SUPPLEMENTARY APPENDIX

Role of the clathrin adaptor PICALM in normal hematopoiesis and polycythemia vera pathophysiology

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Supporting Online Material for

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Methods

Mice

Gene-targeting strategy for *Picalm* mutant strains is described in Supplemental Figure 1b. The targeting vector was generated using a pKOII vector (a kind gift from Dr. DePinho). Both long and short arms of the targeting construct were PCR-amplified using a BAC clone (RP23-204F5, Invitrogen) as a template. A *loxp* sequence, in conjunction with an artificial NcoI site for Southern blot analysis, was introduced into the FseI site in the intron between *Picalm* exons 1 and 2. Targeted mutation of *Picalm* in non-agouti black Bruce4 C57BL/6-Thy1.1 mouse embryonic stem cells (mESCs) was performed with standard methodology. The linearized *Picalm* gene-targeting vector was electroporated into Bruce4 mESCs, and cells were selected for Neomycin and Gancyclovir resistance. A total of 168 clones were isolated, and genotyping identified two homologous recombinant clones. Those clones were expanded, and karyotyping revealed that both clonal cell lines were > 90% euploid. *Picalm* mutant mESCs were microinjected into embryos from the MB6 albino strain of mice [1] and transferred surgically into pseudopregnant recipient females. *Picalm* mutant chimeras were crossed with albino B6 (Cg)-Tyrc-2J/J mice to demonstrate transmission of the mESC-derived genome through the germline. Chimeras produced with one of the *Picalm* mutant mESC lines (#144) produced pigmented F1 offspring carrying the expected *Picalm* mutation ($Picalm^{Neo/+}$). $Picalm^{Neo/+}$ mice were bred with the *FLP*-deleter (stock# 003800, Jackson laboratory) or *EIIa-Cre* (stock# 003724, Jackson laboratory) strain to generate *Picalm* conditional- ($Picalm^{F/+}$) or conventional- ($Picalm^{+/-}$) knockout strains, respectively. We then bred $Picalm^{F/+}$ mice with *Mx1-Cre* transgenic mice (stock# 003556, Jackson

Laboratory) to generate *Picalm*$^{F/+}$*Mx1Cre*+ double transgenic mice. *Picalm*$^{F/+}$*Mx1Cre*+ mice were further inbred with *Picalm*$^{F/+}$ littermates, and offspring were used for experiments. *Picalm*$^{F/F}$ *ERT2-Cre*+ mice were established by crossing *Picalm*$^{F/F}$ mice with *ROSA26*$^{Cre-ERT2}$ mice [2]. Cre recombinase was induced in vivo via intraperitoneal injections of Tamoxifen (75mg/kg/day for 5 consecutive days). The mouse PV model (*Jak2*$^{+/V617F}$ *Vav-Cre*+) is described elsewhere [3,4]. Genotyping of mutant mice was performed by PCR of tail DNA with the following primers; For *Picalm* conditional- and conventional-knockout mice, PicalmFW CATAAGCTAAGATTTCCCCTGTCACA, PicalmFloxFW GTGTGGGATGAATGGTTGGGC, and PicalmRV CCACCATGTAGGGTCTAAAG. For the *Cre* transgene, OIMR1084CreFW-GCGGTCTGGCAGTAAAAACTATC and OIMR1085CreReV-GTGAAACAGCATTGCTGTCACT (Jackson Laboratory).

Fluorescence-Activated Cell Sorting (FACS)

Fetal liver (FL) and bone marrow (BM) cells were incubated with fluorochrome-conjugated (or biotin-conjugated) antibodies. FACS analysis was performed on a LSR II or FACS Canto II flow cytometer (BD), followed by analysis with FlowJo software (Tree Star). Cell sorting was performed on a MoFlo or AriaIII cytometer using DAPI for live/ dead discrimination. Antibodies for FACS were purchased from eBioscience, unless otherwise indicated. For Annexin V staining, FL and spleen cells were suspended in 1 x Annexin Binding Buffer (BD) containing 2% fetal bovine serum and stained with FITC-anti-CD71, PE-anti-Ter119 antibodies, and APC-Annexin V (BD) following the manufacturer's protocol. Cleaved PARP staining was performed as described [5].

Antibodies for FACS

All antibodies were purchased from eBioscience, BD or Biolegend. Fluorochrome-conjugated antibodies included: TER119 (TER-119), CD44 (IM7), CD71 (R17217), CD11b (M1/70), CD41 (eBioMWReg30), CD150 (TC15-12F12.2), B220 (RA3-6B2), Gr-1 (RB6-8C5), CD25 (PC61.5), CD8a (53-6.7), c-kit/CD117 (2B8), CD16/32 (93), CD25 (PC61.5), CD19 (eBio1D3), Sca-1 (E13-161.7), CD4 (RM4-5). The following biotin-conjugated antibodies were used for lineage depletion in erythroid differentiation assay: Biotin-CD3 (145-2C11), Biotin-CD4 (L3T4), Biotin-CD8 (eBio-H35-17.2), Biotin-B220 (RA3-6B2), Biotin-CD19 (eBio1D3), Biotin-Gr1 (RB6-8C5), Biotin-CD11b (M1/70) and Biotin-TER119 (TER-119).

Peripheral blood (PB) analysis

Mice were anesthetized with isoflurane and peripheral blood samples collected from the retro-orbital sinus with heparinized capillary tubes (Fisher Scientific). Blood counts were performed using a HEMAVET 950FS (Drew Scientific) or the ADVIA 120 System (Siemence). For FACS analysis, 30-50 µl of PB was stained with antibodies, washed once with PBS and fixed with FACS Lysing Solution (BD) to eliminate red blood cells.

Bone marrow transplantation

A congenic strain that carries the CD45.1 antigen (B6.SJL-PtprcaPepcb/BoyJ, Jackson Laboratory) served as recipients. One million bone marrow mononuclear cells (BMMNCs) from either control (*Picalm*$^{F/F}$) or knockout (*Picalm*$^{F/F}$ *Mx1Cre*+) mice were

transferred to a lethally-irradiated recipient mouse along with CD45.1^{+} 2 x 10^{5} BMMNCs (for radioprotection). Recipients were injected with pIpC (250μg x 3) one month later and their peripheral blood counts analyzed by a hematology analyzer 1, 3 and 12 months after injection.

Phenylhydrazine treatment and collection of splenic erythroblasts

Phenylhydrazine (PHZ) was purchased from Sigma and injected subcutaneously three times at a dose of 40 mg/kg, as previously described [6]. For erythroblast collection, spleens were isolated 4 days after PHZ treatment. After preparing single-cell suspensions using a cell strainer (BD), cells were incubated with red cell lysis buffer (155mM ammonium chloride, 10mM potassium bicarbonate and 0.1mM EDTA) and then incubated with anti-CD71 biotin conjugated antibody, followed by incubation with anti-Biotin MicroBeads (Miltenyi Biotec). Cell suspensions were subsequently applied onto MACS separation LS columns (Miltenyi Biotec) for positive selection.

Immunohistochemical analysis

14.5 d.p.c. embryos were fixed in phosphate-buffered 10% formalin solution (Fisher Diagnostics) and embedded in paraffin. Picalm staining was performed using anti-Picalm antibody (C-18, Santa Cruz Biotechnology) with standard methodology.

FACS-based transferrin/TfR endocytosis assay

To measure the amount of internalized transferrin in splenic erythroblasts, splenocytes were first incubated with Alexa647-conjugated transferrin (10 μg/ml, Life Technologies)

for 8 min at 37°C. Cells were then washed twice with cold acid buffer (200 mM glycine, 150 mM NaCl, pH 3.0) to remove surface-bound transferrin, followed by a PBS wash. Cells were then stained with fluorophore-conjugated antibodies against lineage-specific markers (TER119, CD71, CD19, CD11b) and subjected to FACS analysis. To measure surface-bound transferrin, splenocytes were incubated with Alexa647-conjugated transferrin (10 μg/ml) for 8 min at 4°C and washed with a neutral pH buffer (PBS, 20mM HEPES, pH 7.4), followed by surface marker staining as described. As a background control, cells were incubated with Alexa647-conjugated transferrin (10 μg/ml) for 8 min at 4°C, followed by washes with acid buffer twice. Control samples were also stained with surface markers and subjected to FACS analysis. Endocytosis assays in K562 cells were performed in the same manner without surface marker staining.

Western blot analysis

Western blot analysis was performed using standard methodology with the following antibodies: Picalm (C-18 and S-19, Santa Cruz Biotechnology), βactin (AC-74, Sigma), HSP90 (clone: 68, BD), AP180 (LP2D11, Fisher Scientific), Eps15 (K-15, Santa Cruz), TfR (H68.4, Life Technologies), H-ferritin (H-53, Santa Cruz Biotechnology), JAK2 (D2E12, Cell Signaling), pSTAT5 (C2E12, Cell Signaling), STAT5 (C-17, Santa Cruz Biotechnology) and αTubulin (DM1A, Sigma).

Wright-Giemsa and benzidine stain

FACS-sorted erythroblasts (10,000) were cyto-spun onto glass slides and prepared for benzidine and/or Wright-Giemsa staining using standard protocols. After fixation with

methanol, cells were stained with 3,3'-diaminobenzidine tetrahydrochloride (Sigma). Wright-Giemsa staining was performed using PROTOCOL Wright-Giemsa Stain Solutions (Fisher Scientific) following the manufacturer's specifications.

Enzyme-linked immunosorbent assay (ELISA) and serum iron measurement

Mouse serum samples were collected 2 months after pIpC injections. Levels of ferritin, transferrin and erythropoietin were measured by ELISA following the manufacturer's instruction. The following kits were used: Mouse Ferritin ELISA kit and Mouse Transferrin ELISA kit (both from Kamiya Biomedical Campany), and Mouse Erythropoietin Quantikine ELISA Kit (MEP00B, R&D). Serum iron levels were measured using a QuantiChrom™ Iron assay Kit (BioAssay System). Total iron binding capacity (TIBC) was calculated as described[7].

Plasmids

Lentivirus vectors expressing shRNA-against human PICALM (pLKO.1-shRNA vectors) and mouse Ap180 (Snap91) were purchased from Sigma. Clone TRCN0000218840, which targets the human *PICALM* 3'UTR, was used for stable *PICALM* knockdown in K562 cells. Five shRNA clones were tested for Ap180 knockdown: TRCN0000113155 (clone #1), TRCN0000113156 (#2), TRCN0000113157 (#3), TRCN0000113158 (#4) and TRCN0000113159 (#5). The pMX-mCherry retrovirus vector was a kind gift from Dr. Davide Robbiani. Human *PICALM* cDNAs (both long- and short-forms) were PCR-amplified using K562 cell cDNA as template and sub-cloned into pMX-mCherry vectors. A series of mutated PICALM expression vectors (see Supplemental Figure 4) was

constructed using a QuikChange Mutagenesis Kit (Agilent Technologies). To make PH-PICALM mutant, the ANTH domain of PICALM was replaced with PCR-amplified PH domain of rat phosphlipase C delta 1 [8].

In vitro erythroid differentiation

In vitro erythroid differentiation was performed as previously described with minor modifications [9,10]. After red cell lysis, bone marrow cells were incubated with a cocktail of biotin-conjugated antibodies against lineage markers (CD19, B220, CD11b, Gr1, CD4, CD8 and TER119) for 15 min on ice. Cells were then washed with PBS and incubated with streptavidin-MicroBeads (Miltenyi Biotec), followed by lineage-depletion with a MACS separation column (Miltenyi Biotec). The resultant lineage-negative progenitors were incubated in 12-well plates ($1x10^5$ cells per well) with 1ml of erythroid differentiation medium [Iscove modified Dulbecco medium (IMDM) containing 15% FBS, 1% bovine serum albumin, holo-transferrin (500 μg/ml, Sigma), recombinant human insulin (10 μg/ml, Sigma), L-glutamine (2 mM) and Epo (2 U/mL, Amgen)]. Colony forming assay was performed as previously described[6].

Real time PCR assay

RNA was extracted using TRIZOL (Life Technologies) and treated with DNase I (Life Technologies). cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (ABI) and Real-time PCR performed using Fast EvaGreen qPCR Master Mix (Biotium) and a ABI7900HT (ABI). Relative mRNA expression levels were

calculated relative to corresponding Hprt levels in each sample. Primer sequences for qRT-PCR were:

Hprt: FW 5'-CACAGGACTAGAACACCTGC-3', Hprt: RV 5'-GCTGGTGAAAAGGACCTCT-3', Ap180 FW: 5'-GCCGAGCAAGTTGGTATTGAT-3', Ap180 RV: 5'-GTTCAAGGGTCTCCATAAGAC TG-3'. Following primer sequences were obtained from PrimerBank (http://pga.mgh.harvard.edu/primerbank/). Bcl-xl FW: 5'-GACAAGGAGATGCAGGTATTGG-3' (PrimerBank ID 31981887a1), Bcl-xl RV: 5'-TCCCGTAGAGATCCACAAAAGT-3'.

Freeze-etch EM

Spleens were harvested 2 months after pIpC injections. Control mice (*Picalm*$^{F/F}$) were treated with PHZ to enrich erythroblasts in spleen. Splenic erythroblasts were enriched via depleting myeloid and lymphoid marker-positive cells using magnetic beads. Freezing, replicating and imaging samples were performed as described previously[11-13].

Lentivirus transduction

The pLKO.1-shRNA lentivirus vector was co-transfected with the packaging plasmid psPAX2 (Plasmid #12260, Addgene) and an envelope plasmid pMD2.G (Plasmid #12259, Addgene) into 293T cells using Lipofectamine 2000 (Life Technologies), and virus supernatant was collected 72hr after transfection. For infection, 5x10^5 K562 cells were spin-infected with 2 ml viral supernatant containing polybrene (5 μg/ml, Sigma) for 2 hours at 32°C. To establish K562 cells stably expressing *PICALM*-shRNA, transduced cells were maintained in medium containing puromycin.

Retrovirus transduction

For PICALM add-back experiments in *PICALM*-knockdown K562 cells, retrovirus encoding a WT or mutant PICALM protein was produced using Phoenix amphotrophic retrovirus packaging cells (http://www.stanford.edu/group/nolan/retroviral_systems/phx.html), as described [14]. For retrovirus transduction of primary mouse BM progenitor cells, 293T cells were transfected with a retroviral vector and a pMCV-Ecopac vector using Lipofectamine 2000. 2×10^5 lineage-depleted BM cells were spin-infected with 1 ml viral supernatant containing polybrene (5 μg/mL). After infection, cells were cultured in maintenance medium [StemSpan™ Serum-Free Expansion Medium (Stem Cell Technologies) containing stem cell factor (100 ng/ml), recombinant insulin like growth factor-I (40 ng/ml, Peprotech), dexamethasone (100nM, Sigma) and Epo (2 U/ml)] for 24hr, and then medium was replaced with erythroid differentiation medium.

Inducible knockout of *Picalm* and ShRNA-mediated *Ap180* knockdown in MEFs

Picalm$^{F/F}$ *ERT2-Cre*+ MEFs, stably transfected with pMSCV-APσ2-EGFP with large-T antigen, were cultured in D-MEM (Invitrogen #11995) supplemented with 10% FBS at 37°C / 5% CO2. *Picalm* deletion was induced upon treatment of the cells with 1uM 4OH-Tamoxifen (Sigma #H7904) in the same medium for 24 hours at 37°C / 5% CO2. The cells were then incubated for additional 48 hours with fresh medium. Control cells were treated similarly with 0.1% DMSO in complete medium as vehicle control. ShRNA-mediated Ap180 knockdown of the MEFs was done in cells grown in 6-well dishes; cells were plated and grown overnight to 60% confluent and then incubated for 15min with 2

ml of medium supplemented with 8ug/ml polybrene followed by addition of 300ul of viral supernatant (shRNA against Ap180 or scrambled shRNA control). Following 24 hr incubation, fresh medium was replaced and the cells grew for an additional 3 days before analysis.

Spinning-disk confocal fluorescence microscopy

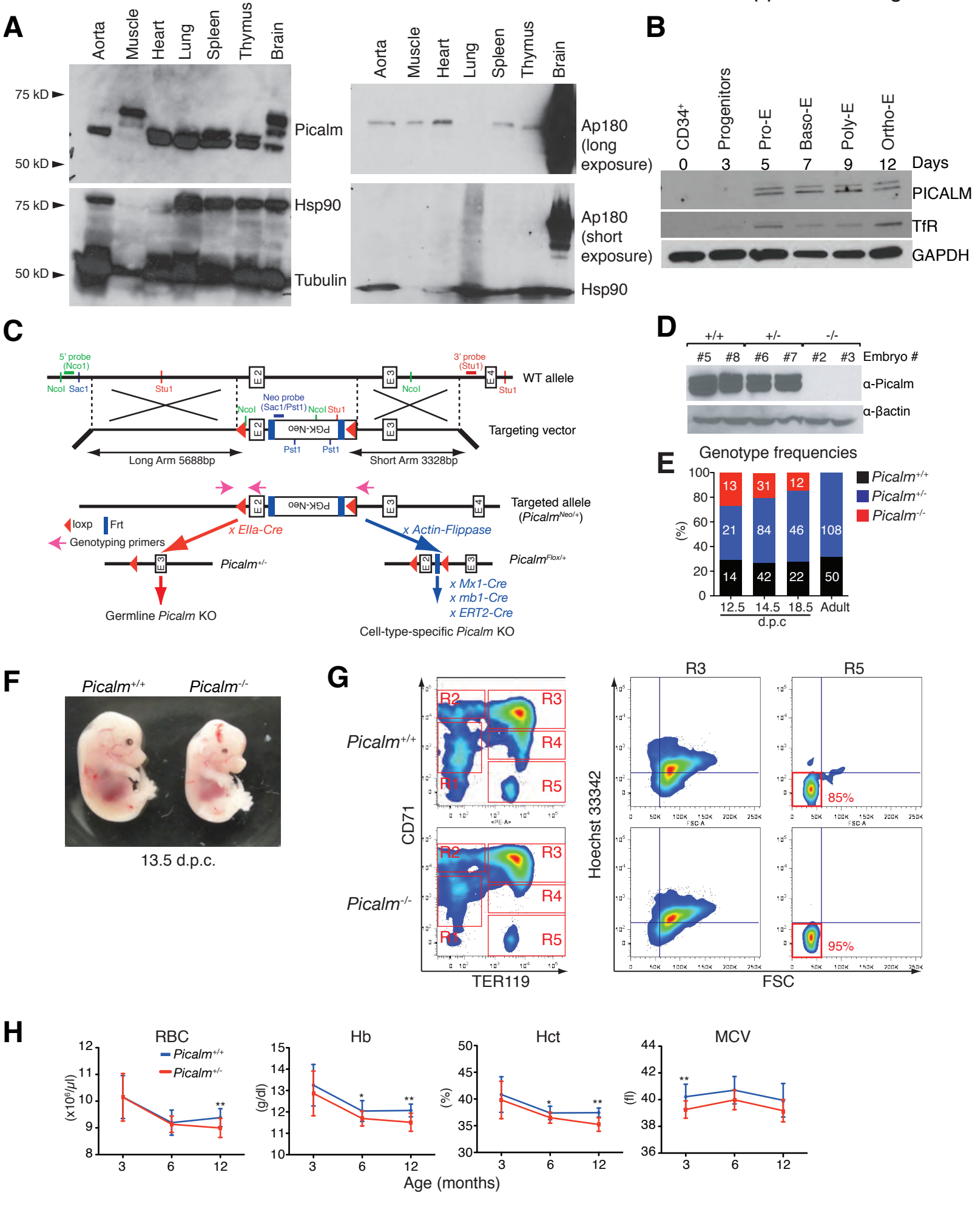
Glass 25mm round #1.5 cover slips (Warner Instruments #W2 64-0715) were sonicated in 70% ethanol for 30 minutes and dried at 100°C and then washed 3 times with sterile water before cell plating. Cells were plated at 30% confluency and imaged in MEM-alpha with no phenol red (Invitrogen #41061037) supplemented with 5%FBS and 20mM HEPES, at 37°C / 5% CO2 5-6 hrs after plating. Imaging from the bottom surface of the cells attached to the cover slip was performed using a fully enclosed, environmentally temperature controlled Zeiss 200M (Carl Zeiss Microimaging, Inc., Thornwood, NY) equipped with a cooled electron multiplication CCD camera (512 by 512 pixels, QuantEM (Photometics, Tuscon, AZ), a CSU-22 spinning-disk confocal head (Yokogawa Electric, Tokyo, Japan) modified with a Borealis high-photon throughput upgrade (Spectral Applied Research, Ontario Canada). The glass coverslips were inserted into an Attofluor Cell Chamber (Invitrogen, Carlsbad, CA) placed in a modified heated stage insert (20/20 Technology, Wilmington, NC) mounted on a computer-controlled piezo Z stage and a linear encoded X&Y platform (Applied Scientific Instruments, Eugene, OR). Images were acquired using a 100x, 1.4NA oil objective (Carl Zeiss Microimaging, Inc., Thornwood, NY), with an additional 1.5x magnification lens placed in front of the camera to create a resolution of 0.11µm / pixel. The control software was SlideBook V5.0

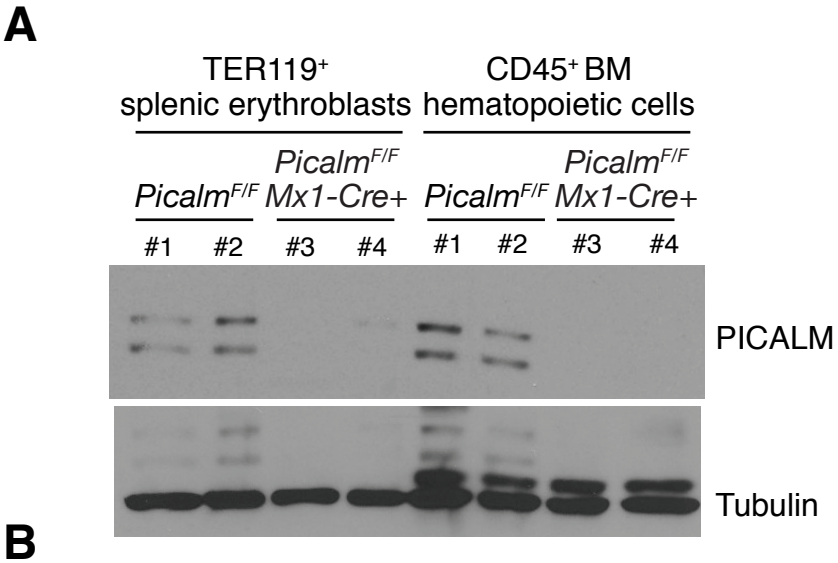
(Intelligent Imaging Inc., Denver, CO) operating on a Windows 7 computer with 12GB of RAM (Dell Corp. Round Rock, TX). The excitation was based on a solid-state laser operating at 491 nm (Cobolt, Solna, Sweden) and the images captured with a 525/50 emission filter (Semrock, Rochester, NY).

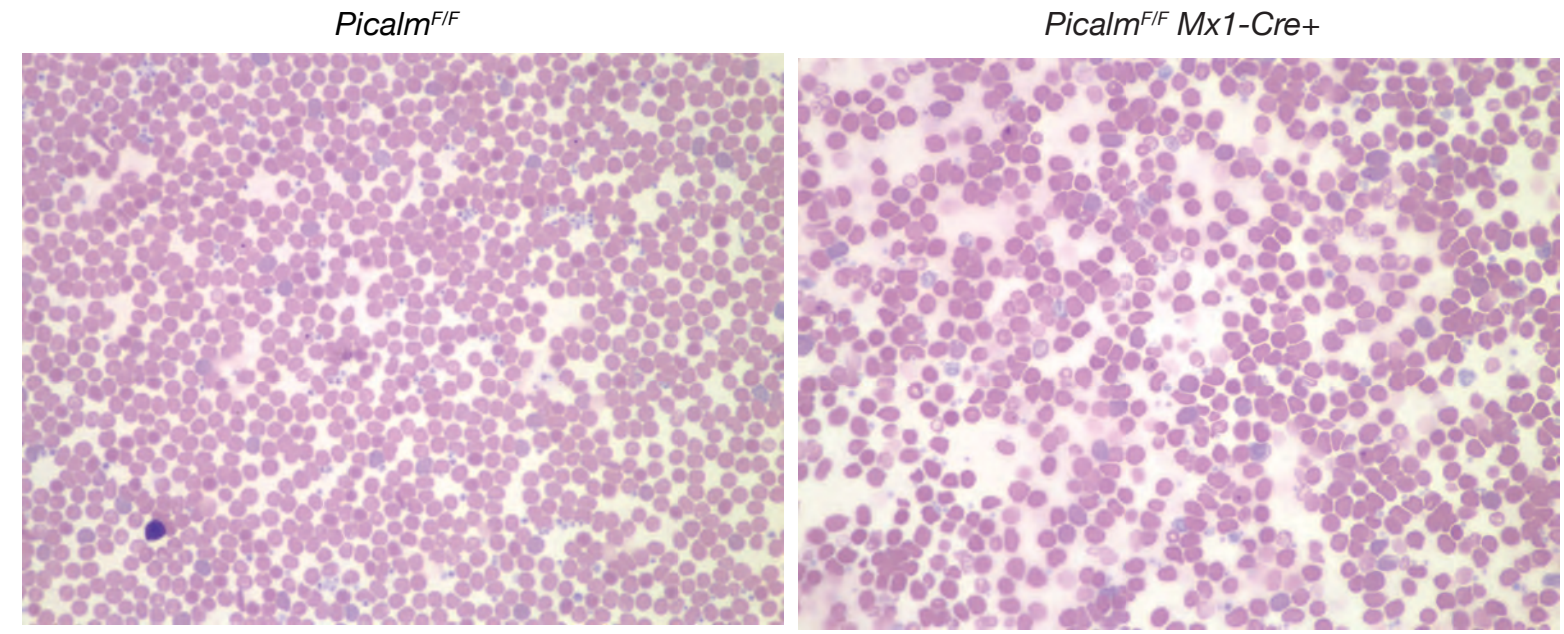
Live-cell imaging and data analysis

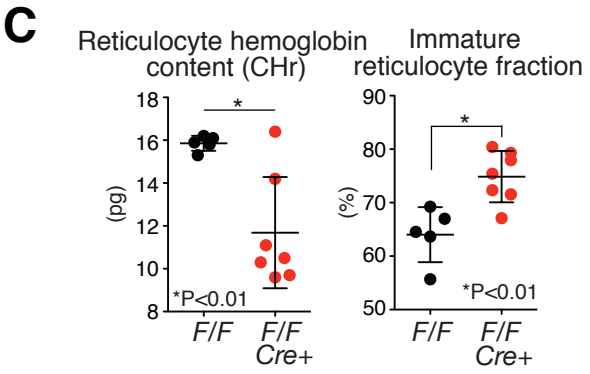
2D time-series were acquired by spinning-disk confocal microscopy using 30 ms exposures per frame every 3 s for 5 min from the attached surfaces of *Picalm*$^{F/F}$ *ERT2-Cre*+ MEFs cells stably expressing AP2 (σ2-EGFP). This duration was sufficient to fully capture the dynamics of coated pits typically lasting 40-120 s while at the same time minimized potential photobleaching and phototoxicity effects. AP2 spots were identified and tracked using the computational framework for Matlab (MathWorks, Natick, MA, USA) as described [15]. Briefly, this framework provides algorithms for the identification of bona fide coated pit trajectories among all fluorescence signals in the data according to the following criteria: the fluorescence signals were diffraction-limited, appeared and disappeared within the time window of the acquisition, and did not collide or intersect with other signals. The resulting trajectories were used to estimate coated pit lifetime distributions, average intensities, and densities of events, as previously described [15]. The density of persistent structures was calculated from trajectories that were present for the full duration of the acquisition.

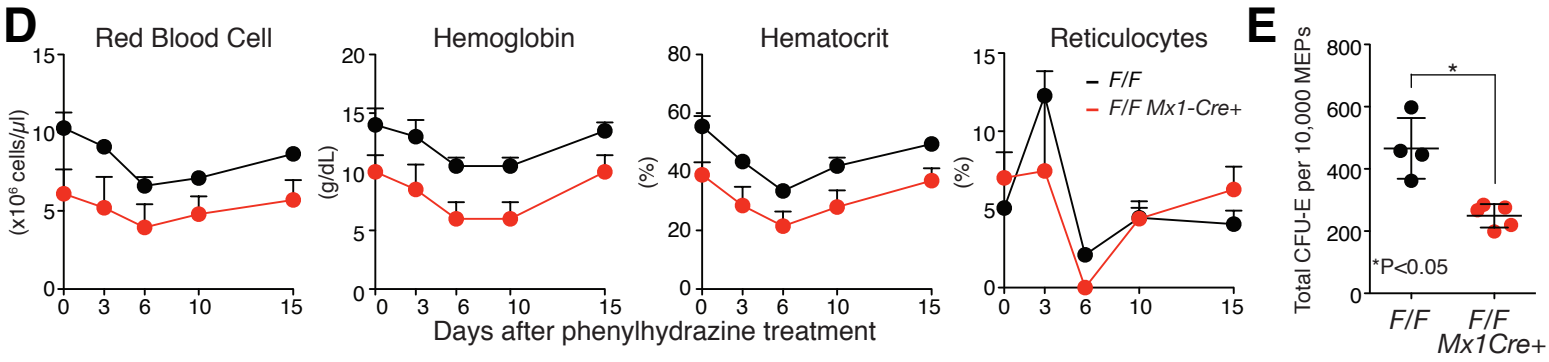
Ishikawa_Supplemental Figure 1

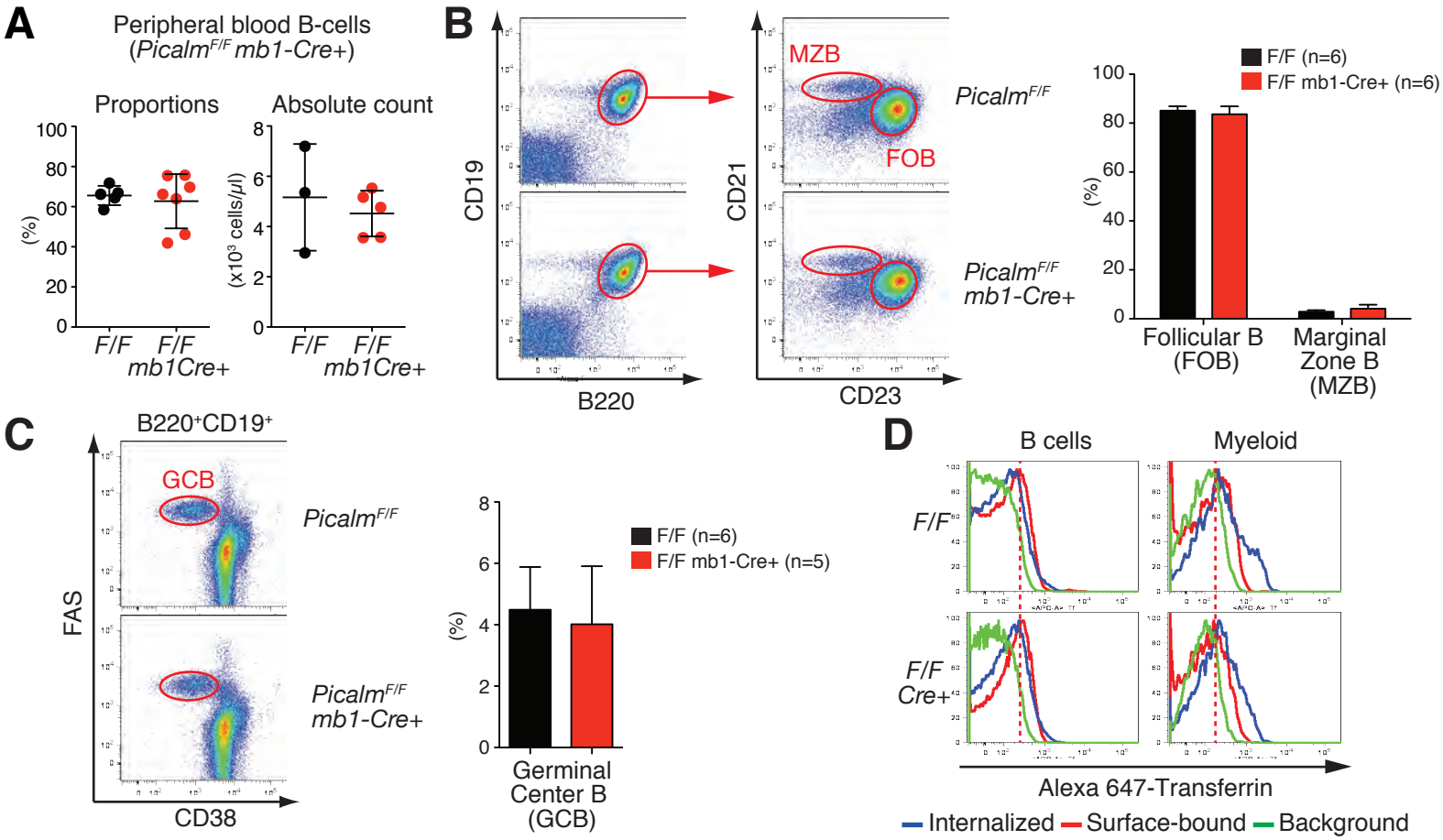


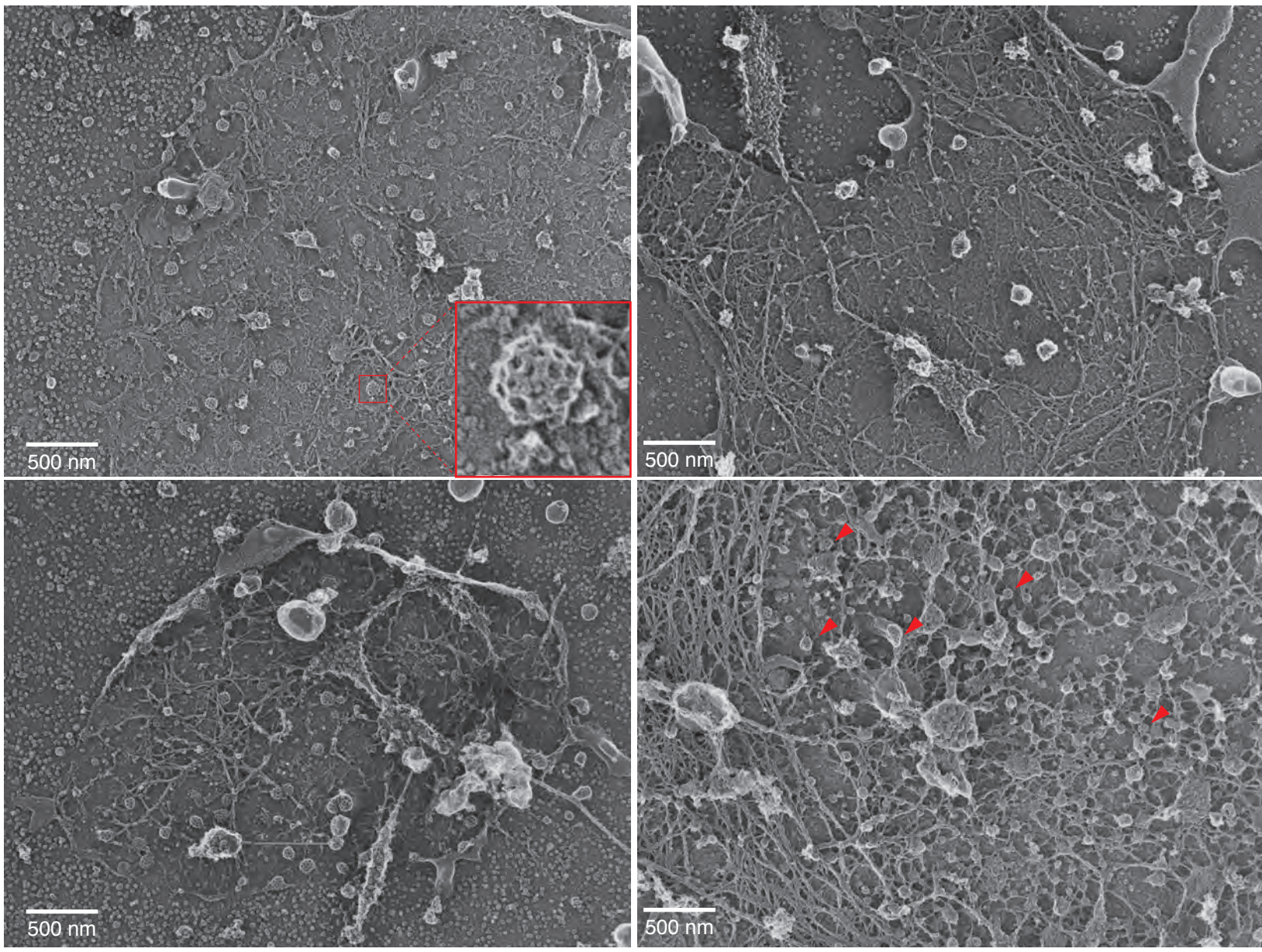

A
TER119+ splenic erythroblasts
CD45+ BM hematopoietic cells
Picalm F/F
Picalm F/F Mx1-Cre+
#1 #2 #3 #4
PICALM
Tubulin

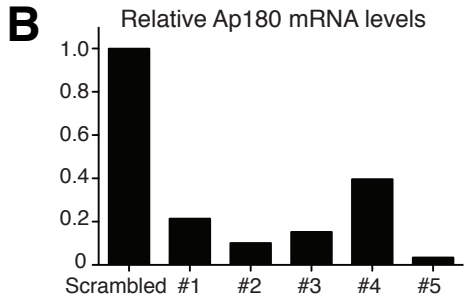

B
Picalm F/F
Picalm F/F Mx1-Cre+

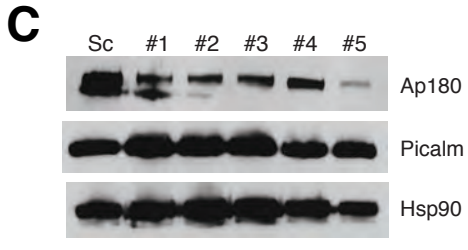

C
Reticulocyte hemoglobin content (CHr)
Immature reticulocyte fraction
(pg)
(%)
*P<0.01
*P<0.01
F/F
F/F Cre+

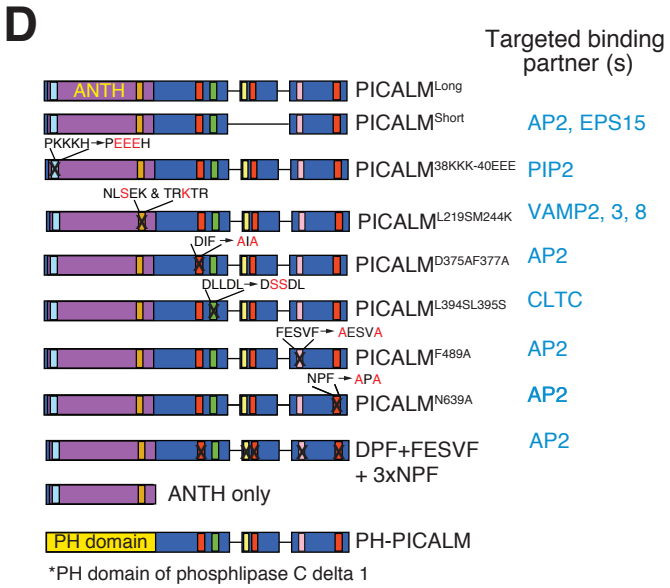

D
Red Blood Cell
Hemoglobin
Hematocrit
Reticulocytes
(x10⁶ cells/μl)
(g/dL)
(%)
(%)
F/F
F/F Mx1-Cre+
Days after phenylhydrazine treatment
E
Total CFU-E per 10,000 MEPs
*P<0.05
F/F
F/F Mx1Cre+

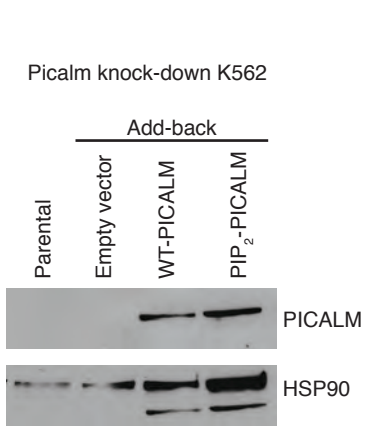

A
Peripheral blood B-cells
(Picalm F/F mb1-Cre+)
Proportions
Absolute count
(%)
(x10³ cells/μl)
F/F
F/F mb1Cre+
B
CD19
B220
MZB
FOB
CD21
CD23
Picalm F/F
Picalm F/F mb1-Cre+
F/F (n=6)
F/F mb1-Cre+ (n=6)
Follicular B (FOB)
Marginal Zone B (MZB)
C
B220⁺CD19⁺
GCB
FAS
CD38
Germinal Center B (GCB)
F/F (n=6)
F/F mb1-Cre+ (n=5)
D
B cells
Myeloid
F/F
F/F Cre+
Alexa 647-Transferrin
Internalized
Surface-bound
Background


A
Picalm F/F Mx1-Cre-
Picalm F/F Mx1-Cre+
500 nm
500 nm
500 nm
500 nm

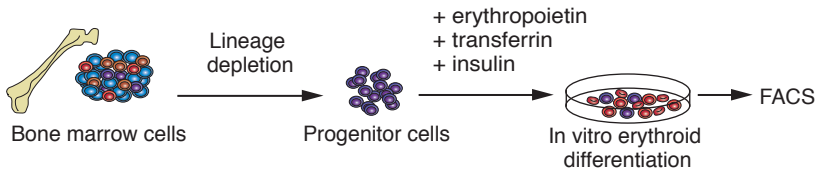

B
Relative Ap180 mRNA levels
1.0
0.8
0.6
0.4
0.2
0
Scrambled
#1
#2
#3
#4
#5


C
Sc
#1
#2
#3
#4
#5
Ap180
Picalm
Hsp90

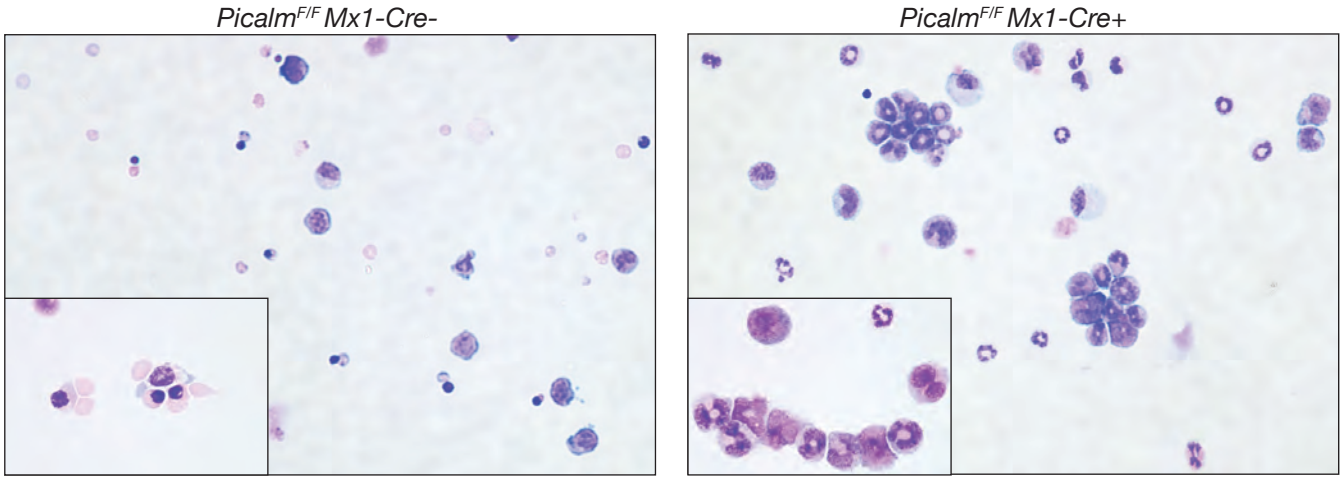

D
Targeted binding partner (s)
ANTH
PICALM Long
PICALM Short
AP2, EPS15
PKKKH→PEEEH
PICALM 38KKK-40EEE
PIP2
NLSEK & TRKTR
PICALM L219SM244K
VAMP2, 3, 8
DIF→AIA
PICALM D375AF377A
AP2
DLLDL→DSSDL
PICALM L394SL395S
CLTC
FESVF→AESVA
PICALM F489A
AP2
NPF→APA
PICALM N639A
AP2
DPF+FESVF
+ 3xNPF
AP2
ANTH only
PH domain
PH-PICALM
*PH domain of phosphlipase C delta 1

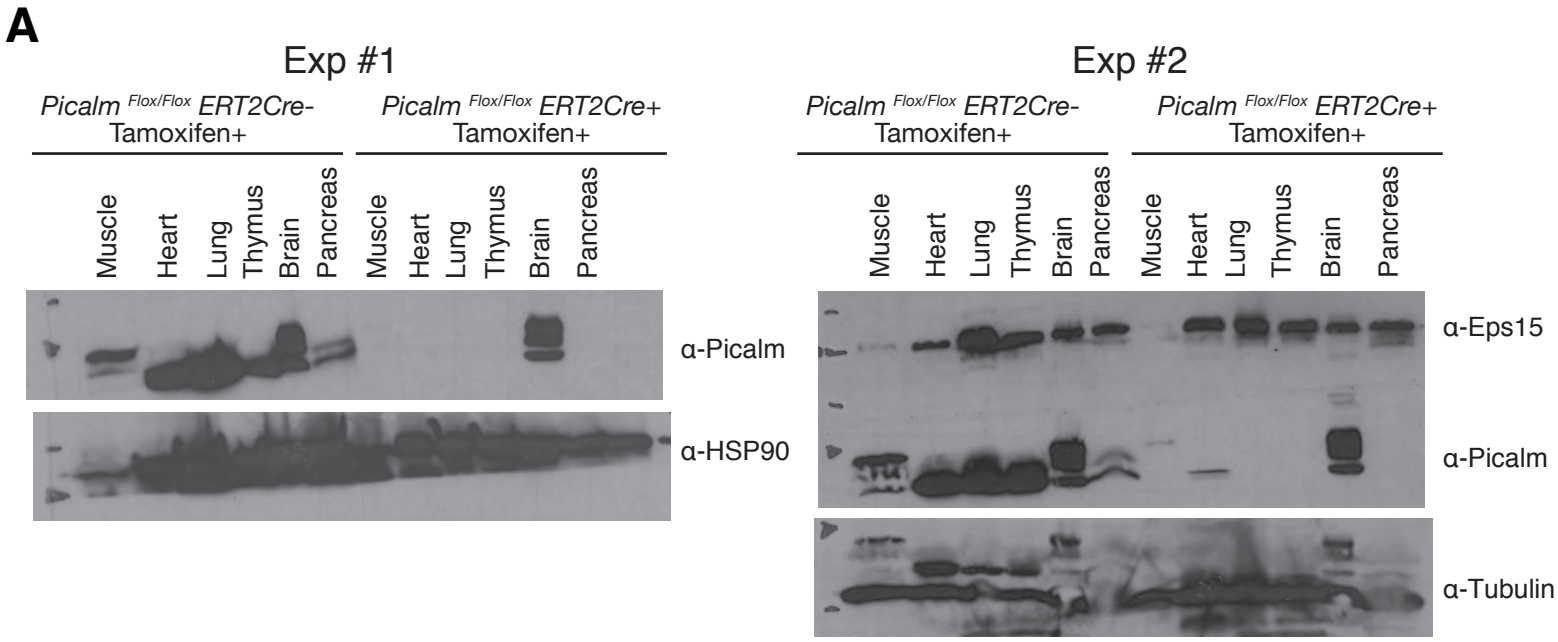

E
Picalm knock-down K562
Add-back
Parental
Empty vector
WT-PICALM
PIP2-PICALM
PICALM
HSP90

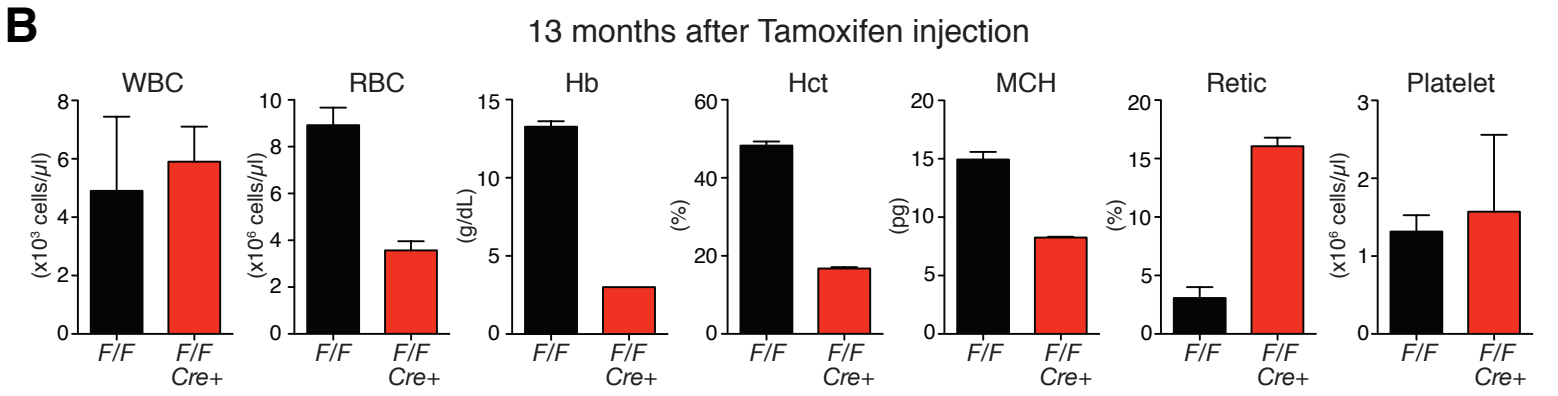
A

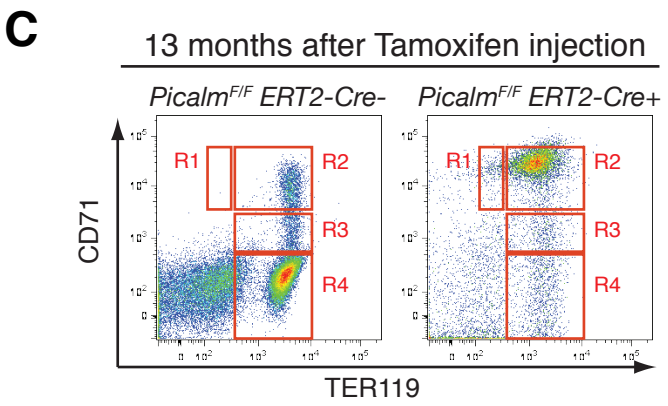

Lineage
depletion
+ erythropoietin
+ transferrin
+ insulin
FACS
Bone marrow cells
Progenitor cells
In vitro erythroid
differentiation

B

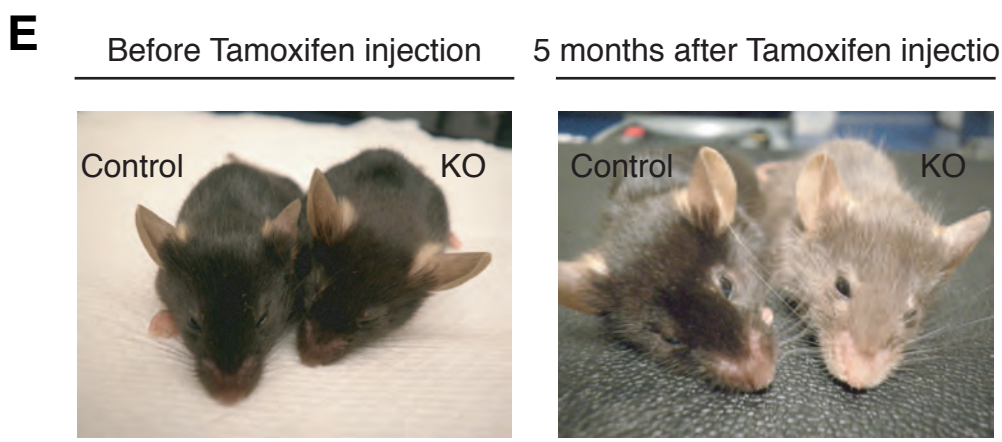

Picalm F/F Mx1-Cre-
Picalm F/F Mx1-Cre+


A
Exp #1
Picalm Flox/Flox ERT2Cre-
Tamoxifen+
Picalm Flox/Flox ERT2Cre+
Tamoxifen+
Muscle
Heart
Lung
Thymus
Brain
Pancreas
α-Picalm
α-HSP90
Exp #2
α-Eps15
α-Picalm
α-Tubulin


B
13 months after Tamoxifen injection
WBC
RBC
Hb
Hct
MCH
Retic
Platelet
F/F
F/F Cre+


C
13 months after Tamoxifen injection
PicalmF/F ERT2-Cre-
PicalmF/F ERT2-Cre+
R1
R2
R3
R4
CD71
TER119


D
Body weight
Male
Female
PicalmF/F ERT2-Cre-
PicalmF/F ERT2-Cre+
(g)
Months after Tamoxifen injection


E
Before Tamoxifen injection
5 months after Tamoxifen injection
Control
KO

Supplemental figure legends

Supplemental Figure 1. Generation of conventional and conditional *Picalm* knockout mice. (**A**) Protein samples from indicated mouse tissues were prepared and subjected to Western blot. Picalm was abundantly expressed in all tissues examined. Multiple isoforms were detected in brain and muscle. Hsp90 or Tubulin expression was examined as a protein-loading control. Ap180 (also known as Snap91) expression was examined by Western blot. Ap180 was predominantly expressed in brain tissue. Blots after short (bottom) and long (top) exposure time are shown. (**B**) G-CSF-mobilized PB CD34+ HSCs were induced into an erythroid lineage as described[16]. Protein samples were prepared on indicated days, and expression of PICALM, TfR and GAPDH was analyzed by Western blot. (**C**) Schematic representation of the *Picalm* knockout strategy. In the targeting construct, a *loxp* sequence was introduced in the intron between *Picalm* exons1 and 2. F1 mice ($Picalm^{Neo/+}$) were bred with either actin-*Flippase*- or *EIIa-Cre*-mice to generate *Picalm* conditional- ($Picalm^{F/+}$) or conventional- ($Picalm^{+/-}$) knockout strains, respectively. $Picalm^{F/+}$ mice were further bred with the *Mx1Cre* transgenic line to generate hematopoietic-specific *Picalm* knockout mice. (**D**) Western blot for Picalm using embryonic tissues (12.5 d.p.c.) confirmed successful depletion of *Picalm in vivo*. β-actin: loading control. (**E**) Embryos were obtained upon timed intercrosses of $Picalm^{+/-}$ mice, and genotypes were determined by PCR using tissue DNA samples. Bar graphs represent proportions of each genotype at indicated embryonic days. A total of 48, 159 and 80 embryos were genotyped at 12.5, 14.5 and 18.5 d.p.c., respectively. (**F**) Images of $Picalm^{+/+}$ (left) and $Picalm^{-/-}$ (right) embryos at 13.5 d.p.c. Note: brain hemorrhage is rarely seen in $Picalm^{-/-}$ embryos. (**G**) Proportions

of enucleated red cells (Hoechst low and FSC low) within the R5 fraction were unaffected in *Picalm*$^{-/-}$ FLs, suggesting that *Picalm* is dispensable for the enucleation process. (**H**) PB analysis of *Picalm* heterozygous mice (*Picalm*$^{+/-}$). *Picalm*$^{+/-}$ mice exhibited a very mild microcytic anemia. RBC: red blood cells; Hb: hemoglobin; Hct: hematocrit; MCV: mean corpuscular volume.

Supplemental Figure 2. Hematopoietic-specific deletion of *Picalm*. (**A**) Western blot for Picalm using purified Ter119+ splenic erythroblasts and CD45+ BM hematopoietic cells confirmed successful and sustained depletion of *Picalm* after pIpC injections. Splenic erythroblasts and BM cells of *Picalm*$^{F/F}$ *Mx1Cre*+ mice were obtained 18 months after pIpC injections. Splenic erythroblasts of control mice were obtained after Phenylhydrazine (PHZ) treatment. Tubulin: loading control. (**B**) Wright-Giemsa staining of PB smears of control and *Picalm*$^{F/F}$ *Mx1Cre*+ mice. (**C**) Dot graphs show reticulocyte hemoglobin content (CHr, left) and proportions of immature reticulocyte fraction (%IRF, right). %IRF was defined as the sum of proportions of high (IRF-H) and medium (IRF-M) fluorescence-intensity regions of reticulocytes. (**D**) PB counts after PHZ challenge. Although *Picalm*$^{F/F}$ *Mx1Cre*+ mice exhibited more severe anemia upon PHZ treatment, they tolerated hemolytic stress and their RBC counts recovered to the pretreatment levels by Day 17. PHZ (40 mg/kg) were injected subcutaneously three times as previously described[17]. (**E**) Dot graph shows absolute counts of CFU-E (colony forming unit erythroid) colonies that derived from FACS-sorted 1x10^4 MEPs in methylcellulose colony forming assay. MEP: megakaryocyte/erythroid progenitors. Horizontal black bars: average value; error bars: standard deviation.

Supplemental Figure 3. B-cell-specific deletion of *Picalm*. (**A**) Dot graph shows proportions (left) and absolute counts (right) of $B220^+$ B-cells in PB of control (*Picalm*$^{F/F}$) and B-cell-specific *Picalm* knockout mice (*Picalm*$^{F/F}$ *mb1Cre*+). Horizontal black bars: average value; error bars: standard deviation. (**B**) Representative FACS profiles of splenic B cells in control and *Picalm*$^{F/F}$ *mb1Cre*+ mice at 8-10 weeks of age (left). Bar graphs show proportions of follicular (FOB) and marginal zone B cells (MZB) in spleen (right). Error bars: standard deviation. (**C**) Representative FACS profiles of germinal center B cells (GCB: $B220^+CD19^+CD38^{low}FAS^+$) upon immunization with sheep red cells. GCB cell development in spleen was analyzed 10-days after immunization. Bar graphs show proportions of GCB cells within $B220^+CD19^+$ splenic B cells. Error bars: standard deviation. (**D**) Representative FACS profiles of transferrin/TfR endocytosis assay in splenic B-lymphoid and myeloid cells.

Supplemental Figure 4. Freeze-etch EM, ShRNA clones against *Ap180* and PICALM mutants (**A**) Additional pictures of freeze-etch EM in primary erythroblasts. Mature vesicles were barely found in *Picalm*-deficient erythroblasts (two right panels). Vesicles in *Picalm*-deficient cells were indicted by arrowheads. Inset: magnified image of a mature vesicle in WT erythroblast. (**B**) Five independent lentivirus-based ShRNA clones targeting mouse Ap180 (Snap91) were validated in Neuro 2a cells. After transduction, cells were selected with puromycin for 48 hours, and Ap180 levels were examined by (**B**) realtime PCR and (**C**) Western blotting. **(D)** *PICALM* mutants used for add-back experiments. The primary interactions of PICALM with other proteins are

indicated (right). Each mutant was predicted to lose (or affect) its binding to other clathrin-coat protein (s) that indicated on the right. **(E)** Levels of exogenously-expressed WT- and PIP_2-PICALM in *PICALM*-knockdown K562 cells were examined by Western blotting with anti-PICALM antibody.

Supplemental Figure 5. In vitro erythroid differentiation assay.

(A) Experimental outline of in vitro erythroid differentiation assay. Mouse bone marrow hematopoietic stem/progenitors were enriched via depleting lineage marker-positive mature cells using magnetic beads. Remaining progenitor cells were cultured in the presence of erythropoietin, holo-transferrin and insulin up to 3 days as described [9,10]. (**B**) Representative images of Wright-Giemsa staining of cytospins. Inset: high magnification image.

Supplemental Figure 6. Global *Picalm* deletion in adult mice. (**A**) *Picalm*$^{F/F}$ *ERT2-Cre*+ mice were intraperitoneally injected with tamoxifen (75mg/kg/day x5) at 1-2 months of age. Protein samples from indicated mouse tissues were prepared 2 months after tamoxifen injections and subjected to Western blot. *Picalm* inactivation was achieved in all tissues examined except brain. HSP90, Tubulin and Eps15: loading control. (**B**) PB counts were examined 13 months after tamoxifen treatment. Tamoxifen-injected *Picalm*$^{F/F}$ *ERT2-Cre*+ mice exhibited a microcytic and hypochromic anemia, as did *Picalm*$^{F/F}$ *Mx1-Cre*+ mice. **(C)** Representative FACS profiles of splenocytes 13 months after tamoxifen injection. Inefficient erythroid differentiation was evident in tamoxifen-treated *Picalm*$^{F/F}$ *ERT2-Cre*+ mice. **(D)** Graphs show body weights of

tamoxifen-treated *Picalm*$^{F/F}$ *ERT2-Cre*- and *Picalm*$^{F/F}$ *ERT2-Cre*+ mice. Body weights were unaffected by global *Picalm* deletion in adult mice. **(E)** Representative pictures of control and *Picalm*$^{F/F}$ *ERT2-Cre*+ mice before and after tamoxifen treatment.

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