Supporting Information

McCracken et al. 10.1073/pnas.1221840110

SI Materials and Methods

Animals. Immunocompetent C57BL/6 and C57BL/6 SJL female mice and immunedeficient NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice were bred and maintained according to the guidelines of the Department of Laboratory Animal Medicine (DLAM) at the University of California, Los Angeles. All animal studies were carried out by using protocols that had been approved by DLAM.

Constructs and Cloning. Codon optimized human deoxycytidine kinase (hdCK) sequence was purchased from DNA 2.0. PCR amplification added EcoR1 and Xho1 restriction sites. hdCK was then placed in murine stem cell virus (pMSCV) vector ahead of an internal ribosomal entry site (IRES) and yellow fluorescent protein (YFP). Amino acid mutations were made by multisite-directed mutagenesis (Stratagene) at the following locations: A100V, R104M, and D133A. A lentiviral vector (2F-X, pCCL based) was constructed for infection of human HSCs. hdCK3mut-IRES-YFP was cut from MSCV, and BamH1 sites were added by phosphate linkers. 2F-X was digested with BamH1 and hdCK3mut-IRES-YFP was ligated into the vector. Helper plasmids for making ecotropic retrovirus was PCL-II. Third-generation nonreplicating lentiviral helper plasmids were used (VSVg, PREV, and PMDL).

Cell Lines. L1210 and L1210-10K mouse leukemia cell lines were a gift from Charles Dumontet (Université Claude Bernard Lyon I, Lyon, France). All stable cell lines were made with retroviral transduction with ecotropic packaging of pMSCV constructs. Cell lines were sorted to equal expression of YFP by FACS and grown in cell-specific media conditions.

In Vitro Uptake Assay. Suspension cell lines: 100,000 cells in 100 μL media were plated in triplicate in a 0.22 micron filter bottom plates. 3H-probe was diluted to 5 μCi/mL in media. A total of 100 μL (0.5 μCi) was added per well. Cells were incubated at 37 °C for 1 h, washed five times, and dried. Scintillation fluid was added and counted for counts per minute (cpm) for 1 min on a BetaMax plate reader (PerkinElmer).

Recombinant Protein Production. For bacterial expression of His-tagged human dCK, 5′ and 3′ primers (5′-CATGGATCCATGG-CCACCCCGCCCAAGAG-3′ and 5′-GTAGGTACCTCACAA-AGTACTCAAAAACTCTTTGACCTTTTC-3′) were designed introducing BamHI and KpnI sites for cloning into pQE-80L vector (Qiagen). TOP10 bacteria were transformed with pQE-80L-hdCK3mut, dCKDM, or hdCK. A single colony was used for an overnight starter culture. A large-scale 1-L culture was inoculated and induced at OD_{600}: 0.6 with isopropylthio-β-galactoside (IPTG) to 1 mM. Cells were harvested 4 h later and cleared lysate was prepared according to Qiagen instructions. Lysate was combined with 1 mL bed volume Ni-NTA agarose and transferred to a 20-mL column. Beads were washed extensively and eluted in PBS 150 mM NaCl, 250 mM imidazole, 5% (wt/vol) glucose.

Enzyme Kinetic Assay. A coupled spectrophotometric kinase assay was adapted to determine kinetics for L-FMAU (1). Reads were performed in ∼30-s intervals at 37 °C for 30 min. An NADH standard curve was constructed. Nonlinear regression analysis and Michaelis–Menten plots determined the K_m values.

Grafts. Cells were counted, washed, and resuspended in a 1:1 mixture of sterile RPMI and Matrigel. Each graft contained 2×10^5 cells in 100 μL total volume. Grafts were implanted s.c. in 6- to 8-wk-old female NSG mice. [^{18}F]-FDG (2-deoxy-2-^{18}fluoro-D-glucose) was performed on day 7 or 8, with sequential [^{18}F]-L-FMAU (1-(2-deoxy-2-^{18}fluoro-β-L-arabinofuranosyl)-5-methyl-uracil) scans on day 8 or 9 depending on tumor growth rate. Grafts were removed after [^{18}F]-L-FMAU imaging, weighed, and total cpm/g was determine by the amount of radioactivity in each graft measured using a Wallac Wizard 3″ 1480 Automatic Gamma Counter (PerkinElmer).

Mouse HSC Transplant. Six- to 10-wk-old C57BL/6 SJL mice were injected i.v. with 150 mg/kg 5-fluorouracil (APP Pharmaceuticals). BM was harvested 5 d after treatment and cultured with IL-3 (6 ng/mL), IL-6 (10 ng/mL), stem cell factor (SCF) (100ng/mL), and 5% (vol/vol) conditioned media from WeHi-3 cells (WeHI) as growth factors. After 24 h, the cells were infected with Murine Stem Cell Viruses (MSCV), MSCV-hdCK3mut-IRES-YFP or MSCV-IRES-YFP and 1.6 μg/mL of polybrene under spin conditions (2,500 rpm, 90 min, 30 °C, Beckman CS-6R centrifuge) and then incubated overnight at 37 °C. Cells were superinfected the next day, washed, and counted for total cell number. Six- to 8-wk-old C57BL/6 mice were lethally irradiated (900 rad) before i.v. injection of 5×10^5 transduced BM cells on the same day. Each population was ∼40–60% transduced, with each animal transplanted with a mixed population of reporter and nonreporter cells.

Human HSC Transplant. Hematopoietic stem and progenitor cells were enriched from Ficoll fractionated cord blood or bone marrow with CD34+MACS beads (Miltenyi). Cells were thawed and prestimulated for 24 h in RetroNectin (TaKaRa)-coated nontissue culture treated 24-well plates (Falcon) in X-VIVO 15 medium (Lonza) containing SCF (50 ng/mL), fms-like tyrosine kinase-3 (Flt-3) ligand (50 ng/mL), Thrombopoietin (TPO) (50 ng/mL), and IL-3 (20 ng/mL). The next day, cells were transduced at a lentiviral vector concentration of 2×10^8 transduction units/mL. Cells were injected 24 h after transduction. Neonatal (1-3 days old) NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ mice were conditioned with 150 cGy total body irradiation. Twenty-four hours postconditioning, mice were injected intrahepatically with CD34$^+$-enriched cord blood or bone marrow cells at a dose of 3×10^5 cells in 50 μL of medium.

Anti-hdCK Antibody Generation. Six His-tagged human dCK was produced in bacteria, purified by Ni-NTA (nickel-nitrilotriacetic acid) resin chromatography and used as immunogen. Four mice (BALB/c females 6–8 wk) were immunized by i.p. injection of 200 μg 6 His-hudCK in RIBI adjuvant (Sigma) followed by four monthly boosts of 100 μg immunogen i.p. in RIBI. Antibody titer was determined in the serum by ELISA. Spleen of the highest titer mouse was excised and dissociated. Isolated splenocytes were fused to the myeloma cell line sp2/0 at a ratio of 5:1 splenocytes/myeloma using PEG1500 (Roche). Twenty percent of fusion was plated in hypoxanthine-aminopterin-thymidine medium (HAT medium) onto 10× flat bottom 96-well plates at 200 μL/well, the remaining fusion was frozen down. Fusion was cultured until clones appeared in the wells and covered 25–50% of well. Supernatant was collected and ELISA performed: 96-well flat bottom assay plates (Nunc Maxisorp) were coated with 10 μg/μL immunogen, blocked with PBS 1% (wt/vol) BSA. Supernatants were applied to wells, goat antimouse HRP was used to detect binding, and reaction was developed with 2,2′-Azinobis [3-ethyl-benzothiazoline-6-sulfonic acid]-diammonium salt (ABTS). Positive wells were replated in 24-well plates in hypoxanthine-thymidine

(HT) medium. ELISA was repeated on previously positive wells when cultures got 50% confluent. Positive supernatants were tested for ability to detect immunogen by Western blot at 1:10 dilution in PBST-5% (wt/vol) milk. Positive wells were subcloned by limiting dilution to obtain single clones per well in 96-well flat bottom plates. Subclones were tested by ELISA and highest positives were tested on Western blot. Clones were tested for IHC staining ability and clone 9D4 (plate 9 well D4) was determined to be the best in overall performance. Preparative amounts of antibody were produced in CELLine-1000 flasks (Integra Bioscience) and purified by affinity chromatography (protein G sepharose, Prosep-G; Millipore). Anti-dCK clone 9D4 is now commercially available with Millipore.

Antibodies and Western Blot. A total of 20 μg of total cell lysate in RIPA buffer was run on SDS/PAGE using Precise Tris·Hepes protein gels, 4–20%. Gels were transferred onto 0.22 micron nitrocellulose and blocked for 1 h at room temperature in 5% (wt/vol) milk in PBS with 0.05% Tween. Antibodies were diluted in 5% (wt/vol) milk in PBS with 0.05% Tween as follows: dCK (Witte Laboratory-9D4, see *SI Materials and Methods*; Millipore) 1:1,000, YFP (Witte Lab, polyclonal rAb) 1:5,000, ERK2 (Santa Cruz; SC-154) 1:5,000, goat antimouse IgG HRP (Bio-Rad; 172–1011) 1:10000, goat antirabbit IgG HRP (Bio-Rad; 170–6515)1:15000. ECL substrate (Millipore) was used for detection and development on GE/Amersham film.

Immunohistochemistry. Tissue was fixed in 10% phosphate-buffered formalin overnight. Sections were fixed in paraffin and cut at 0.4 μm, with staining for hematoxylin and eosin for representative histology every five slides. Tissue sections were heated at 65 °C for 1 h to melt the paraffin followed by rehydration. Antigen retrieval was performed using citric acid buffer and visualization was performed using a liquid DAB$^+$ kit (Dako). Slides were blocked for endogenous peroxidase activity with 3% (vol/vol) H_2O_2 in PBS for 5 min, then blocked for mouse IgG by using Vector Labs Mouse-on-Mouse (M.O.M.) kit (BMK-2202). Primary antibodies were diluted with M.O.M. diluent as follows: dCK (Witte Lab-94D, 1:2,000), YFP (Witte Lab-Mouse Monoclonal, 1:200), HLA (Santa Cruz; 1:100), mouse IgG (Santa Cruz; 0.4 μg/μL), and incubated at 4 °C overnight. Secondary antibody was added (ImmPRESS antimouse Ig (peroxidase) Polymer Detection kit).

Flow Cytometry and Fluorescent-Activated Cell Sorting. Single-cell suspensions from spleen, thymus, bone marrow, and peripheral blood were stained with the following fluorochrome-conjugated antibodies: anti-CD45.1, anti-CD4, anti-CD8, anti-Ter119, anti-CD71, anti-CD11b, anti-GR1, anti-B220, anti-CD19, anti-IgM, anti-CD43, and Hoechst for cell cycle analysis. Human engraftment was monitored with the following fluorochrome-conjugated antibodies: anti-hCD45, anti-hCD3, anti-hCD19, and anti-hCD33. In all reporter BM chimera animals, reporter-labeled cells were defined as YFP (FITC channel) positive. Cell sorting was performed on the FACSAria automated cell sorter (BD Biosciences), and flow cytometry was performed on the BD FACScanto II.

MicroPET and Image Analysis. Mice were warmed under gas anesthesia (2% (vol/vol) isoflurane) and injected i.v. with 200 μCi of either [^{18}F]-FDG or [^{18}F]-L-FMAU (radiochemical synthesis described in ref. 2), followed by 1-h unconscious or 3-h conscious uptake. Mice were then positioned in an imaging chamber for sequential imaging with the Siemens Preclinical Solutions MicroPET Focus 220 and MicroCAT II CT systems (Siemens). MicroPET data were acquired for 10 min and reconstructed with a filtered background projection probability algorithm. MicroPET and CT images were coregistered. Quantification of PET signal was performed by drawing 3D region of interests (ROIs) around the area of interest using AMIDE software (http://amide.sourceforge.net/). The mean intensity of the ROI, based on the percent injected dose per gram, was normalized to control grafts of L1210-10K ROI drawn around untransduced grafts in the same animal. Data are presented as fold change over L1210-10K grafts. Images are presented here using a false-color scale that is proportional to tissue concentration (% injected dose/gram, %ID/g) of positron-labeled probe. Red represents the highest with yellow, green, and blue corresponding to lower concentrations.

In Vivo Uptake Assay. BM chimera animals were placed under anesthesia with 2% (vol/vol) isoflurane in heated chambers. One microcurie of [^{18}F]-L-FMAU was injected i.v. with 1-h unconscious probe uptake. Animals were euthanized and spleen, femur, tibia, and thymus were removed. Cells were then dissociated into single cell suspension and stained for CD45.1. In each tissue 200,000 cells of CD45.1, YFP$^+$ (donor and reporter labeled), or CD45.1 (donor) were sorted. The amount of radioactivity in each cell type was measured using a Wallac Wizard 3-Inch 1480 Automatic Gamma Counter (PerkinElmer).

Peripheral Blood Analysis. Peripheral blood was collected through serial retroorbital bleeds into Capiject EDTA collection tubes (T-MQK). Complete blood counts were performed by Department of Laboratory Animal Medicine, University of California, Los Angeles core laboratory with Hemavet. Engraftment was determined by flow cytomerty.

Methylcellulose Assay. One hdCK3mut and one YFP BM chimera animal 6 wk posttransplant were compared with a wild-type SJL (B6.SJL-Ptprc) mouse for each experiment in three independent experiments. Bone marrow from both femurs and tibia was extracted. Total bone marrow was plated in duplicate. FACS was used to isolate donor cells through sorting CD45.1 positive and YFP (reporter) positive or YFP negative; cells were plated in duplicate. Commercial methylcellulose for CFC was used (R&D Systems). Colonies were analyzed on day 11, and fluorescent analysis was analyzed by flow cytometry.

Integration Site Analysis. DNA was isolated from FACS-sorted cells using the PureLink Genomic DNA Mini kit (Invitrogen). Dependent on availability, 1–100 ng of DNA was used to perform nonrestrictive linear amplification-mediated PCR (3). Briefly, 100 cycles of linear amplification were performed with primer HIV3linear (biotin-AGTAGTGTGTGCCCGTCTGT). Linear reactions were purified using 1.5 volumes of AMPure XP beads (Beckman Genomics) and captured onto M-280 streptavidin Dynabeads (Invitrogen Dynal). Captured single strand DNA was ligated to read 2 linker (Phos-AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-3C spacer) using CircLigase II (Epicentre) in a 10-μL reaction at 65° for 2 h. PCR was performed on these beads using primer HIV3-right (AATGATACGGCGACCACCGAGATCTACACTGA-TCCCTCAGACCCTTTTAGTC) and an appropriate indexed reverse primer (CAAGCAGAAGACGGCATACGAGAT-index-GTGACTGGAGTTCAGACGTGT). PCR products were mixed and quantified by probe-based qPCR and appropriate amounts were used to load Illumina v3 flow cells. Paired-end 50-bp sequencing was performed on an Illumina HiSEq 2000 instrument using a custom read 1 primer (CCCTCAGACCCTTTTAGTC-AGTGTGGAAAATCTCTAGCA). Reads were aligned to the hg19 build of the human genome with Bowtie (4) and alignments were condensed and annotated using custom Perl and Python scripts to locate vector integrations. A custom Python script was written to assess for overlapping integration sites between different samples, and a conservative estimate of 5% FACS sorting impurity was used to set a cutoff to eliminate overlaps with technical causes.

Graphs, Statistics, and Survival Analysis. Graphs are plotted as mean with SE of mean (SEM) for error bars. Statistics were analyzed

using a Student's nonpaired *t* test. Survival analysis was plotted in Graphpad and determined based on overall length of animal survival. (Two animals were excluded from survival analysis due to premature death caused from malocclusion.)

1. Iyidogan P, Lutz S (2008) Systematic exploration of active site mutations on human deoxycytidine kinase substrate specificity. *Biochemistry* 47(16):4711–4720.
2. Campbell DO, et al. (2012) Structure-guided engineering of human thymidine kinase 2 as a positron emission tomography reporter gene for enhanced phosphorylation of a non-natural thymidine analog reporter probe. *J Biol Chem* 287(1):446–454.
3. Paruzynski A, et al. (2010) Genome-wide high-throughput integrome analyses by nrLAM-PCR and next-generation sequencing. *Nat Protoc* 5(8):1379–1395.
4. Langmead B, Schatz MC, Lin J, Pop M, Salzberg SL (2009) Searching for SNPs with cloud computing. *Genome Biol* 10(11):R134.

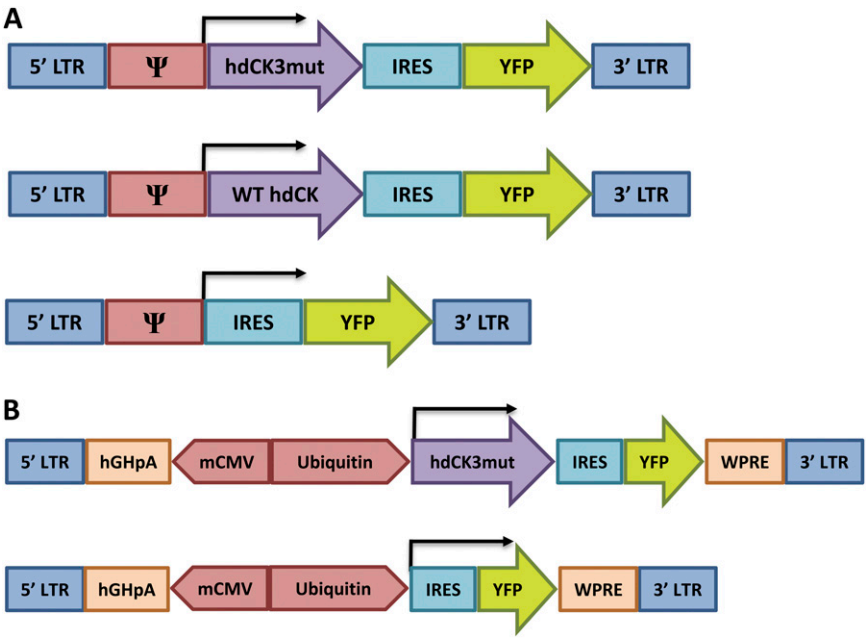

Fig. S1. (*A*) Vector maps of pMSCV retroviruses used in generating stable ectopic expressing L1210-10K cell lines, and mouse HSC experiments. (*B*) 2F-X lentivirus vector maps used in human HSC experiments.

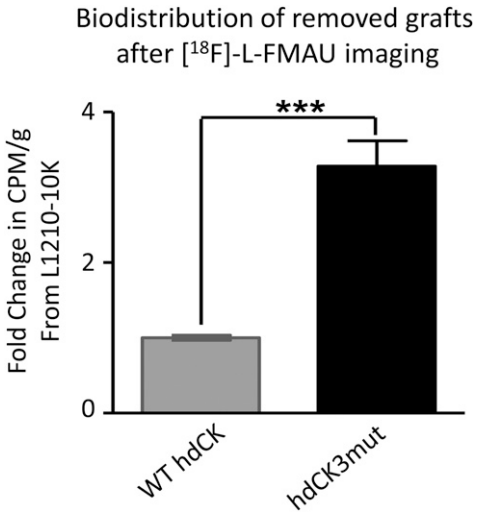

Fig. S2. Grafts were excised after [^{18}F]-L-FMAU MicroPET and were weighed and placed in a gamma counter. Counts were normalized to counts per minute per gram (cpm/g) and compared with nontransduced tumors for background cpm ($P < 0.0001$).

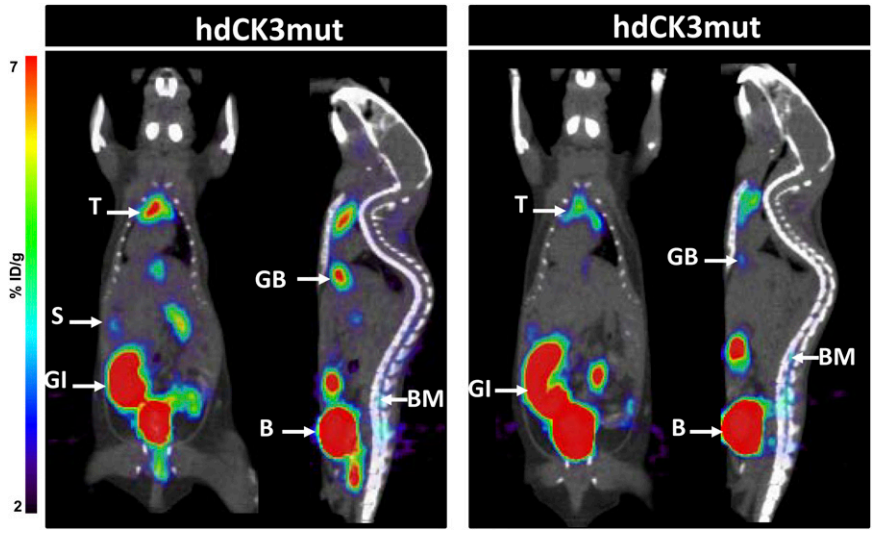

Fig. S3. Additional [^{18}F]-L-FMAU MicroPET scans at 4 wk postmouse BMT in reporter chimeric mice.

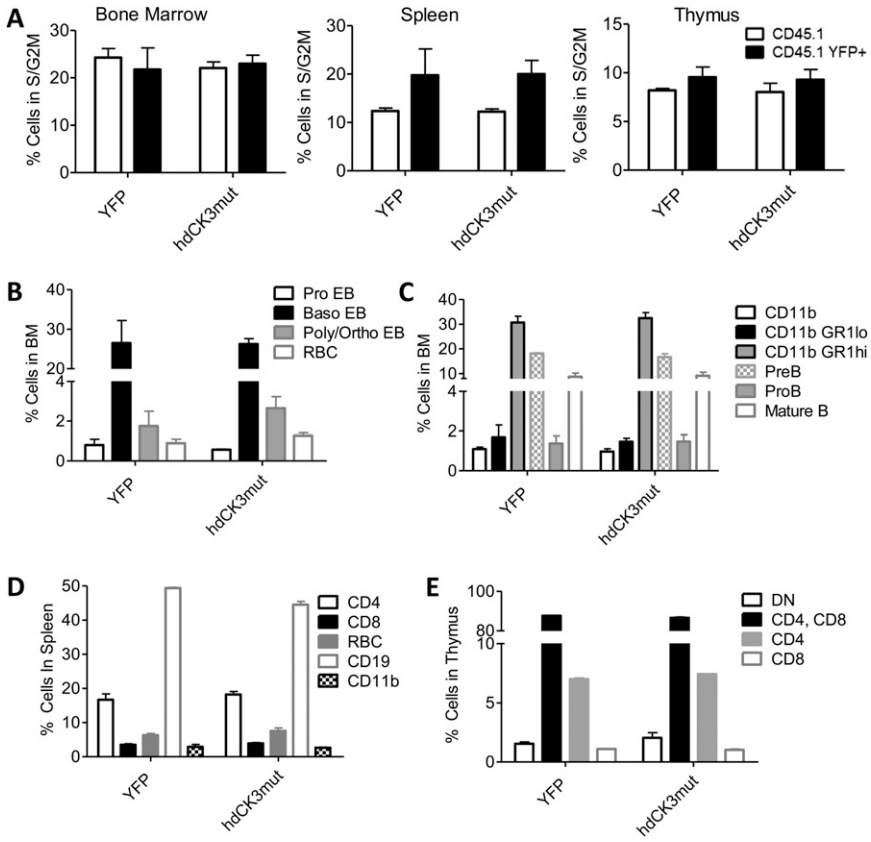

Fig. S4. Quantification of flow cytometry from hdCK3mut and YFP mHSC recipient mice. (*A*) Cell cycle analysis by Hoechst staining. (*B* and *C*) Analysis of bone marrow (BM) lineage distribution. (*B*) Red blood cell development with CD71 and Ter119 staining. Pro EB-CD71^{+}, Ter119^{-}; Baso EB-CD71^{+}, Ter119^{+}; Poly/Ortho EB-CD71 low, Ter119^{+}; RBC-CD71^{-}, Ter119^{+}. (*C*) Myeloid and B-cell development Pre B-B220^{+}, CD43^{-}; Pro B-B220^{+}, CD43^{+}; mature B-B220^{+}, IgM^{+}. (*D*) Total engraftment within the spleen. (*E*) Thymus engraftment.

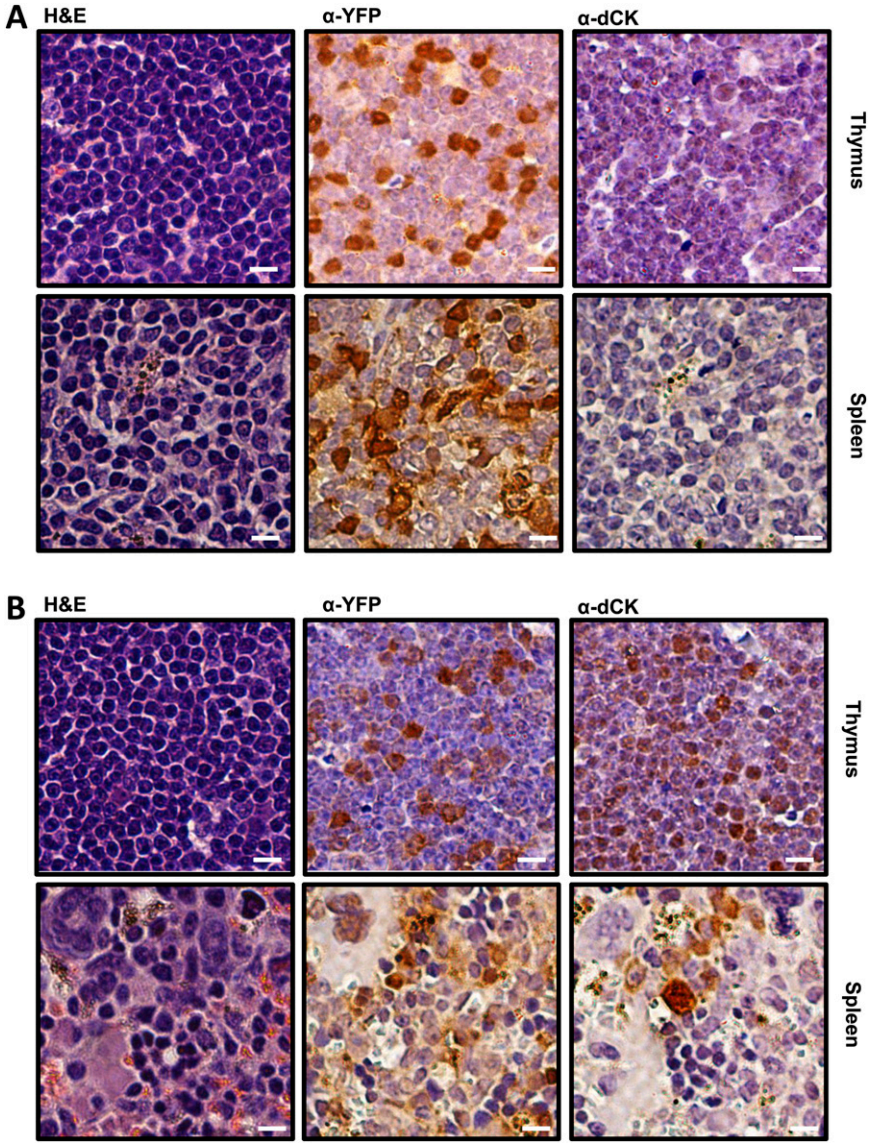

Fig. S5. IHC of mHSC recipient animals of (*A*) YFP and (*B*) hdCK3mut at 8 wk post-BMT. Spleen and thymus were analyzed for normal tissue architecture through H&E. IHC of α-YFP detected vector positive cells in YFP and hdCK3mut. α-dCK detected reporter cells only within hdCK3mut recipient mice. (Scale bar, 10 μm.)

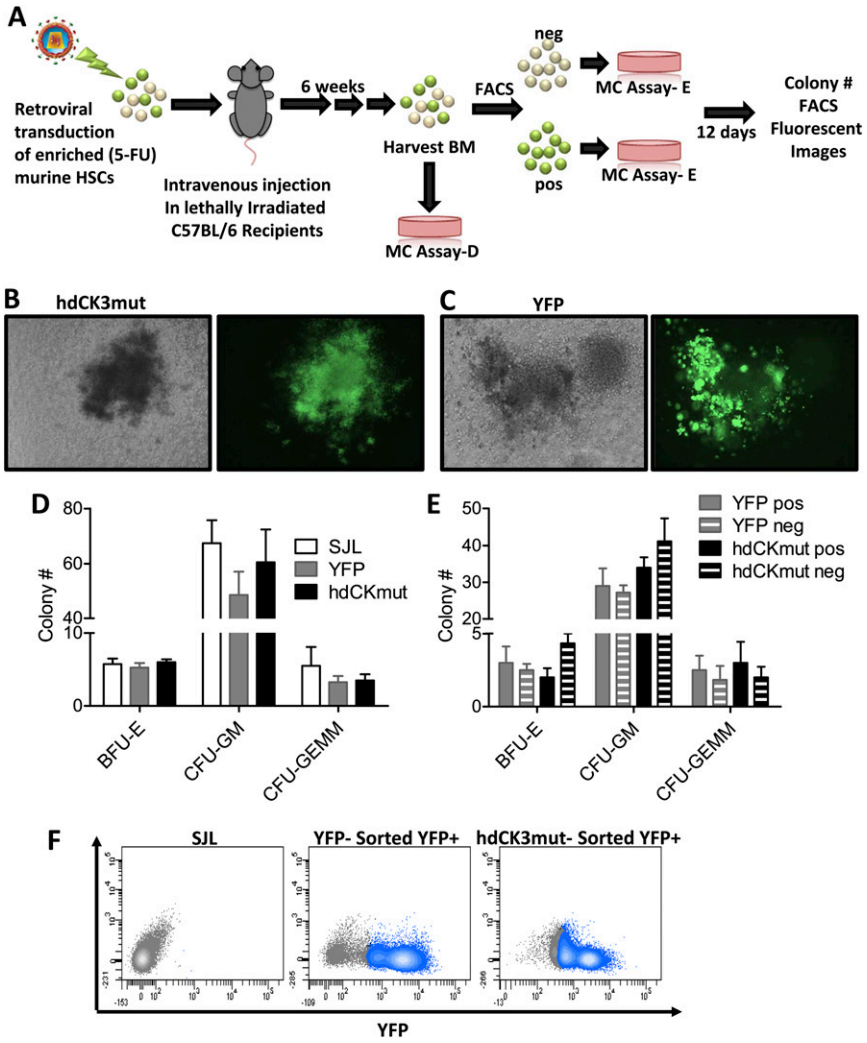

Fig. S6. (*A*) Schematic of MC assay. hdCK3mut or YFP recipients at 6 wk post-BMT were harvested for total bone marrow. (*B* and *C*) Representative white and fluorescent images demonstrate the normal morphology of methylcellulose colonies for hdCK3mut and YFP. (*D*) Cells were placed in a MC assay, with an aged matched normal BL6 (CD45.1). (*E*) Remaining bone marrow was sorted based on CD45.1 YFP^{+} or CD45.1 YFP^{-} and placed in MC assay. Total colonies were counted at 12 d postplating. (*F*) Cells from *C* were analyzed for retained YFP expression through flow cytometry.

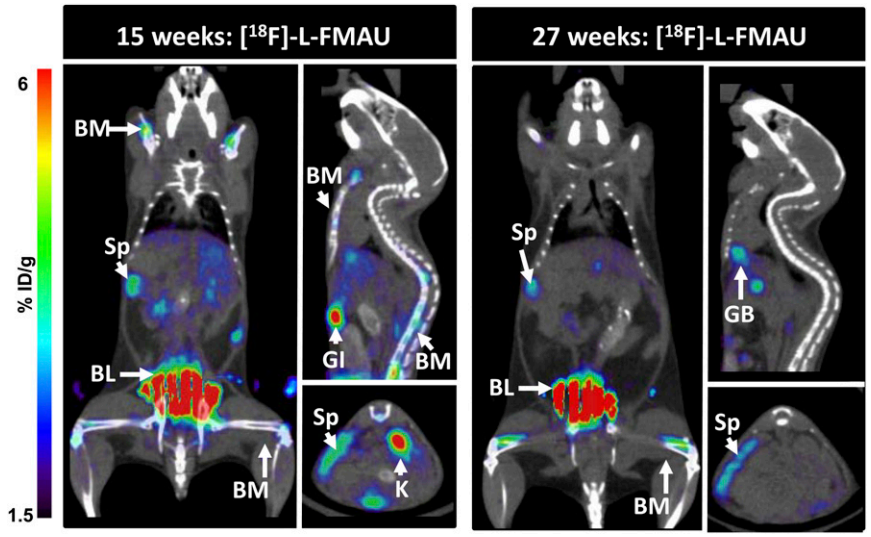

Fig. S7. Sequential scans of hdCK3mut recipient mice at 15 and 27 wk post-BMT. Reporter signal is observed within the spleen (Sp) and bone marrow (BM). Probe metabolism is seen within the kidneys (K), gallbladder (GB), and bladder (Bl).

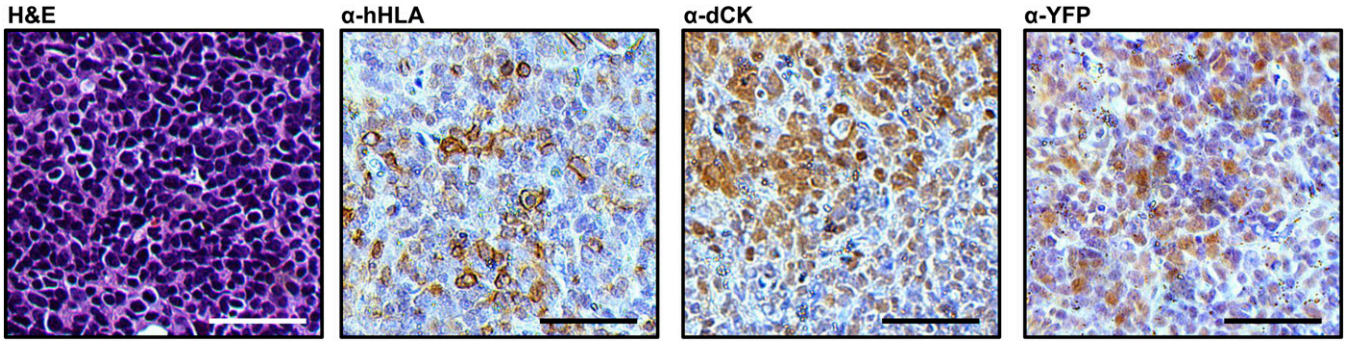

Fig. S8. IHC of hdCK3mut recipient hHSC recipient animal. Thymus was analyzed for normal tissue architecture through H&E. IHC of α-hHLA detected total human engraftment. α-YFP detected vector positive cells with α-dCK detecting hdCK3mut reporter cells. (Scale bar, 50 μm.)

Table S1. Enzyme kinetics for hdCK3mut and hdCKDM with L-FMAU

	L-FMAU		
Enzyme	K_m (μM)	V_{max}, nmol/min/μg	R^2
hdCK3mut	12.681 ± 3.893	0.724 ± 0.217	0.899
hdCKDM	55.970 ± 6.880	1.671 ± 0.312	0.975

Enzymatic studies were performed using His-tagged recombinant dCK in a coupled enzyme assay with LDH/PK. Values are an average of three independent experiments.