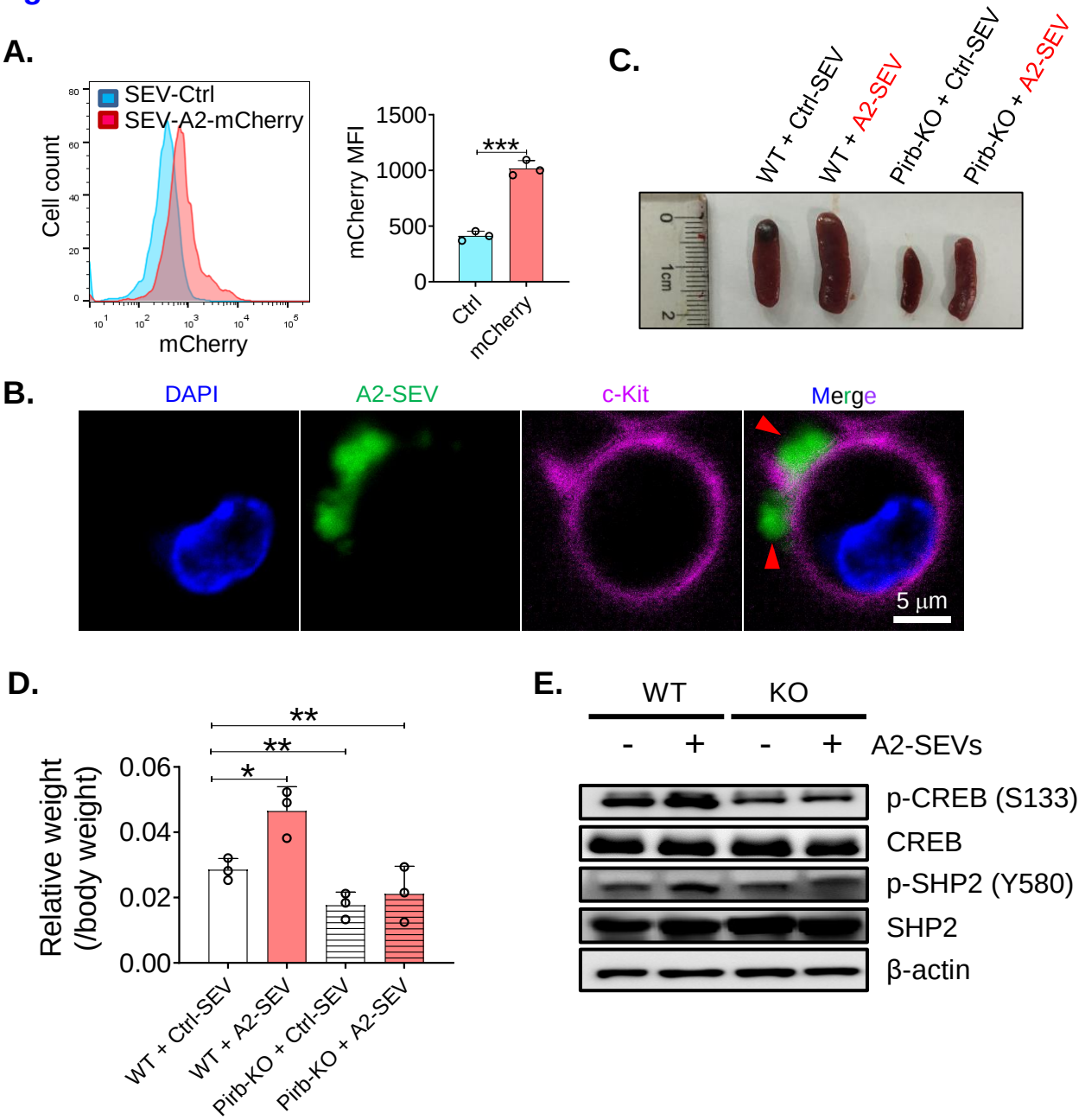

A.
SEV-Ctrl
SEV-A2-mCherry
Cell count
mCherry
mCherry MFI
***
Ctrl
mCherry
C.
WT + Ctrl-SEV
WT + A2-SEV
Pirb-KO + Ctrl-SEV
Pirb-KO + A2-SEV
B.
DAPI
A2-SEV
c-Kit
Merge
5 μm
D.
Relative weight (/body weight)
WT + Ctrl-SEV
WT + A2-SEV
Pirb-KO + Ctrl-SEV
Pirb-KO + A2-SEV
E.
WT
KO
A2-SEVs
p-CREB (S133)
CREB
p-SHP2 (Y580)
SHP2
β-actin

Figure S11

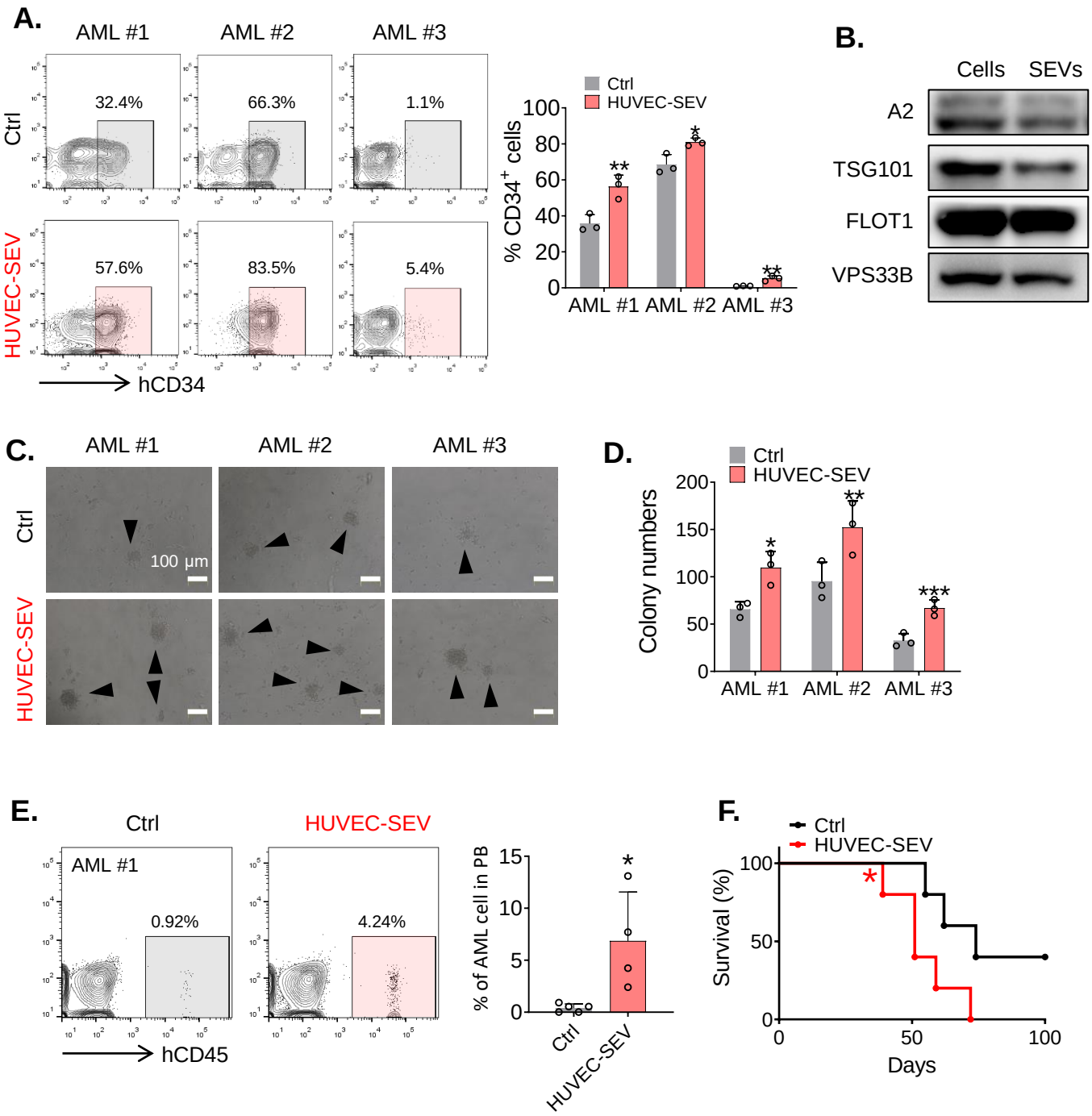
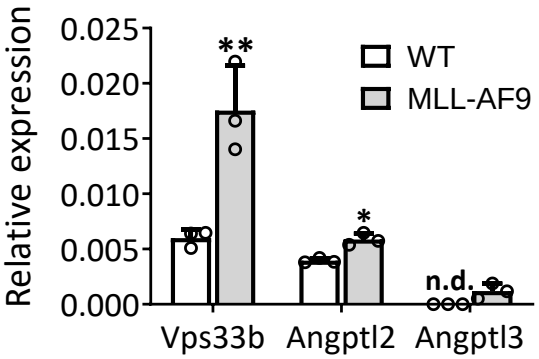

A.
AML #1
AML #2
AML #3
Ctrl
HUVEC-SEV
32.4%
66.3%
1.1%
57.6%
83.5%
5.4%
hCD34
Ctrl
HUVEC-SEV
% CD34+ cells
AML #1 AML #2 AML #3
B.
Cells
SEVs
A2
TSG101
FLOT1
VPS33B
C.
AML #1
AML #2
AML #3
Ctrl
HUVEC-SEV
100 μm
D.
Ctrl
HUVEC-SEV
Colony numbers
AML #1 AML #2 AML #3
E.
Ctrl
HUVEC-SEV
AML #1
0.92%
4.24%
hCD45
% of AML cell in PB
Ctrl
HUVEC-SEV
F.
Ctrl
HUVEC-SEV
Survival (%)
Days

Figure S12

A.



B.