Supporting Information

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SI Materials and Methods

Mice. Mice carrying the atypical protein kinase C aPKCλ$^{f/f}$ allele (1), and Mx1-Cre (2, 3), Vav1-Cre (4, 5), aPKCζ$^{-/-}$ mice (6) have been described previously. The aPKCλ$^{f/f}$ mice were crossed with Mx1-Cre (2, 3) or Vav1-Cre (4, 5) mice to get aPKCλ$^{f/f}$; Mx1-Cre or aPKCλ$^{f/f}$; Vav1-Cre genotypes. The aPKCλ$^{f/f}$; Mx1-Cre or aPKCλ$^{f/f}$; Vav1-Cre mice were further bred with aPKCζ$^{-/-}$ mice to get the triple transgenic aPKCζ$^{-/-}$λ$^{f/f}$; Mx1-Cre or aPKCζ$^{-/-}$λ$^{Δ/Δ}$; Vav1-Cre mice. Mx1-Cre mice were maintained in a mixed SV/129 background, whereas Vav1Cre mice were maintained in a pure C57B/6 background. CD45.2^{+} C57BL/6 and CD45.1^{+} B6.SJL$^{Ptprca\ Pep3b/BoyJ}$ mice were purchased from The Jackson Laboratory. Littermate mice from the same breeding were used in all experiments. All mouse strains were maintained at an Association for Assessment and Accreditation of Laboratory Animal Care-accredited, specific-pathogen–free animal facility at Cincinnati Children's Research Foundation, Cincinnati, under an Institutional Animal Care and Use Committee approved protocol. All mice were between 6 and 12 wk of age at the time of experimentation. Mice genotypes were determined by PCR analysis. Sequences of genotyping primers are available upon request.

Deletion of aPKCλ. WT; Mx1-Cre, aPKCλ$^{f/f}$; Mx1-Cre or aPKCζ$^{-/-}$λ$^{f/f}$; Mx1-Cre mice or CD45.1^{+} B6.SJL$^{Ptprca\ Pep3b/BoyJ}$ mice competitively or noncompetitively transplanted with CD45.2^{+} WT; Mx1-Cre, aPKCλ$^{f/f}$; Mx1-Cre or aPKCζ$^{-/-}$λ$^{f/f}$; Mx1-Cre hematopoietic stem cells (HSCs) were treated intraperitoneally with seven injections of 300 μg polyinosinic:polycytidylic acid (pI-pC) (Amersham) on every other day (2). For competitive repopulation experiment, the day of the last injection of pI-pC was considered to be day 0 for normalization. A multiplex PCR amplifying the WT allele, the aPKCλ floxed allele, and the aPKCλ gene-deleted allele was performed in conditions of linear amplification (28 cycles) using previously described primers (1).

Long-Term HSC Repopulation. Adult recipient mice were lethally irradiated with a Cs^{37} γ-irradiator, as previously described (2, 3, 7). For competitive repopulation experiments, CD45.2^{+} bone marrow nucleated cells (BMNC) were mixed with CD45.1^{+} BMNC and were transplanted into lethally irradiated CD45.1^{+} B6.SJL$^{Ptprca\ Pep3b/BoyJ}$ recipient mice. Short- and long-term HSC engraftment was measured by chimera assessment using flow cytometry (FACS) (2, 7).

5-Fluorouracil Treatment. WT; Vav1-Cre, PKCζ$^{-/-}$; Vav1-Cre, aPKCλ$^{Δ/Δ}$; Vav1-Cre or aPKCζ$^{-/-}$λ$^{Δ/Δ}$; Vav1-Cre mice and WT or p62$^{-/-}$ mice were injected intravenously with a single dose of (150 mg/kg body weight) 5 fluorouacil (5-FU) (Pfizer Inc.) and the hematopoietic response was monitored as described previously (4, 8, 9). In a separate experiment, CD45.1^{+} B6.SJL$^{Ptprca\ Pep3b/BoyJ}$ secondary WT recipient mice, lethally irradiated and competitively transplanted with HSCs from CD45.2^{+} aPKCζ$^{-/-}$ mice, were treated with 5-FU on weeks 16 and 22 and the hematopoietic response was monitored (8).

Proliferation and Survival Assay. HSC survival and proliferation in vivo were determined as previously described (2, 7). For in vivo proliferation analysis, mice received single intraperitoneal injections of BrdU (300 μg). Forty-five minutes later, BM cells were harvested and stained for surface markers. The cells were then fixed and stained with allophycocyanin-conjugated anti-BrdU antibody (Cytofix/Cytoperm Kit, BD Biosciences) according to the manufacturer's instructions.

Mobilization Assay. Peripheral blood was isolated from WT; Vav1-Cre or PKCζ$^{-/-}$; Vav1-Cre or aPKCλ$^{Δ/Δ}$; Vav1-Cre or aPKCζ$^{-/-}$λ$^{Δ/Δ}$; Vav1-Cre mice by retro-orbital bleeding. To quantify mobilization, peripheral blood hematopoietic progenitors were cultured in methycellulose medium supplemented with cytokines (Methocult GF M3434; Stem Cell Technologies) (2, 7). To assess G-CSF–induced mobilization, recombinant human G-CSF (Neupogen, Amgen) was injected intraperitoneally every day for 5 d at a dose of 200 μg/kg and peripheral blood was collected on the sixth day after starting its administration (2).

Homing, Adhesion, and Migration Assays. HSC/P homing in vivo and adhesion to fibronectin (CH-296; Takara Bio) and migration to CXCL12 in vitro were determined as previously described (2–4, 7, 10).

Polarity Staining of HSC. Freshly sorted LT-HSCs [Lineage^{-}Sca1^{+}c-Kit^{+} (LSK) CD34^{-}Flk2^{-} BM cells] were collected in HBSS supplemented with 10% FBS and seeded on fibronectin-coated glass coverslips. After 12 h of incubation at 37 °C, 5% CO_2, 3% O_2, long-term (LT)-HSCs were fixed with BD Cytofix Fixation Buffer (BD Biosciences) for 15 min at 4 °C and gently washed with PBS. For immunofluorescence staining, cells were permeabilized with 0.2% Triton X-100 (Sigma) in PBS for 20 min and blocked with 10% Donkey Serum (Sigma) for 30 min. Incubations with primary and secondary antibodies were performed for 1 h at room temperature. Coverslips were mounted with ProLong Gold Antifade Reagent (Invitrogen). The cells were coimmunostained with an anti–α-tubulin antibody (rat monoclonal ab6160; Abcam) together with either an anti-PKCζ antibody (rabbit monoclonal 9368; Cell Signaling Technologies) or with an anti-Par6 antibody (goat polyclonal sc-14405; Santa Cruz Biotechnology). Primary antibodies were stained with an anti-rat AMCA-conjugated secondary antibody and an anti-goat DyLight 488-conjugated antibody or an anti-rabbit DyLight 549-conjugated antibody (Jackson ImmunoResearch Inc.). Immunofluorescence samples were analyzed using fluorescence microscopy (AxioObserver Z1 equipped with a 63× PH objective and appropriate fluorescence filters; Zeiss). The localization of each single stained protein was considered polarized when a clear asymmetric distribution was visible when a line was drawn across the middle of the cell. A total of 50 to 100 individual LT-HSCs were analyzed per sample. Data represent percentage of the total number of cells scored. The experiment was repeated four times.

FACS Analysis of HSC/P. BM cells were stained using a mixture of biotin-conjugated monoclonal anti-mouse lineage antibodies CD45R (B220, Clone RA3-6B2), Gr-1 (Ly6G, Clone RB6-8C5), CD4 (L3T4, Clone GK1.5), CD8a (Ly-2, Clone 53–6.7), Mac-1 (CD11b, CloneM1/70), CD3ε (Clone 145–2C11), and TER119 (Ly-76) (all from Pharmingen). In a second step, the cells were incubated with streptavidin-allophycocyanin (APC)-Cy7, Alexafluor 700-anti-mouse Sca-1 (Ly6A/E, clone D7; e-Bioscience), PerCP-Cy5.5 anti-mouse CD117 (c-kit, Clone 2B8; Pharmingen), FITC anti-mouse CD34 (RAM 3–4; Pharmingen), Pacific Blue anti-mouse IL7Rα (A7R34; e-Bioscience), phycoerythrin (PE) anti-mouse Flk2 (Pharmingen), APC anti-mouse CD150 (mShad; Pharmingen), and PE Cy7 anti-mouse FcRγII/III (2.4G2; Pharmingen) antibodies. LT-HSC, short-term HSC, and multipotent progenitors were identified as follows: IL7Rα^{-} LSKCD34^{-} Flk2^{-}CD150^{+}, and IL7Rα^{-} LSK CD34^{+}Flk2^{-}, and LSKCD34^{+} Flk2^{+}, respectively. Myeloid and lymphoid progenitors were iden-

tified as follows: IL7Rα$^-$ Lin$^-$Sca1$^-$c-kit$^+$CD34$^+$FcγRII/III^{low} (common myeloid progenitors), IL7Rα$^-$ Lin$^-$Sca1$^-$c-kit$^+$CD34$^+$ FcγRII/III^{high} (granulocyte-monocyte progenitors), IL7Rα$^-$ Lin$^-$ Sca1$^-$c-kit$^+$CD34$^-$FcγRII/III^{low} (megakaryocyte-erythroid progenitors), or IL-7Rα$^+$Lin$^-$Sca1^{low}c-kit^{lo} (common lymphoid progenitors). Flow cytometry (FACS Canto; FACS Aria, BD) were used for all HSC analysis.

Methylcellulose Assay for Hematopoietic Progenitors. Hematopoietic progenitors isolated from BM, spleen, or peripheral blood were grown on methylcellulose medium supplemented with cytokine mixtures (Methocult GF M3434; Stem Cell Technologies) and colony-forming progenitors were scored on day 10.

RT-PCR Analysis. Total RNA was isolated from LSKCD34$^-$Flk2$^-$ HSCs from WT; Mx1-Cre or aPKCλ$^{f/f}$; Mx1-Cre mice, pre-treated with pI-pC (8–10 wk) and from WT; Vav1-Cre or aPKCλ$^{Δ/Δ}$; Vav1-Cre mice. Isolated RNA was treated with RNase-free DNase (Roche Diagnostics) and was reverse-transcribed with Multiscribe Reverse Transcriptase (Applied Biosystems). cDNA was PCR-amplified (Product: 600 bp; 35 cycles; 95 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min 30 s) using TaqRed DNA Polymerase (Sigma Diagnostics). aPKCλ-F: 5′-CGGCATGTGTAAGGAAGGAT-3′ and aPKCλ-R: 5′-GGCAAGCAGAATCAGACACA-3′. GAPDH was used as an internal control.

Statistical Analysis. Statistical analyses were performed using Student *t* test (GraphPad Prism Version 5.0, GraphPad Software Inc.). For the Kaplan-Meier analysis of survival, a log-rank test was performed.

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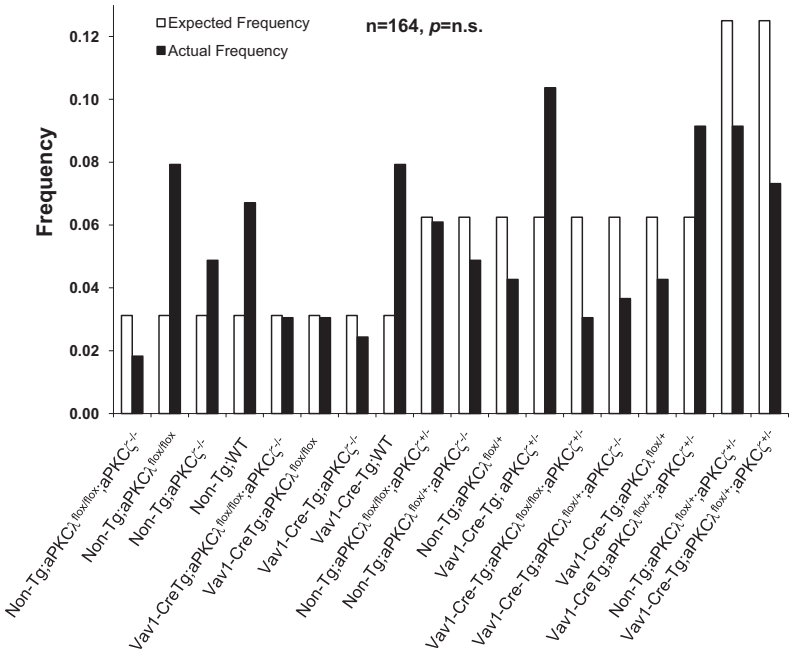

Fig. S1. Frequency of expected and actual frequencies of alive mice corresponding to 18 genotypes obtained by crossing Vav1-Cre;aPKCλ$^{f/+}$ females with non-Tg;aPKCζ$^{+/-}$ males (n = 164 mice). P = not significant (N.S., χ^2 test), for the deficiency of aPKCζ, aPKCλ (Vav1-Cre;aPKCλ$^{flox/flox}$) or both aPKCζ and aPKCλ (Vav1-Cre;aPKCλ$^{flox/flox}$;aPKCζ$^{-/-}$).

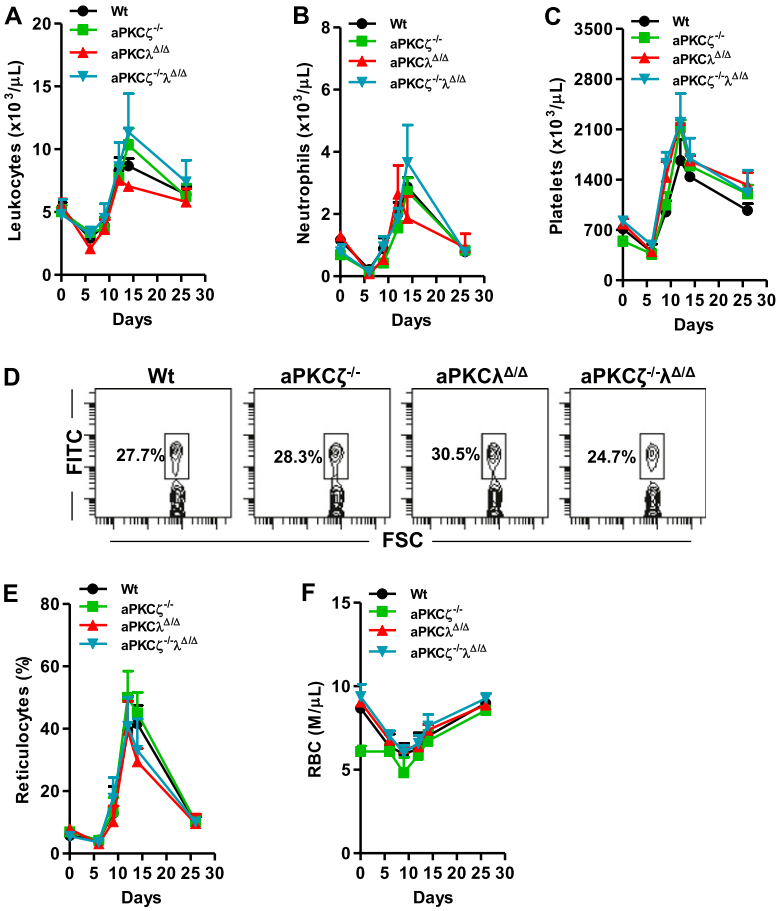

Fig. S2. aPKCζ and aPKCλ are dispensable for 5-FU–induced stressed hematopoiesis. (*A–C*) 5-FU–induced hematopoietic recovery in WT; Vav1-Cre or PKCζ$^{-/-}$; Vav1-Cre or aPKCλ$^{\Delta/\Delta}$; Vav1-Cre or aPKCζ$^{-/-}$λ$^{\Delta/\Delta}$; Vav1-Cre mice. Evolution of (*A*) leukocyte, (*B*) neutrophil, and (*C*) platelets counts in the peripheral blood for 3 wk (n = 3–4 mice per group). Error bars represent SEM. (*D*) Representative FACS contour diagram showing frequency of reticulocytes (Thiazole orange, Retic Count; BD) in the peripheral blood of 5-FU–treated WT; Vav1-Cre or PKCζ$^{-/-}$; Vav1-Cre or aPKCλ$^{\Delta/\Delta}$; Vav1-Cre or aPKCζ$^{-/-}$λ$^{\Delta/\Delta}$; Vav1-Cre mice on day 12. (*E*) Evolution of reticulocyte content in the peripheral blood of 5-FU–treated WT; Vav1-Cre or PKCζ$^{-/-}$; Vav1-Cre or aPKCλ$^{\Delta/\Delta}$; Vav1-Cre or aPKCζ$^{-/-}$λ$^{\Delta/\Delta}$; Vav1-Cre mice for 3 wk (n = 3–4 mice per group). Error bars represent SEM. (*F*) Changes in red blood cell counts in the peripheral blood of 5-FU–treated WT; Vav1-Cre or PKCζ$^{-/-}$; Vav1-Cre or aPKCλ$^{\Delta/\Delta}$; Vav1-Cre or aPKCζ$^{-/-}$λ$^{\Delta/\Delta}$; Vav1-Cre mice for 3 wk (n = 3–4 mice per group). Error bars represent SEM.

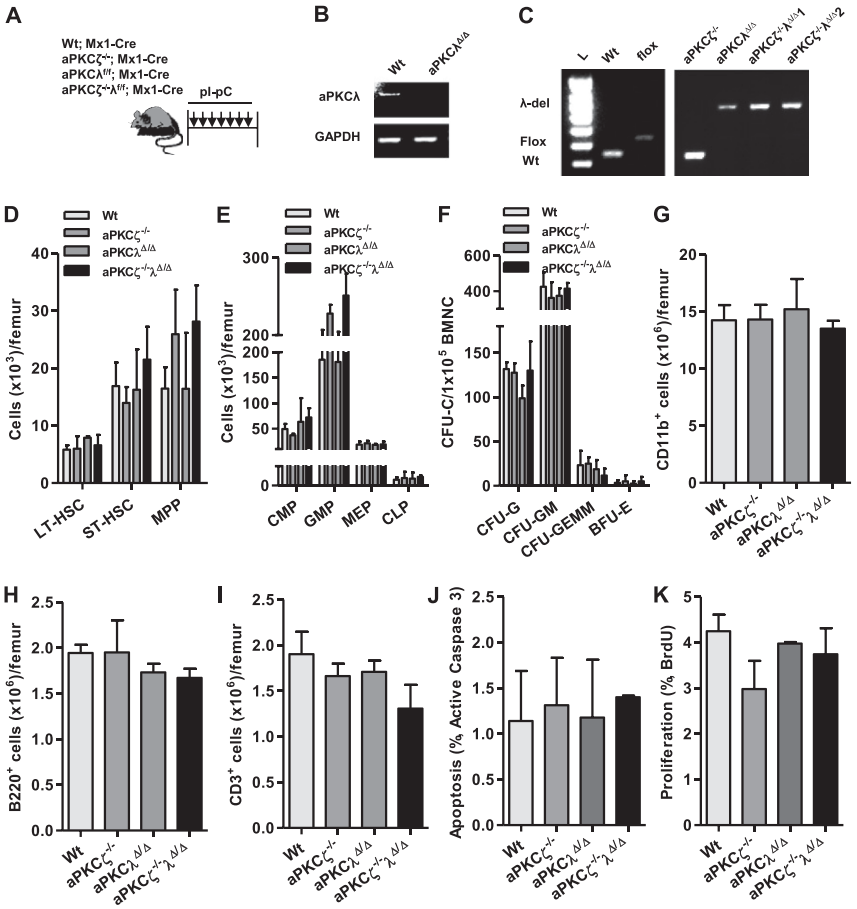

Fig. S3. Inducible deletion of aPKCζ and aPKCλ does not affect steady-state hematopoiesis. (*A*) Experimental set up. WT; Mx1-Cre or aPKCζ$^{-/-}$; Mx1-Cre, or aPKCλ$^{f/f}$; Mx1-Cre or aPKCζ$^{-/-}$λ$^{f/f}$; Mx1-Cre mice were treated with pI-pC (5–7 intraperitoneal injections) to delete aPKCλ alleles in vivo. BM HSC/P and hematopoiesis were analyzed after 8 to 12 wk of the final pI-pC injection. (*B*) mRNA expression (RT-PCR) analysis of aPKCλ in LSKCD34^{-}Flk2^{-} cells isolated from the BM of WT; Mx1-Cre or aPKCλ$^{f/f}$; Mx1-Cre mice. (*C*) Genomic DNA PCR analysis of WT, floxed, aPKCλ-deleted, and aPKCζ alleles in the peripheral blood of WT; Mx1-Cre or aPKCζ$^{-/-}$; Mx1-Cre or aPKCλ$^{f/f}$; Mx1-Cre or aPKCζ$^{-/-}$ aPKCλ$^{f/f}$; Mx1-Cre mice. (*D*) Absolute numbers of HSCs present in the BM of WT; Mx1-Cre or aPKCζ$^{-/-}$; Mx1-Cre or aPKCλ$^{f/f}$; Mx1-Cre or aPKCζ$^{-/-}$ aPKCλ$^{f/f}$; Mx1-Cre mice (n = 3 mice per group). Error bars represent SD. (*E*) Absolute numbers of hematopoietic progenitors (HPC) present in the BM of WT; Mx1-Cre or aPKCζ$^{-/-}$; Mx1-Cre or aPKCλ$^{f/f}$; Mx1-Cre or aPKCζ$^{-/-}$ aPKCλ$^{f/f}$; Mx1-Cre mice (n = 3 mice per group). Error bars represent SD. (*F*) Absolute numbers of colony-forming progenitors; granulocyte (CFU-G), granulocyte-monocyte (CFU-GM), granulocyte-erythroid-monocyte-megakryocyte (CFU-GEMM), and burst-forming units (BFU-E) present in the BM of WT; Mx1-Cre or aPKCζ$^{-/-}$; Mx1-Cre or aPKCλ$^{f/f}$; Mx1-Cre or aPKCζ$^{-/-}$ aPKCλ$^{f/f}$; Mx1-Cre mice (n = 3 mice per group). Error bars represent SD. (*G*) Absolute numbers of CD11b^{+} myeloid cells present in the BM of WT; Mx1-Cre or aPKCζ$^{-/-}$; Mx1-Cre or aPKCλ$^{f/f}$; Mx1-Cre or aPKCζ$^{-/-}$ aPKCλ$^{f/f}$; Mx1-Cre mice (n = 3 mice per group). Error bars represent SD. (*H*) Absolute numbers of B220^{+} B-lymphoid cells present in the BM of WT; Mx1-Cre or aPKCζ$^{-/-}$; Mx1-Cre or aPKCλ$^{f/f}$; Mx1-Cre or aPKCζ$^{-/-}$ aPKCλ$^{f/f}$; Mx1-Cre mice (n = 3 mice per group). Error bars represent SD. (*I*) Absolute numbers of CD3^{+} T-lymphoid cells present in the BM of WT; Mx1-Cre or aPKCζ$^{-/-}$; Mx1-Cre or aPKCλ$^{f/f}$; Mx1-Cre or aPKCζ$^{-/-}$ aPKCλ$^{f/f}$; Mx1-Cre mice (n = 3 mice per group). Error bars represent SD. (*J*) Apoptosis (active caspase 3) of BM-LSK cells in WT; Mx1-Cre or aPKCζ$^{-/-}$; Mx1-Cre or aPKCλ$^{f/f}$; Mx1-Cre or aPKCζ$^{-/-}$ aPKCλ$^{f/f}$; Mx1-Cre mice in vivo (n = 3 mice per group). Error bars represent SD. (*K*) Proliferation of BM-LSK cells in WT; Mx1-Cre or aPKCζ$^{-/-}$; Mx1-Cre or aPKCλ$^{f/f}$; Mx1-Cre or aPKCζ$^{-/-}$ aPKCλ$^{f/f}$; Mx1-Cre mice in vivo (n = 3 mice per group). Error bars represent SD.

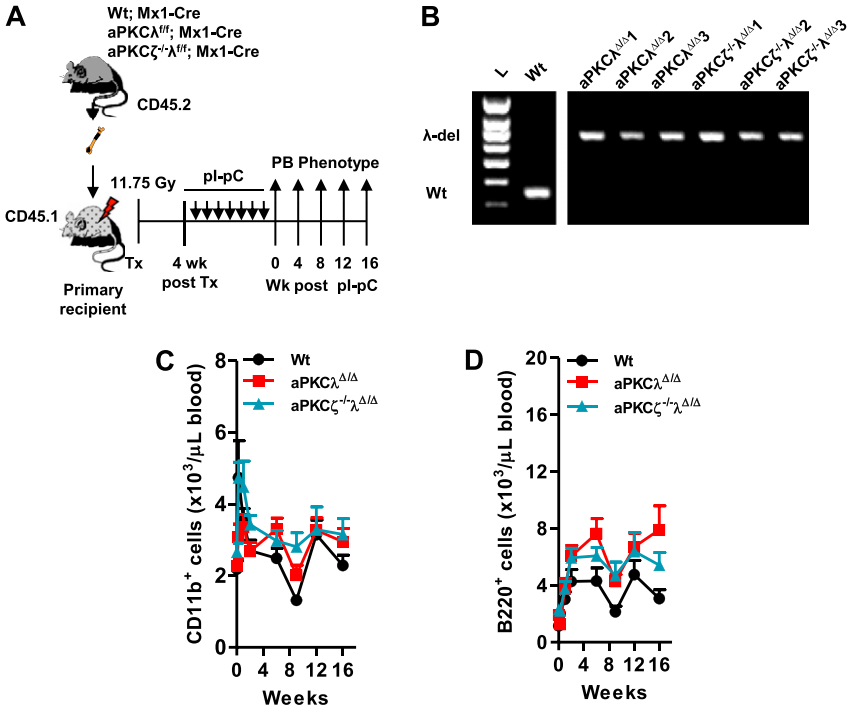

Fig. S4. Inducible deletion of aPKCζ and aPKCλ do not affect HSC lineage repopulation activity. (*A*) Experimental set up. BM cells from CD45.2$^+$ WT; Mx1-Cre or aPKCλ$^{f/f}$; Mx1-Cre or aPKCζ$^{-/-}$λ$^{f/f}$; Mx1-Cre mice were noncompetitively transplanted into lethally irradiated CD45.1$^+$ B6.SJL$^{Ptprca\ Pep3b/BoyJ}$ recipient mice. After BM engraftment (4 wk), aPKCλ deletion was induced by administration of pI-pC and CD45.2$^+$ chimera was monitored at different time points. (*B*) Genomic DNA PCR analysis of WT, floxed, and aPKCλ-deleted alleles in the BM of CD45.1$^+$ B6.SJL$^{Ptprca\ Pep3b/BoyJ}$ recipient mice after 16 wk of transplantation from *A*. (*C*) Evolution of peripheral blood myeloid-cell content (CD11b$^+$ cells gated on CD45.2$^+$ graft) in the recipient mice (n = 6–8 mice per group). Error bars represent SEM. (*D*) Evolution of peripheral blood B-cell content (B220$^+$ cells gated on CD45.2$^+$ graft) in the recipient mice (n = 6–8 mice per group). Error bars represent SEM.

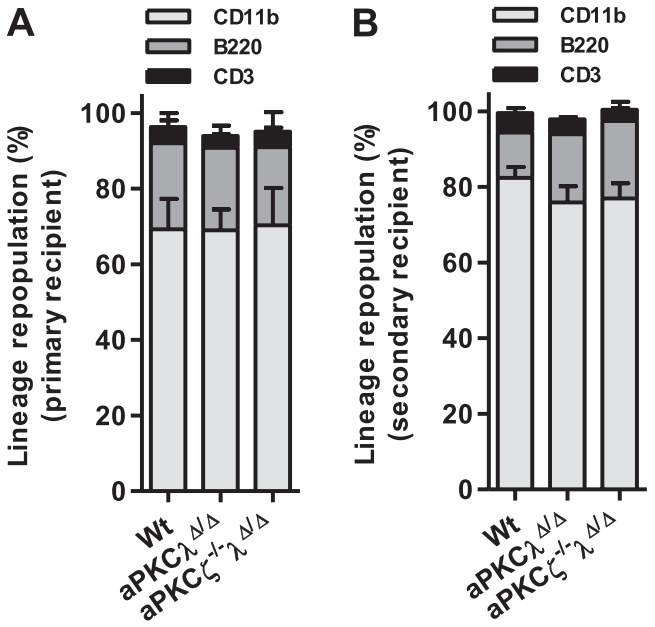

Fig. S5. Deficiency of aPKCζ and aPKCλ do not affect lineage repopulation during serial BM transplantation. (*A*) Frequency of lineage-repopulating myeloid (CD45.2$^+$CD11b$^+$) or B (CD45.2$^+$B220$^+$) or T (CD45.2$^+$CD3$^+$) cells present in the BM of the primary recipient mice (n = 7–9 mice per group). Error bars represent SD. (*B*) Frequency of lineage-repopulating myeloid (CD45.2$^+$CD11b$^+$) or B (CD45.2$^+$B220$^+$) or T (CD45.2$^+$CD3$^+$) cells present in the BM of the secondary recipient mice (n = 8 mice per group). Error bars represent SD.

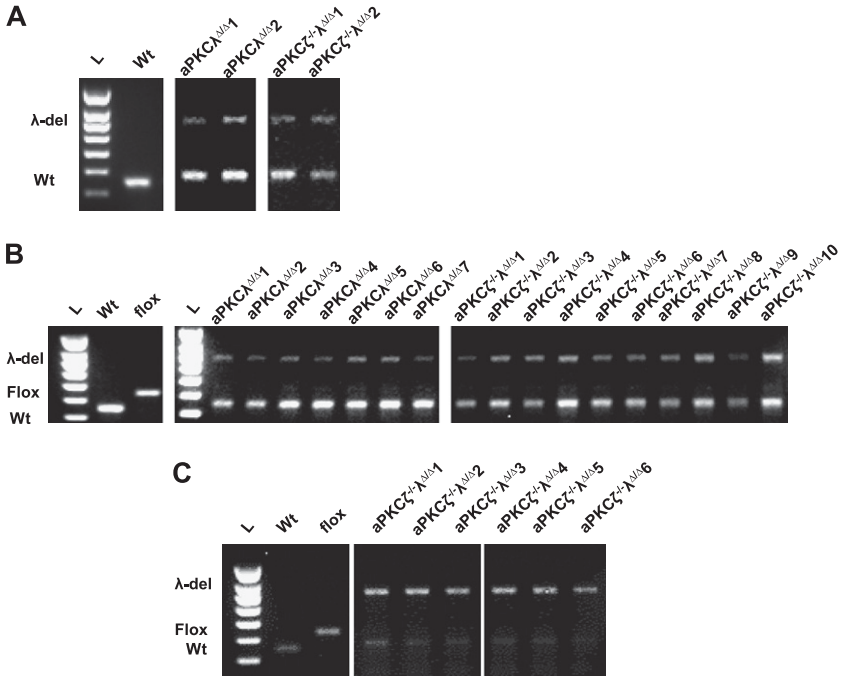

Fig. S6. PolyI-polyC (pI-pC) induces deletion of aPKCλ in the recipient mice. Genomic DNA PCR analysis of WT, floxed, and aPKCλ-deleted alleles in the peripheral blood of CD45.1^{+} B6.SJL$^{Ptprca\ Pep3b/BoyJ}$ (*A*) primary, (*B*) secondary, and (*C*) tertiary recipients. Analysis of gene deletion of recipient mice was performed after 8 to 10 wk of pI-pC administration (primary recipients) or serial BM transplantation (secondary and tertiary recipients). BM cells from CD45.2^{+} WT; Mx1-Cre or aPKCλ$^{f/f}$; Mx1-Cre or aPKCζ$^{-/-}$λ$^{f/f}$; Mx1-Cre mice were mixed with CD45.1^{+} B6.SJL$^{Ptprca\ Pep3b/BoyJ}$ WT BM cells and competitively transplanted into lethally irradiated recipient mice. After BM engraftment (4 wk), aPKCλ deletion in primary recipients was induced by administration of pI-pC.

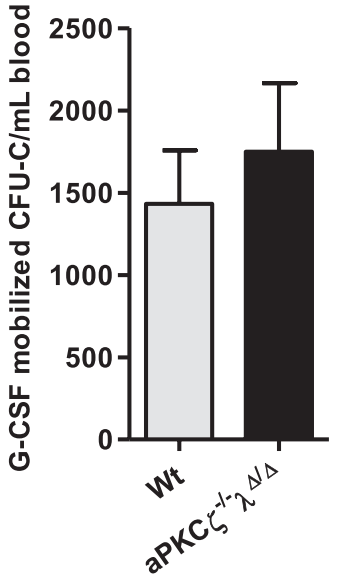

Fig. S7. Deficiency of aPKCζ and aPKCλ do not affect G-CSF–induced HSC/P mobilization. Absolute numbers of circulating CFU-Cs present in WT or aPKCζ- and aPKCλ-deficient mice, treated with G-CSF. (*n* = 3–4 mice per group). Error bars represent SD.

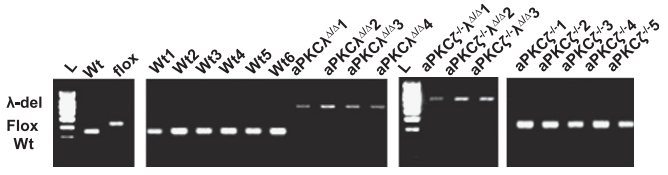

Fig. S8. Deletion of aPKCλ in noncompetitively transplanted mice. Genomic DNA PCR analysis of WT, floxed, and aPKCλ-deleted alleles in the peripheral blood of CD45.1^{+} B6.SJL$^{Ptprca\ Pep3b/BoyJ}$ recipient mice. BM cells from CD45.2^{+} WT; Vav1-Cre or PKCζ$^{-/-}$; Vav1-Cre or aPKCλ$^{\Delta/\Delta}$; Vav1-Cre or aPKCζ$^{-/-}$λ$^{\Delta/\Delta}$; Vav1-Cre mice were noncompetitively transplanted into lethally irradiated CD45.1^{+} B6.SJL$^{Ptprca\ Pep3b/BoyJ}$ recipient mice.