## Supplementary Information

## Supplementary Methods

## Genotyping

GO-ATeam2 mice were genotyped by PCR of tail DNA or by transdermal GFP fluorescence. The PCR protocol was as follows: $94^{\circ} \mathrm{C}$ for $5 \mathrm{~min} ; 34$ cycles of $94^{\circ} \mathrm{C}$ for $30 \mathrm{~s}, 56^{\circ} \mathrm{C}$ for $30 \mathrm{~s}, 72^{\circ} \mathrm{C}$ for 30 s ; $72{ }^{\circ} \mathrm{C}$ for 5 min ; and $4^{\circ} \mathrm{C}$ hold. Primers are listed in Table S6.

## Cell preparation

For C57BL/6 mice, BM cells were isolated from bilateral femurs and tibiae by flushing with PBS + $2 \%$ fetal calf serum (FCS) (Gibco) using a 21-gauge needle (Terumo Corporation, Tokyo, Japan) and a 10 mL syringe (Terumo). As an exception, for $\mathrm{U}^{-13} \mathrm{C}_{6}$-labeled glucose tracer experiments using C57BL/6 mice, BM was flushed with PBS $+0.1 \%$ bovine serum albumin (BSA, Cat\# A4503). The BM plug was dispersed by refluxing through the needle, and the suspension was centrifuged $680 \times g$ for 5 min at $4^{\circ} \mathrm{C}$. Cells were lysed with lysis buffer ( $0.17 \mathrm{M} \mathrm{NH}_{4} \mathrm{Cl}, 1 \mathrm{mM}$ EDTA, 10 mM NaHCO 3 ) at room temperature (RT) for 5 min , washed with two volumes PBS $+2 \% \mathrm{FCS}$ (or PBS $+0.1 \%$ BSA for tracer experiments), and centrifuged at $680 \times g$ for 5 min at $4^{\circ} \mathrm{C}$. Cells were resuspended in PBS $+2 \%$ FCS (or PBS $+0.1 \%$ BSA for tracer experiments) and filtered through $40 \mu \mathrm{~m}$ nylon mesh (BD Biosciences). Cells were again centrifuged $680 \times g$ for 5 min at $4^{\circ} \mathrm{C}$ and treated with anti-CD16/32 antibody for Fc-receptor block ( $2 \mu \mathrm{~L} /$ mouse; BD Biosciences, Cat $\# 553152$ ) for 10 min at $4^{\circ} \mathrm{C}$. Anti-c-Kit magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany, Cat\# 130-091-224) were added at a $1: 5 \mathrm{v} / \mathrm{v}$ ratio for 15 min at $4{ }^{\circ} \mathrm{C}$. After removing the antibody with two PBS $+2 \%$ FCS (or PBS + $0.1 \%$ BSA for tracer experiments) washes, c-Kit-positive cells were isolated using Auto-MACS Pro (Miltenyi Biotec) with the Possel-s or Possel-d2 program. Isolated cells were centrifuged once at 340 $\times g$ for 5 min and stained with an antibody cocktail for flow cytometry.
For analysis of the GO-ATeam2 hematopoietic cells, BM from GO-ATeam2 mice was flushed with PBS $+0.1 \%$ BSA to minimize exposure to nutrients in FCS. Hemolysis, centrifugation, filtering, and Fc receptor blocking were performed in the same manner as for cell preparation using C57BL/6 mice. Cells were stained for 30 min with an antibody cocktail at $4^{\circ} \mathrm{C}$ and then washed and suspended in $1000 \mu \mathrm{~L}$ PBS $+0.1 \%$ BSA and centrifuged at $340 \times g$ at $4{ }^{\circ} \mathrm{C}$ for 5 min . Supernatants were discarded in preparation for flow cytometry.

Flow cytometry and cell sorting

Murine hematopoietic stem and progenitor fractions were labeled as follows: To stain cells from C57BL/6 mice, lineage (Lin) markers (CD4, CD8a, Gr-1, Mac-1, Ter-119, B220)-PerCP-Cy5.5 (BD Biosciences for CD4 (Cat\# 550954), Gr-1 (Cat\# 552093), Mac-1 (Cat\# 550993), B220 (Cat\# 552771) and BioLegend for CD8a (Cat\# 100734) and Ter-119 (Cat\# 116228) antibodies), c-Kit-APC-Cy7 (BioLegend, Cat\# 105826), Sca-1-PE-Cy7 (BioLegend, Cat\# 122514), CD150-PE (BioLegend, Cat\# 115904), CD48-FITC (BioLegend, Cat\# 103404), and Flt3-APC (BioLegend, Cat\# 135310) were used. For HSC collection after 5-FU administration (intraperitoneally or intravenously), Mac-1 antibody was excluded from the antibody cocktail ${ }^{1}$ and the LSK gate was expanded to include c-Kit-high to -dim Lin ${ }^{-}$cells ${ }^{2}$. When sorting or analyzing $\mathrm{EPCR}^{+} \mathrm{CD} 150^{+} \mathrm{CD} 48{ }^{-} \mathrm{LSK}$ cells from C57BL/6 mice or mVenus-p27K-mice, CD150-BV421 (BioLegend, Cat\# 115926), CD48-APC (BioLegend, Cat\# 103412), and EPCR-PE (Biolegend, Cat\# 141503) were used in addition to LSK for staining, and FLT3 staining was excluded. To stain cells from GO-ATeam2 mice, lineage markers (CD4, CD8a, Gr-1, Mac-1, Ter-119, B220)-PerCP-Cy5.5, c-Kit-APC-Cy7, Sca-1-PE-Cy7, CD150-BV421, and CD48APC were used. In the analysis using GO-ATeam2 mice, Flt3 was not used to define HSCs because the fluorescence of the FRET sensor (EGFP, mKO) limits the available fluorochromes for surface marker staining. In analysis using the AMPK inhibitor dorsomorphin (Cayman Chemical, Cat\# 21207), CD150-APC (BioLegend, Cat\# 115910) and CD48-AlexaFluor700 (BioLegend, Cat\# 103426) were used to stain LSK-SLAM to eliminate effects of dorsomorphin fluorescence on cell staining. Cells were resuspended in $0.5-2 \mathrm{~mL}$ of $\mathrm{PBS}+2 \% \mathrm{FCS}+0.1 \%$ propidium iodide (PI) (Invitrogen, Cat\# P3566) (for C57BL/6 mice) or PBS $+0.1 \%$ BSA (for GO-ATeam2 mice) and sorted using the FACSAria IIIu Cell Sorter (BD Biosciences) into RPMI1640 (without glucose) (Nacalai Tesque, Cat\# 09892-15) containing 4\% w/v BSA or GO-ATeam2 basal medium (Ba-M, Table S1 with $4 \% \mathrm{w} / \mathrm{v}$ BSA) (custom made by Gmep Inc.). Murine HSCs were defined as CD150 ${ }^{+}$CD48-Flt3-LSK (for C57BL/6 mice) or CD150 ${ }^{+}$CD48-LSK (for GO-ATeam 2 mice and mVenus-p27K- mice, and when EPCR was included in the antibody cocktail against C57BL/6 mice) cells. MPPs were defined as CD150 CD48 ${ }^{+}$Flt3- ${ }^{-}$LSK (for C57BL/6 mice) or CD150 ${ }^{-}$CD48 ${ }^{+}$LSK (for GO-ATeam 2 mice) cells. Among myeloid progenitors (MyPs), GMPs/MEPs/CMPs were defined as follows: GMPs (CD16/32 ${ }^{+}$CD34 ${ }^{+}$), MEPs (CD16/32- CD34 ${ }^{+}$), and CMPs (CD16/32- CD34 ${ }^{+}$). CLPs were defined as Lin ${ }^{-} \mathrm{Sca}^{-} \mathrm{1}^{\text {low }} \mathrm{c}$ -

Kit $^{\text {low }}{ }^{\text {Flt3 }}{ }^{+}$IL7R $\alpha^{+}$cells. Data were analyzed using FlowJo ${ }^{\text {TM }}$ V10 (Tree Star) software.

## Intracellular staining for phosphorylated Rb (pRb)

$\mathrm{EPCR}^{+}$or EPCR ${ }^{-}$LSK-SLAM cells from PBS- or 5-FU-treated C57BL/6 mice were purified separately
(see "Flow cytometry and cell sorting" for details). Anti-phospho-Rb (Ser807/811) antibody (CST, Cat\# 8516T) was used as the primary antibody and Anti-rabbit $\operatorname{IgG}(\mathrm{H}+\mathrm{L}), \mathrm{F}(\mathrm{ab}$ ') Fragment (Alexa Fluor488 Conjugate) (CST, Cat\# 4412) was used as the secondary antibody. Fixation and permeabilization were performed according to the manufacturer protocol. pRb and DNA content (stained with PI) were analyzed by flow cytometry.

## Analysis of mVenus-p27K-mouse-derived BM cells

mVenus-p27K-mice were provided by Dr. Yosuke Tanaka and Dr. Toshio Kitamura. Surface-markerstained BMMNCs (see "Flow cytometry and cell sorting" for details) were analyzed by flow cytometry to determine the frequency of G0 marker positivity for $\mathrm{EPCR}^{+}$or $\mathrm{EPCR}^{-} \mathrm{CD} 150^{+} \mathrm{CD} 48{ }^{-}$ LSK or progenitor cells.

## In vivo 2-NBDG assay

C57BL/6 mice treated with PBS or 5-FU were subjected to an in vivo 2-NBDG assay as reported by Jun et al. ${ }^{3}$. Mice received a bolus dose of $375 \mu \mathrm{~g} 2-\mathrm{NBDG}$ (Cayman Chemical, Cat\# 11046) intravenously and were euthanized by cervical dislocation after 1 h . Mice were immediately placed on ice, and all subsequent cell preparation processes were performed while the cells were chilled on ice The 2-NBDG positive cell fraction was detected by flow cytometry.

## Conversion of GO-ATeam 2 fluorescence to ATP concentration

A study presenting the significance of measuring the absolute concentration of ATP at the single-cell level is currently in preparation for submission, but briefly, the FRET efficiency was converted to the absolute concentration of ATP using the following method (Watanuki et al., in preparation). To permeabilize BM cells, $\alpha$-hemolysin stock solution (Sigma-Aldrich, St. Louis, MO, USA) was diluted in permeabilization buffer ( 140 mM KCl (Wako, Cat\# 163-03545), 6 mM NaCl (Wako, Cat\# 19101665), 0.1 mM EGTA (Wako, Cat\# QB-6401), and 10 mM HEPES (Wako, Cat\# 342-01375) [pH 7.4]) to a final concentration of $50 \mu \mathrm{~g} / \mathrm{mL} \alpha$-hemolysin. GO-ATeam2-knock-in BMMNCs were added to the buffer and permeabilized for 30 min at $37{ }^{\circ} \mathrm{C}$ under $5 \% \mathrm{CO}_{2}$. To calibrate ATP concentration, calibration buffer ( $140 \mathrm{mM} \mathrm{KCl}, 6 \mathrm{mM} \mathrm{NaCl}, 0.5 \mathrm{mM}$ MgCl2 (Wako, Cat\# 136-03995), and 10 mM HEPES [pH 7.4]) and Mg-ATP stock solution (Sigma-Aldrich, Cat\# A9187) were prepared. After washing GO-ATeam2-knock-in BMMNCs with calibration buffer, fresh calibration buffer without ATP was added. Mg-ATP was gradually added to increase ATP concentration in the cell suspension, and FRET values of the GO-ATeam2 biosensor at defined ATP concentrations were analyzed by flow
cytometry. The FRET value (relative ratio of FRET to EGFP fluorescence intensities) was calculated by the following equation.

$$
\text { FRET value }=\frac{\text { Fluorescence of FRET }}{\text { Fluorescence of EGFP } \ldots(1)}
$$

The excitation wavelength of FRET and EGFP was set at 488 nm .
The FRET value was then fitted to Hill's formula ${ }^{4}$ as a function of ATP concentration:

$$
\begin{equation*}
\theta=\frac{[L]^{n}}{\left[K_{A}\right]^{n}+[L]^{n}} \cdots \tag{2}
\end{equation*}
$$

where $\theta$ is the original percentage of receptor proteins occupied by the ligand, $[L]$ is the free (unbound) ligand concentration, $K_{A}$ is the concentration of ligand at half saturation, and $n$ is Hill's coefficient.

Equation (2) was transformed as $\log \left(\frac{\theta}{1-\theta}\right)=n \log [L]-n \log K_{A} \ldots$ (3)
such that $\theta$ could be expressed by the FRET value as follows:

$$
\begin{equation*}
\theta=\frac{\text { FRET value }-1.4}{6} \ldots \tag{4}
\end{equation*}
$$

We estimated parameters $n$ and $K_{A}$ by fitting observed FRET values to the linear regression model represented in equation (3). In our experiment, $n=3.1234$, and $K_{A}=0.84699$. Using these parameters, cellular ATP concentration, [L], was estimated.

## Time-course analysis of FRET values

Surface-marker-stained BMMNCs from GO-ATeam2 mice were dispensed into $\mathrm{Ba}-\mathrm{M}$ containing minimal salts, vitamins, and buffers (HEPES and sodium bicarbonate), but no glucose or mitochondrial substrates (Table S 1 ), or into PLFA medium. The FRET/EGFP ratio was analyzed in a real-time manner using the BD FACSAria IIIu Cell Sorter under ambient pressure. Depending on their purpose, experiments were conducted in the presence or absence of various nutrients or metabolic modulators (Supplemental Figure 4A). For this platform, 2 mL of $\mathrm{Ba}-\mathrm{M}$ or PLFA medium per tube was presaturated with $1 \% \mathrm{O}_{2} / 5 \% \mathrm{CO}_{2} / 94 \% \mathrm{~N}_{2}$ to stabilize ATP levels of BMMNCs (Supplemental Figure 4B) and mimic the hypoxic BM environment; when medium was not pre-saturated, ATP concentrations rapidly decreased, even in the presence of glucose, pyruvate, or lactate (Supplemental Figure 4C).
To reduce the effect of autofluorescence as much as possible, the top $40-50 \%$ of EGFP and FRET fractions of MFI were used in the analysis (MFI > 1000 for EGFP and FRET). Then, data reporting EGFP and FRET fluorescence values in individual cells from each gating (e.g. HSCs, MPPs) were extracted along with time course data. Relevant nutrients and inhibitors were added to medium with
samples for analysis. Data acquired by the FACSAria IIIu device and retrieved as FCS files were analyzed by the flowCore package in R software. The FRET/EGFP ratio of each set of single cells was fitted to a generalized additive model using the 'gam' function in the 'mgcv' package with 's', a splinebased smoothing function, in default settings as a function of time, then smoothened using the 'predict' function. Pseudocolor plots of the FRET/EGFP ratio were created using the ' $k d e 2 d$ function'. If needed, fitted data were converted to ATP concentration using the model described above.

To compare changes in ATP concentrations in PBS- and 5-FU-treated groups in Figure 4, Figure 6F-G, we corrected differences in baseline ATP concentrations by multiplying all data from the PBS-treated group by the following value: ATP concentration at 0 s in the 5 -FU group/ATP concentration at 0 s in the PBS group.

## Ki67/Hoechst staining

Ki67 (BD Biosciences, Cat\# 558617) and Hoechst 33432 (Invitrogen, Cat\# H3570) were used for cell cycle analysis of fixed cells from C57BL/6 mice. A total of $4 \times 10^{6} \mathrm{BMMNCs} /$ sample were stained with anti-CD150-APC, anti-CD48-FITC, anti-lineage (CD4, CD8a, Gr-1, Mac-1, Ter-119, B220)-PerCPCy5.5, anti-c-Kit-APC-Cy7, and anti-Sca-1-PE-Cy7 antibodies. To stain samples after 5-FU treatment, Mac-1 was excluded from the antibody cocktail. Stained samples were centrifuged at $340 \times g$ and $4^{\circ} \mathrm{C}$ for 5 min . Next, $250 \mu \mathrm{~L}$ of BD Cytofix/Cytoperm (BD Biosciences, Cat\# 555028) was added, and samples were incubated for 20 min at $4^{\circ} \mathrm{C}$ for fixation. Fixed cells were centrifuged and washed twice at $340 \times g$ at $4{ }^{\circ} \mathrm{C}$, with 1 mL BD Perm/Wash buffer (BD Biosciences, Cat\# 554723) diluted 10 -fold. Each sample was stained with $10 \mu \mathrm{~L}$ of Ki67-Alexa555 for 1 h at RT, shaded from light. Ki67-stained cells were centrifuged and washed twice at $340 \times g$ and $4^{\circ} \mathrm{C}$ with PBS. Samples were resuspended in $500 \mu \mathrm{~L}$ of PBS $+10 \mu \mathrm{~g} / \mathrm{mL}$ Hoechst 33432, filtered, and analyzed with the BD FACSAria IIIu instrument.

## Analysis of peripheral blood and BM chimerism

Periorbitally collected peripheral blood from BMT recipients was centrifuged for 3 min at $340 \times g$ and the supernatant discarded. Samples were subjected to hemolysis with $1000 \mu \mathrm{~L}$ of $0.17 \mathrm{M} \mathrm{NH}_{4} \mathrm{Cl}$ for $40-50 \mathrm{~min}$ and centrifuged at $340 \times g$ for 5 min . The supernatant was discarded, and samples were again subjected to hemolysis with $1000 \mu \mathrm{~L}$ of $0.17 \mathrm{M} \mathrm{NH}_{4} \mathrm{Cl}$ for $10-20 \mathrm{~min}$. Samples were centrifuged again at $340 \times g$ for 5 min and the supernatant was discarded. Pellets were then resuspended in $50 \mu \mathrm{~L}$ PBS and $0.3 \mu \mathrm{~L}$ Fc receptor block and incubated at $4^{\circ} \mathrm{C}$ for 5 min . Surface antigen staining was performed using the following antibody panel: Gr-1-PE-Cy7 (BioLegend, Cat\# 108416), Mac-1-PE-

Cy7 (BioLegend, Cat\# 101216), B220-APC (BioLegend, Cat\# 103212), CD4-PerCP-Cy5.5, CD8a-PerCP-Cy5.5, CD45.1-PE (BD Biosciences, Cat\# 553776), and CD45.2-FITC (BD Biosciences, Cat\# 553772). An antibody cocktail was prepared by mixing $0.3 \mu \mathrm{~L}$ of each antibody. The frequency (\%) of donor-derived cells was calculated as follows:

The frequency (\%) of donor-derived cells $=100 \times$ Donor-derived $\left(\right.$ Ly $5.2^{+}$Ly5.1 $\left.{ }^{\circ}\right)$ cells $(\%) /($ Donorderived cells [\%] + Competitor- or recipient-derived [Ly5.2-Ly5.1 ${ }^{+}$] cells [ $\%$ ]].
Myeloid, B , and T cells were identified by $\mathrm{Gr}-1^{+}$or $\mathrm{Mac}-1^{+}, \mathrm{B} 220^{+}$, or $\mathrm{CD}^{+}$or $\mathrm{CD} 8^{+}$, respectively.
Four months after BM transplant, the frequency of donor-derived cells in BM was determined using one femur and tibia per recipient. Anti-CD150-BV421, anti-CD48-PE (BD Biosciences, Cat\# 557485), anti-lineage (CD4, CD8a, Gr-1, Mac-1, Ter-119, B220)-PerCP-Cy5.5, anti-c-Kit-APC-Cy7, anti-Sca-1-PE-Cy7, anti-Ly5.1-Alexa-Fluor700 (BioLegend, Cat\# 110724), and anti-Ly5.2-FITC antibodies were used for surface antigen detection. An antibody cocktail was prepared by mixing $1 \mu \mathrm{~L}$ of each antibody.

## Comparison of metabolite levels before and after sorting

c-Kit-positive cells were isolated using Auto-MACS Pro (Miltenyi Biotec) with the Possel-s or Posseld2 program as described above (see "Cell preparation" for details). Isolated cells were counted, and $1 \times 10^{5}$ viable cells were dispensed into methanol containing an internal standard as a pre-sorting cell sample and stored at $-80^{\circ} \mathrm{C}$ until IC-MS analysis. To the isolated cell suspension, $0.1 \% \mathrm{PI}$ was added and samples were sorted using the FACS Aria IIIu. A total of $1 \times 10^{5}$ viable cells ( $\mathrm{PI}^{-}$cells) were sorted directly into methanol containing an internal standard as a post-sorting cell sample and stored at $-80^{\circ} \mathrm{C}$ until IC-MS analysis. The detected metabolites were quantified based on calibration curve data (see "Ion chromatography mass spectrometry (IC-MS) analysis" in "Methods" for details).

## Preparation and storage of in vitro $\mathbf{U -}{ }^{13} \mathbf{C}_{\mathbf{6}}$-glucose tracer samples

For tracer analysis, C57BL/6 mice were euthanized to obtain 25,000-50,000 cells of each fraction (HSC, MPP, GMP, CLP) from BM using the FACSAria IIIu instrument. Numbers of mice used to obtain each sample were as follows: 30-35 each for steady state HSCs and MPPs, 60-65 each for 5FU treated HSCs, 10 each for GMPs and CLPs. In addition, bone and BM cells were chilled by placing dishes and tubes on ice during the cell preparation process; samples were washed with ice-cold buffer throughout the entire process before cell sorting. Experiments and experimental manipulations regarding the sampling of mouse femurs and tibias were also performed in the shortest amount of time possible by skilled personnel. Sorted cells were centrifuged at $340 \times g$ and $4^{\circ} \mathrm{C}$ for 5 min . After
discarding the supernatant, cells were added to 1 mL pre-saturated GO-ATeam $2 \mathrm{Ba}-\mathrm{M}+0.1 \% \mathrm{BSA}+$ $200 \mathrm{mg} / \mathrm{dL} \mathrm{U-}{ }^{13} \mathrm{C}_{6}$ - (Sigma-Aldrich, Cat\# 389374) or U- ${ }^{12} \mathrm{C}_{6}$-glucose and incubated 10 or 30 min . If the process of pre-saturation was omitted, ATP levels dropped rapidly within a short time (Supplementary Figure 5G).When using oligomycin ( $1 \mu \mathrm{M}$ ) (Cell Signaling Technology, Cat\# 9996), exposure time was set to 10 min . Samples were then immediately centrifuged at $1000 \times g$ and $4{ }^{\circ} \mathrm{C}$ for 3 min . After discarding supernatants, cells were frozen and stored at $-80^{\circ} \mathrm{C}$.

## Preparation and storage of in vivo $\mathrm{U}-{ }^{13} \mathrm{C}_{6}$-glucose tracer samples

U- ${ }^{13} \mathrm{C}_{6}$-glucose administration to C57BL/6 mice was performed based on the methods of Jun et al. ${ }^{3}$, with some modifications. Mice were intraperitoneally administered medetomidine hydrochloride, midazolam, and butorphanol tartrate at $0.75 \mathrm{mg} / \mathrm{kg}, 4 \mathrm{mg} / \mathrm{kg}$, and $5 \mathrm{mg} / \mathrm{kg}$, respectively. After anesthesia, mice were kept warm on a hot plate set at $37^{\circ} \mathrm{C}$ while a 27 -gauge needle was placed in the external tail vein and $\mathrm{U}_{-}{ }^{13} \mathrm{C}_{6}$-glucose was continuously administered. The dose and duration of U- ${ }^{13} \mathrm{C}_{6}$ glucose administration followed Jun et al. ${ }^{3}$, and $0.4125 \mathrm{mg} / \mathrm{g}$ body mass was administered in 1 min , followed by $0.008 \mathrm{mg} / \mathrm{g}$ body mass per minute for 3 h . After $\mathrm{U}^{13} \mathrm{C}_{6}$-glucose administration, mice were euthanized by cervical dislocation and immediately placed on ice. For in vivo tracer analysis, BMMNCs from the bilateral femur, tibia, pelvis, and sternum of each mouse were used to prepare sufficient numbers of HSCs, and pre-chilled $0.1 \%$ BSA + PBS was used for BM flushing and washing. HSCs were directly sorted in methanol and stored at $-80^{\circ} \mathrm{C}$ until IC-MS analysis. A total of $1-3 \times 10^{4}$ HSCs were purified from one or two mice in the PBS group and from two or three mice in the 5-FU group.

When generating the heat map of labeling rates in each metabolite, 1 was added as a pseudo number to the labeling rate of all metabolites. When calculating the total amount of ${ }^{13} \mathrm{C}$ labeled metabolites for each pathway, metabolites other than $\mathrm{M}+0$ were summed in each metabolite.

## Metabolite extraction

Frozen samples were mixed with $500 \mu \mathrm{~L}$ methanol containing internal standards and sonicated for 10 s. Then, $200 \mu \mathrm{~L} \mathrm{ddH} \mathrm{H}_{2} \mathrm{O}$ (Invitrogen, Cat\# 10977-015) and $400 \mu \mathrm{~L}$ chloroform (Nacalai tesque, Cat\# 08402-55) were added and samples were centrifuged at $10000 \times g$ and $4^{\circ} \mathrm{C}$ for 3 min . The aqueous phase was transferred to an Amicon ultrafiltration system (Human Metabolome Technologies, Inc., Cat\# UFC3LCCNB-HMT) and centrifuged at $9100 \times g$ and $4{ }^{\circ} \mathrm{C}$ for 3 h . Filtered samples were analyzed by IC-MS.

## Quantitative ${ }^{13}$ C-MFA with OpenMebius

OpenMebius (Open source software for ${ }^{13} \mathrm{C}$-MFA) provides the platform to simulate isotope labeling enrichment from a user-defined metabolic model setup worksheet developed in MATLAB (MathWorks, Natick, MA, USA) 5 . Quantitative ${ }^{13} \mathrm{C}$-MFA was performed according to a manual prepared by the software developer (http://www-shimizu.ist.osakau.ac.jp/hp/en/software/OpenMebius.html), but some metabolic model modifications were made to more faithfully reflect our measured data. Specifically, the model was modified to include (a) the conversion of pyruvate to lactate catalyzed by lactate dehydrogenase, (b) the formation of citrate from acetyl CoA and oxaloacetate catalyzed by citrate synthase, (c) the synthesis of alpha-ketoglutarate from citrate catalyzed by aconitase and isocitrate dehydrogenase, and (d) the synthesis of fumarate from succinate by succinate dehydrogenase. Reactions with pyruvate formate lyase performed by Escherichia coli, Streptococcus spp., and ethanol fermentation of acetyl CoA were excluded from the default metabolic network sheet.

The lactate efflux values in ${ }^{13} \mathrm{C}$-MFA were determined using the following trial and error method. First, various values $(0-100)$ were entered as candidate lactate efflux values and simulations were run to determine the optimal lactate efflux. When the lactate efflux value was set low (below 50), either the simulation could not be run and an error occurred, or the simulation resulted in the glycolytic system progressing in the opposite direction. These results suggested that the appropriate solution was not obtained because the lactate efflux was unnatural compared to the level of glycolytic metabolites. This was validated by experimental data showing that isotopic labeling rates for most glycolytic metabolites were close to $100 \%$ at short labeling times (Supplementary Figure 2C). Therefore, we ran the simulation with a higher lactate efflux value. Finally, we set the lactate efflux to 65 , which yielded reasonably satisfactory results for nearly $100 \%$ labeling of glycolytic and PPP metabolites in PBS- or DMSO-treated HSCs.

The rate of lactate efflux 5-FU-treated HSCs with the rate of glucose uptake set to 100 was defined using the following equation, with the flux in stationary phase HSC set to 65: $65 \times$ (Percentage of glycolytic metabolites labeled with ${ }^{13} \mathrm{C}$ in the total ${ }^{13} \mathrm{C}$-labelled metabolites [5-FUtreated HSCs] $) /\left(\right.$ Percentage of glycolytic metabolites labeled with ${ }^{13} \mathrm{C}$ in the total ${ }^{13} \mathrm{C}$-labelled metabolites [PBS-treated HSC])

In the metabolic flux measurements of HSCs under mitochondrial stress, the lactate efflux determined by the above method exceeded the maximum value that could be modeled ( $85>$ ), so we decreased the lactate efflux flux by 5 and adopted the maximum value, 80 , at which modeling became possible. For values of efflux other than those of lactate efflux flux, the values specified by the OpenMebius manual
were used to eliminate arbitrary factors as much as possible.
The metabolic substrate used for labeling was set to $100 \% \mathrm{U}-{ }^{13} \mathrm{C}_{6}$ glucose. Metabolites used in the analysis included the first intermediate metabolite produced when $\mathrm{U}-{ }^{13} \mathrm{C}_{6}$ glucose is metabolized (e.g., G6P or F6P with all carbons labeled, the labeled metabolite of the first cycle of the TCA cycle) and the unlabeled metabolite that was measured. Some of the labeled metabolites in the TCA cycle (e.g., citrate [M2]) and erythrose 4-phosphate (M4) in PPP were detected with non-negligible amounts of natural isotopes ( $>5 \%$ even when labeled with $\mathrm{U}-{ }^{12} \mathrm{C} 6$ glucose compared to $\mathrm{U}-{ }^{13} \mathrm{C}_{6}$ glucose). The presence of such natural isotopes may result in overestimation of the amount of increased labeling with U- ${ }^{13} \mathrm{C}_{6}$ glucose. In such cases, the amount of natural isotope detected when labeled with $\mathrm{U}-{ }^{12} \mathrm{C}_{6}$ glucose was subtracted from the amount of labeled metabolite detected with $\mathrm{U}-{ }^{13} \mathrm{C}_{6}$ glucose. If the resulting true labeled isotope abundance was negative, the labeled amount was modeled as zero. When analyzing in MATLAB, the number of modeling cycles was set to 100 , and the iteration time was set to a maximum of 2000 cycles.

## Luminometric ATP measurement

HSCs were sorted from C57BL/6 mice treated with PBS or 5-FU and dispensed into pre-saturated GOATeam 2 medium with $0.1 \% \mathrm{BSA}$ in a $1 \% \mathrm{O}_{2} / 5 \% \mathrm{CO}_{2}$ incubator. HSCs were then exposed to $15 \mu \mathrm{M}$ of PFKFB3 inhibitor (AZ PFKFB3 26) or DMSO and placed in a $1 \% \mathrm{O}_{2} / 5 \% \mathrm{CO}_{2}$ incubator for 10 min . Cells were centrifuged at $4^{\circ} \mathrm{C}$ and $340 \times g$ and the supernatant was removed. ATP measurements were performed according to manufacturer instructions using Cell ATP Assay Reagent Ver. 2 (Toyo B- Net Corporation). The amount of ATP per cell was calculated by dividing the amount of ATP detected by the number of cells used for analysis.

## Apoptosis assay of HSC after 2-DG or oligomycin treatment

Purified C57B6/J mouse-derived HSCs were exposed to 2-DG ( 50 mM ) and oligomycin ( $1 \mu \mathrm{M}$ ) in pre-saturated $0.1 \%$ BSA+GO-ATeam 2 medium under $1 \% \mathrm{O}_{2} / 5 \% \mathrm{CO}_{2}$ conditions for 10 min and subjected to apoptosis assay using the PE Annexin V Apoptosis Detection Kit I (BD Biosciences, Cat\# 559763) according to manufacturer instructions.

## CRISPR/Cas9 knockout (KO) of Pfkfb3

Target sequences of single guide RNA (sgRNA) were provided in a previous report ${ }^{6}$ and identified using the web tool GenScript (https://www.genscript.com) for Pfkfb3. sgRNAs were synthesized using
a CUGA7 gRNA Synthesis Kit (Nippon Gene, Tokyo, Japan, Cat\#314-08691) following manufacturer instructions, diluted to $1.5 \mu \mathrm{~g} / \mu \mathrm{L}$, and cryopreserved at $-80^{\circ} \mathrm{C}$ until use. CD150 ${ }^{+} \mathrm{CD} 48^{-}$Flt3 ${ }^{-}$LSK cells sorted by FACSAria IIIu were cultured in SF-O3 medium supplemented with stem cell factor (SCF) $(50 \mathrm{ng} / \mathrm{mL})$ (Peprotech, Cat\# 250-03) and thrombopoietin (TPO) (Peprotech, Cat\# 300-18) ( $50 \mathrm{ng} / \mathrm{mL}$ ) (S50T50 medium) and incubated under $20 \% \mathrm{O}_{2} / 5 \% \mathrm{CO}_{2}$ conditions for $16-24 \mathrm{~h}$, enabling subsequent HSC-specific gene editing with the CRISPR-Cas9 system. Ribonucleoprotein complex preparation and electroporation were conducted as previously reported ${ }^{7}$. Briefly, $3 \mu \mathrm{~g}$ Cas 9 protein (TrueCut Cas 9 Protein v2, Thermo Fisher Scientific, Cat\# A36496) plus $3 \mu \mathrm{~g}$ of sgRNA were incubated in Buffet T (Invitrogen, Cat\# MPK10096) for 20 min at RT in a volume $6 \mu \mathrm{~L}$. Cultured cells were resuspended in $30 \mu \mathrm{~L}$ Buffer T and added to ribonucleoprotein at a total volume of $36 \mu \mathrm{~L}$. Cells were electroporated using the Neon Transfection System (Thermo Fisher Scientific) at 1700 V for 20 ms with one pulse. The cell suspension was transferred to S 50 T 50 medium and cultured under $20 \% \mathrm{O}_{2} / 5 \% \mathrm{CO}_{2}$ conditions. To evaluate gene editing efficiency, genomic DNA from LSK cells was extracted using the NucleoSpin system (Macherey-Nagel, Dürin, Germany) 2-3 dafter electroporation. PCR was performed using the following settings: $95^{\circ} \mathrm{C}$ for 2 min ; 35 cycles of $95^{\circ} \mathrm{C}$ for $30 \mathrm{~s}, 60^{\circ} \mathrm{C}$ for 30 s , and $72{ }^{\circ} \mathrm{C}$ for 30 s ; followed by final extension at $72{ }^{\circ} \mathrm{C}$ for 5 min . PCR products were purified using Wizard SV Gel and the PCR Clean-Up System (Promega Corporation, Madison, WI, USA, Cat\# A9281) following manufacturer instructions. A tracking of indels by decomposition (TIDE) assay ${ }^{8}$ or inference of CRISPR edits analysis ${ }^{9}$ was performed to analyze the sequence data of each PCR product obtained by Sanger sequencing. Among five sgRNAs, Pfkfb3-sg1 displayed the best editing efficiency and was used for subsequent transplant and culture experiments.

## BM transplant of Pfkfb3-KO HSCs

Either Rosa26 (control) or Pfkfb3 sequences in HSCs were targeted using CRISPR/Cas9. After electroporation, HSCs were incubated for $2-3 \mathrm{~h}$ in S 50 T 50 medium under $5 \% \mathrm{CO}_{2} / 20 \% \mathrm{O}_{2}$ conditions, and then counted using a TC10 Automated Cell Counter (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Subsequently, 500 gene-edited HSCs together with $2 \times 10^{6} \mathrm{BM}$ cells from Ly5.1 congenic mice were transplanted retro-orbitally into lethally ( 9.5 Gy using MBR-1520R with a $125 \mathrm{kV} 10 \mathrm{~mA}, 0.5$ $\mathrm{mm} \mathrm{Al}, 0.2 \mathrm{~mm} \mathrm{Cu}$ filter)-irradiated Ly5.1 mice. During Pfkfb3 KO using the vector-free CRISPRCas9 system, the KO efficiency was not $100 \%$, so the transplanted cells were a mixture of Pfkfb3-KO cells and wild-type cells. Therefore, after 2, 8 , and 16 weeks, peripheral blood was collected and donor-derived chimerism was assessed by a TIDE assay based on a recent study by Shiroshita et al. ${ }^{10}$.

The following oligonucleotides for sgRNA synthesis and primers for post-knockout genomic PCR were used.

For Rosa region KO:
sgRNA target: $5^{\prime}$-ACTCCAGTCTTTCTAGAAGA-3'
Forward primer 1: 5'-CCAAAGTCGCTCTGAGTTGTTATCAGT-3'
Reverse primer 1: $5^{\prime}$-GGAGCGGGAGAAATGGATATGAAG-3'
Forward primer 2: 5'-CCAAAGTCGCTCTGAGTTGTTATCAGT-3'
Reverse primer 2: 5'-GGAGCGGGAGAAATGGATATGAAG-3'
Sequence primer: 5'-ACATAGTCTAACTCGCGACAC-3'
For Pfkfb3 KO:
sgRNA target: 5'-GTTGGTCAGCTTCGGCCCAC-3
Forward primer: $5^{\prime}$-AATTGTGTAGCACAGGATCACC-3'
Reverse primer: $5^{\prime}$-GCCACTAAAGGAAGGCTAGTTAC-3'
Sequence primer: 5'-CTCAATCTTCCCGAGTCTGTCTC-3'
For CD45 KO:
sgRNA target: $5^{\prime}$-GGGTTTGTGGCTCAAACTTC-3'
Forward primer: 5'-AGAAGCCATTGCACTGACTTTG-3'
Reverse primer: 5'-GTGTGATCTTTCCCCGAAACAT-3'
Sequence primer: $5^{\prime}$-CTGCAAAGAGGACCCTTTACAGT-3'
To calculate the KO efficiency of the Rosa locus, primer 1 or primer 2 was used for PCR amplification.

## Pfkfb3 overexpression in GO-ATeam2 ${ }^{+}$HSCs and time-course analysis of FRET values

cDNA encoding Pfkfb3 was subcloned into pMY-IRES-hCD8 upstream of IRES-hCD8. To produce a recombinant retrovirus, plasmid DNA was transfected into Plat-E cells using FuGENE® HD Transfection Reagent (Promega, Cat\# E2311). Cell supernatants were then used to transduce GOATeam $2^{+}$HSCs pre-cultured with SCF and TPO for 16 h . At 48 h post-transduction, surface-markerstained, retrovirally $p f k f b 3$-overexpressed GO-ATeam $2^{+}$cells were used for time-course analysis of FRET values as described above subsection "Time-course analysis of FRET values." Cells transduced with pMY-IRES-hCD8 retrovirus served as controls. Transduced cells were stained with the following antibody panel: lineage markers (CD4, CD8a, Gr-1, Mac-1, Ter-119, B220)-PerCPCy5.5, c-Kit-APC-Cy7, Sca-1-PE-Cy7, CD150-BV421, CD48-BV510 (BD Biosciences, Cat\# 563536), and hCD8-APC (BioLegend, Cat\# 980904). FRET value data for hCD8-positive cells were used for subsequent conversion to ATP concentration.

## Pfkfb3/Pfkfb3CA overexpression in HSCs and BMT

cDNA encoding Pfkfb3 or the constitutively active S461E Pfkfb3 mutant ( $P f k f b 3 \mathrm{CA})^{11}$ was subcloned into pMY-IRES-hCD8 upstream of IRES-hCD8 or into pMY-IRES-EGFP upstream of IRES-EGFP ${ }^{12}$, respectively. To produce a recombinant retrovirus, plasmid DNA was transfected into Plat-E cells using the FuGENE® HD Transfection Reagent. Cell supernatants containing virus were then filtered with Millex-HV Syringe Filter Unit ( $0.45 \mu \mathrm{~m}$, PVDF, 33 mm , gamma sterilized, Millipore) and used to transduce Ly5.1+ HSCs pre-cultured in SCF and TPO for 16 h .

At 48 h post-transduction, 2000 transduced $\mathrm{GFP}^{+}$cells were sorted and transplanted, together with $4 \times 10^{5}$ BMMNCs from C57BL/6-Ly5.2 mice, into lethally (9.5Gy using MBR-1520R with a 125 $\mathrm{kV} 10 \mathrm{~mA}, 0.5 \mathrm{~mm} \mathrm{Al}, 0.2 \mathrm{~mm}$ Cu filter)-irradiated C57BL/6-Ly5.2 mice. Cells transduced with pMY-IRES-EGFP retrovirus served as controls. After 1-4 months, peripheral blood was collected and donorderived chimerism was analyzed by flow cytometry. The frequency (\%) of donor-derived cells was calculated as follows:
$100 \times$ Donor-derived (Ly5.2-Ly5.1+) cells (\%) / (Donor-derived cells [\%] + Competitor- or recipientderived [Ly5.2 ${ }^{+}$Ly5.1] cells [\%])

## Knockout and overexpression of Pfkfb3 in HSPC and non-competitive BMT

PFKFB3 was knocked out and overexpressed in FACS-sorted $\mathrm{Lin}-\mathrm{Sca}-1^{+} \mathrm{c}-\mathrm{Kit}^{+}$and $\mathrm{Ly} 5.2^{+}$cells, respectively. Methods were partially modified from those described in the "CRISPR/Cas9 KO of Pfkfb3" and "Pfkfb3/Pfkfb3CA overexpression in HSCs and BMT" sections.
For KO of Pfkfb3, triple-gRNA purchased from Synthego (Redwood City, CA, USA) was used. After gene editing, Ly $5.2^{+}$HSPCs were collected and cultured in S50T50 medium under 5\% $\mathrm{CO}_{2} / 20 \% \mathrm{O}_{2}$ conditions for $2-3 \mathrm{~h}$, and $3 \times 10^{5}$ HSPCs were transplanted retro-orbitally into lethally-irradiated (8.5Gy using MBR-1520R-3 (Hitachi Power Solutions) with a $125 \mathrm{kV} 10 \mathrm{~mA}, 0.5 \mathrm{~mm} \mathrm{Al}, 0.2 \mathrm{~mm} \mathrm{Cu}$ filter) recipient Ly5.1 mice noncompetitively.
The sequences of triple-gRNA and the primer set used to confirm KO efficiency were as follows.
sgRNA sequences:
5'-AGACCUGGCUUACCUUUCGU-3'
5'-UGGAGAUGUAAGUCUUACCC-3'
5’-GUUGGUCAGCUUCGGCCCAC-3'
Forward Primer: ${ }^{\prime}$ '-CAAAGGAAAAGTCCCATGGAGA-3'
Reverse Primer: 5'-GGGCTTTGGCATGTGGAATG-3'

Sequencing Primer: 5'-CAAAGGAAAAGTCCCATGGAGAATG-3'
For Pfkfb3 overexpression, HSPCs were cultured in S50T50 medium under $5 \% \mathrm{CO}_{2} / 20 \% \mathrm{O}_{2}$ conditions for $8-16 \mathrm{~h}$ after retroviral transduction, and the equivalent of $3 \times 10^{5} \mathrm{HSPCs}$ were noncompetitively transplanted retro-orbitally into lethally-irradiated (8.5Gy using MBR-1520R-3) recipient Ly5.1 mice. After transduction, a group of the cells was cultured in S50T50 medium for 48 $h$ to confirm that transduction (GFP positivity) had been established.

## Cell cycle analysis and apoptosis assay of Pfkfb3-KO/overexpressing HSPCs after noncompetitive BMT

BMMNCs were collected from the bilateral femur, tibia, pelvic bone, and sternum of each individual recipient mouse on day 2 after noncompetitive BMT. Recipient BMMNCs were then stained with Lineage-marker-PerCP5.5, Ly5.1-PerCP5.5, and Ly5.2-PE (cell cycle analysis) or Lineage-markerFITC, Ly5.1-FITC, and Ly5.2-Alexa700 (apoptosis assay). For the analysis, all BMMNCs from each recipient were used in one analysis, and all lineage-marker negative Ly $5.2^{+}$cells were analyzed. Cell cycle analysis (Ki67/Hoechst33432 staining) was performed as described in the "Ki67/Hoechst33432 staining" section. In vivo BrdU labeling assays were performed as reported by Jun et al. ${ }^{3}$ using the FITC BrdU Flow Kit (BD Biosciences, Cat\# 559619). Apoptosis assays were performed using the PE Annexin V Apoptosis Detection Kit I according to manufacturer instructions.

Cell cycle analysis (Ki67/Hoechst33432 staining) of Pfkfb3-overexpressing HSPCs after transplantation was also performed using all BMMNCs from each recipient mouse, and the analysis was performed on all $P f k f b 3$-overexpressing cells $\left(\mathrm{GFP}^{+}\right)$.

## 5-FU administration after BM recovery in Pfkfb3-KO HSPCs

PFKFB3 was gene-edited in HSPCs using triple-gRNA as described above, and the equivalent of $3 \times$ $10^{5}$ LSK cells were transplanted retro-orbitally into lethally-irradiated (8.5Gy using MBR-1520R-3) recipient Ly5.1 mice noncompetitively. After 2 months, recipient mice were treated with $150 \mathrm{mg} / \mathrm{kg}$ of 5-FU intraperitoneally. Peripheral blood was collected on the day of 5-FU administration (day 1), and on days $4,6,9$, and 16. The dynamics of Pfkfb3- or Rosa-KO cell abundance (as control group) were analyzed by Sanger sequencing as described above.

## Immunocytochemistry

HSCs from PBS- or 5-FU-treated C57BL/6 mice were subjected to immunocytochemistry using antibodies for PFKFB3 (Abcam, Cat\# ab181861), phosphorylated-PFKFB3 (Bioss, Cat\# bs-3331R),
and methylated-PFKFB3 (developed by Takehiro Yamamoto) ${ }^{13}$. Purified HSCs were resuspended in 50\% FCS-PBS and cytospun using the Thermo Scientific Cytospin 4 system (Thermo Fisher Scientific). When using 2-NBDG-positive or -negative HSCs, C57BL/6 mice were given 2-NBDG intravenously (see "In vivo 2-NBDG assay" for details) and subjected to cytospinning. Cytospun cells were fixed using 4\% paraformaldehyde in PBS pH 7.4 for 10 min at RT. Fixed cells were washed twice with ice-cold PBS. For permeabilization, cells were incubated for 5 min with PBS containing $0.1 \%$ Triton X-100. Permeabilized cells were washed once with ice-cold PBS. After blocking with 3\% BSAPBS for 30 min , cells were incubated in the diluted antibody with $0.3 \%$ BSA-PBS in a humidified chamber overnight at $4{ }^{\circ} \mathrm{C}$. A dilution factor of 1:100 was used for all antibodies. The next day, cells were incubated with Goat anti-Mouse IgG2a Secondary Antibody, Alexa Fluor ${ }^{\text {TM }} 555$ (Thermo Fisher Scientific, Cat\# A-21137) and DAPI in 0.3\% BSA-PBS for 1 h at RT. After two washes with ice-cold PBS, samples were coverslipped with a drop of mounting medium and imaged with a Zeiss LSM 880 microscope (ZEISS, Jena, Germany). Images were acquired at room temperature under darkened conditions using a 100x oil immersion lens. The obtained image data was analyzed using Imaris software (Bitplane) to calculate the MFI of the target for each cell.

## RNA sequencing

Library preparation for RNA-seq was performed on 3000-3500 HSCs derived from mice after 5-FU or PBS administration. Total RNA was prepared using Rneasy Micro kit (QIAGEN, Hilden, Germany). cDNA was synthesized and amplified using SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing (Takara Bio, Inc., Shiga, Japan). RNA-seq libraries were prepared using the Nextera XT Kit (Illumina, San Diego, CA, USA). Single-end 75 bp sequencing was performed on a NextSeq 500 platform (Illumina). RNA-seq data were obtained from three independent experiments (biological duplicates) for each cell type. TopHat (version 2.0.13; with default parameters) was used for mapping to the reference genome (UCSC/mm10) with annotation data from iGenomes (Illumina). Then, gene expression levels were quantified using Cuffdiff (Cufflinks version 2.2.1; with default parameters).

## MACSQuant analysis of cell number

After single GO-ATeam 2 knock-in HSC culture, most of the medium ( $150-170 \mu \mathrm{~L}$ ) in wells of a 96well plate was aspirated and samples were stained with $10 \mu \mathrm{~L}$ antibody cocktail for 30 min at $4{ }^{\circ} \mathrm{C}$. Antibodies used were anti-lineage markers (CD4, CD8a, Gr-1, Mac-1, B220, Ter-119)-PerCP-Cy5.5, anti-c-Kit-APC-Cy7, anti-Sca-1-PE-Cy7, anti-CD150-BV421, and anti-CD48-APC for LSK-SLAM analysis. Antibody cocktail was prepared by mixing $0.1 \mu \mathrm{~L}$ of each antibody. After incubation, $100 \mu \mathrm{~L}$

PBS $+2 \%$ FCS was added to wells, and the plates were centrifuged for 5 min at $4{ }^{\circ} \mathrm{C}$ and $400 \times g$ with low acceleration and medium deceleration. Then, $100 \mu \mathrm{~L}$ supernatant was aspirated and cell pellets were resuspended in $200 \mu \mathrm{LPBS}+2 \%$ FCS $+0.1 \%$ PI $+0.25 \%$ Flow-Check Fluorospheres (Beckman Coulter, Brea, CA, USA, Cat\# A69183). Samples were acquired in fast mode in the MACSquant analysis settings, and volumes of $100 \mu \mathrm{~L}$ (large colonies) or $150-170 \mu \mathrm{~L}$ (small colonies) were analyzed. Data were exported as FCS files and analyzed using FlowJo software. Cell number was corrected by bead count of Flow-Check ( $\sim 1000$ cells $/ \mu \mathrm{L}$ ). HSCs were counted using CD150 ${ }^{+}$CD48 ${ }^{-}$ LSK cell counts. Megakaryocytes were identified as cells with high forward scatter and side scatter, as well as high CD150 and CD41 expression.

## cDNA synthesis and quantitative RT-PCR

cDNA synthesis and RT-PCR using PFKFB3CA overexpressing cells were performed as previously reported. The primers used were as follows:
MA069663-F: $5^{\prime}$-GGGCATGGCGAGAATGAGTACAA-3'
MA069663-R: $5^{\prime}$-TTCAGCTGGGCTGGTCCACAC-3'

Custom RPMI medium for ATP analysis

| Amino Acids | Concentration (mg/L) | Vitamins | Concentration (mg/L) |
| :---: | :---: | :---: | :---: |
| L-Arginine HCl | - | D-Biotin | 0.2 |
| L-Asparagine H2O | - | D-Calcium pantothenate | 0.25 |
| L-Aspartic acid | - | Choline chloride | 3 |
| L-Cystine 2 HCl | - | Folic acid | 1 |
| L-Glutamic acid | - | i-Inositol | 35 |
| L-Glutamine | - | Niacinamide | 1 |
| Glycine | - | p-Aminobenzonic acid (PABA) | 1 |
| L-Histidine HCl H 2 O | - | Pyridoxine HCl | 1 |
| Hydroxy-L-proline | - | Riboflavin | 0.2 |
| L-Isoleucine | - | Thiamine HCl | 1 |
| L-Leucine | - | Vitamin B12 | 0.005 |
| L-Lysine HCl |  | Inorganic salts | Concentration (mg/L) |
| L-Methionine | - | Calcium nitrate (Ca(NO3)2 4H2O) | 100 |
| L-Phenylalanine | - | Potassium chloride (KCI) | 400 |
| L-Proline | - | Magnesium sulfate (MgSO4) | 48.84 |
| L-Serine | - | Sodium chloride ( NaCl ) | 6000 |
| L-Threonine | - | Sodium bicarbonate ( NaHCO 3$)$ | 2000 |
| L-Tryptophan |  | Sodium phosphate (Na2HPO4) | 800 |
| L-Tyrosine 2Na 2H2O |  | Other componemts | Concentration (mg/L) |
| L-Valine |  | Glutathione reduced | 1 |
| Supplements | Concentration (mg/L) | HEPES | 5960 |
| Sodium lactate |  | Thymidine | 0.3633 |
| BSA | - | Phenol red |  |
| Cholesterol | - | Sodium pyruvate |  |
| Oleic acid |  | Sugars | Concentration (mg/L) |
| Palmitic acid |  | D-Glucose | - |

Table S1. Custom RPMI medium for culture and ATP analysis Composition of custom RPMI medium for culture (upper) and ATP analysis (lower). "-" means $0 \mathrm{mg} / \mathrm{L}$.

Table S2. In vitro tracer analysis for 5-FU-treated HSCs (uploaded separately as an Excel file) Results of tracer analysis using $\mathrm{U}^{13} \mathrm{C}_{6}$-glucose with HSCs from mice treated with PBS or 5-FU. Each section contains raw data from the glycolytic system, TCA cycle, and PPP~NAS from top to bottom. Data from three individual experiments are described for each. All values represent average metabolite levels in single HSCs obtained by dividing the metabolite levels detected in HSCs (compared to internal standards) by the number of HSCs used in the analysis.

Table S3. In vitro tracer analysis for oligomycin-treated HSCs (uploaded separately as an Excel file) Results of tracer analysis using $\mathrm{U}^{13} \mathrm{C}_{6}$-glucose with HSCs treated with DMSO (Oligomycin-) or oligomycin (Oligomycin + ). Each section contains raw data from the glycolytic system, TCA cycle, and PPP~NAS from top to bottom. Data from four individual experiments are described for each. All values represent average metabolite levels in single HSCs, obtained by dividing the metabolite levels detected in HSCs (compared to internal standards) by the number of HSCs used in the analysis.

Table S4. ${ }^{13} \mathrm{C}$ quantitative metabolic flux analysis (uploaded separately as an Excel file) Metabolic flux values of each enzyme obtained from 100 trials of ${ }^{13} \mathrm{C}$ quantitative metabolic flux analysis for PBS-treated (left), 5-FU-treated (middle), and OXPHOS-inhibited HSCs (right).

Table S5. In vivo tracer analysis for 5-FU treated mice (uploaded separately as an Excel file) Results of tracer analysis during continuous in vivo administration of $\mathrm{U}-{ }^{13} \mathrm{C}_{6}$-glucose to mice treated with 5-FU or PBS. A sheet is prepared for each metabolite and each contains two tables. The A.U. table (left) shows the metabolite levels detected in the four biological replicates in the 5-FU and PBS groups, obtained by dividing the metabolite levels detected in HSCs (compared to internal standards) by the number of HSCs used in the analysis. The ratio table (right) shows the calculated percentage of labeled metabolites among detected metabolites, where 12 C indicates unlabeled metabolites and 13 Cn indicates n -carbon labeled metabolites by $\mathrm{U}^{13} \mathrm{C}_{6}$-glucose.

| GO-ATeam2 genotyping |  |
| :--- | :--- |
| Primer name | Sequence |
| 55_CAGGS-5F | 5'-AGAGCCTCTGCTAACCATGTTCATGCCTTC-3' |
| 570_KusabiraOrange-141R | 5'-GTGACACTAAGTCAAACGCGAAA-3' |

Table S6. Primer list for genotyping PCR

Figure S1


Figure S1. Dependence on glycolysis increases with cell cycle progression of HSCs; related to Figure 1
(A) Schematic illustration of 5-FU administration and analysis. (B) Representative staining plot of BM cells derived from mice 5 d (day 6) after treatment with PBS or 5-FU (day 1 ); note the gating of c-Kit high-dim cells in the LSK staining. (C) Frequency of Ki67-positive and -negative HSCs after 5-FU administration. $\mathrm{n}=5$ biological duplicates for each group. (D) Changes in ATP concentration in HSCs after 5-FU administration ( $\mathrm{n}>70$ single HSCs for each group). Data are representative results of pooled samples of two biological replicates. (I) Ki-67 positivity in HSCs by route of 5-FU administration (i.p. or i.v.). $\mathrm{n}=4$ biological duplicates for each group. (F-G) Intracellular staining of pRb in $\mathrm{EPCR}^{+}$or EPCR ${ }^{-}$HSCs derived from PBS- or 5-FU-treated mice. Representative plot of pRb and DNA content in $\mathrm{EPCR}^{+}$HSCs from both groups (F). Summary of results (G). $\mathrm{n}=3$ biological duplicates for each group. (H-J) Analysis of mVenus-p27K- mice treated with PBS or 5-FU. Experimental schema (H). Representative G0 marker distribution in HSC in PBS (blue) or 5-FU (red) groups (I). Percentage of G0 marker-positive cells in total HSCs and EPCR ${ }^{+}$or EPCR ${ }^{-}$HSCs in PBS (blue bars) or 5-FU group (red bars). $\mathrm{n}=4-5$ biological replicates for each group. The data for each panel is extracted from the same individual. (K) Relative percentage of HSCs remaining after culture under quiescencemaintaining or proliferative conditions in the presence of oligomycin. HSC number for the control (Ctl) vehicle (DMSO)-treated group was set to $100 \% ; \mathrm{n}=4$ technical replicates for each group. The data are representative results from three independent experiments. (See "MACSQuant analysis of cell number" under "Supplementary Methods" for more information.)
Data are presented as mean $\pm$ SD. ${ }^{*} \mathrm{p} \leq 0.05,{ }^{* *} \mathrm{p} \leq 0.01,{ }^{* * *} \mathrm{p} \leq 0.001$ as determined using Student's $t$-test (G, J, K) or one-way ANOVA followed by Tukey's test (C-E).

Figure S2


Figure S2. Quantified metabolite pool in HSCs under quiescence, proliferation, or OXPHOSinhibition; related to Figure 1, 2
(A) Comparison of metabolite levels in c-Kit enriched cells before and after sorting. $\mathrm{n}=3$ biological replicates for each group. (B) Metabolic overview of U-13C6-glucose tracing among pathways related to glycolysis, PPP, NAS, and the TCA cycle in HSCs from 5-FU- (blue) or PBS-treated (red) mice, and DMSO- (black) or oligomycin (Oligo)-treated (orange) HSCs. Fates of carbons derived from $\mathrm{U}^{13} \mathrm{C}_{6}$-glucose in each metabolite are shown as yellow circles. Each graph indicates relative amounts of $\mathrm{U}-{ }_{-}^{13} \mathrm{C}_{6}$-glucose-derived metabolites. (C-E) Ratio of $\mathrm{U}^{13} \mathrm{C}_{6}$-glucose-labelled to nonlabelled metabolites in glycolysis (C), the first round of the TCA cycle (D), and the PPP plus nucleic acid synthesis (E) in PBS- (red bars) and 5-FU-treated (blue bars) HSCs. (F-H) Ratio of U- ${ }^{13} \mathrm{C}_{6}$ -glucose-labelled to non-labelled metabolites in glycolysis (F), the first round of the TCA cycle (G), and the PPP plus nucleic acid synthesis (H) in HSCs treated with vehicle (black bars) or oligomycin (orange bars).
In (B-H), data are extracted from three biological replicates for HSCs derived from PBS- or 5-FUtreated mice and from four for HSCs after DMSO or oligomycin treatment. Data are presented as mean $\pm$ SD. ${ }^{*} \mathrm{p} \leq 0.05, * * \mathrm{p} \leq 0.01,{ }^{* * *} \mathrm{p} \leq 0.001$ as determined using two-way ANOVA with Sidak's test (A) and Student's $t$-test (B) by comparing PBS and 5-FU groups or DMSO and oligomycin groups.
Abbreviations: G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; F1,6BP, fructose-1,6bisphosphate; G3P, glycerol-3-phosphate; DHAP, dihydroxyacetone phosphate; 3PG, 3phosphoglycerate; 2PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate; PYR, pyruvate; LAC, lactate; Ac-CoA; acetyl-CoA; CIT, citrate; ACO, cis-aconitic acid, isocitrate; 2OG, 2-oxoglutarate; SUC, succinate; FUM, fumarate; MAL, malate; OAA, oxaloacetate; 6PG, glucose-6-phosphate; Ru5P, ribulose-5-phosphate; Xu5P, xylulose-5-phosphate; R5P, ribose-5-phosphate; S7P, sedoheptulose-7-phosphate; E4P, erythrose-4-phosphate; PRPP, phosphoribosyl pyrophosphate; IMP, inosine monophosphate; ATP, adenosine triphosphate; GTP, guanine triphosphate; UMP, uridine monophosphate; UTP, uridine triphosphate; TTP, thymidine triphosphate.

Figure S3


Figure S3. Quantitative ${ }^{13}$ C-MFA of HSCs under quiescence, proliferation, and OXPHOS inhibition; related to Figure 3
(A-C) Enzyme reaction flux values for each simulation (100 times in total) in PBS-treated (A), 5-FU-treated (B), and OXPHOS-inhibited HSCs (C). Flux values calculated in the same simulation are connected by lines; note the small variation in flux values calculated in different simulations in 5-FU-treated (B) or OXPHOS-inhibited HSCs (C) compared to that in PBS-treated HSCs (A). (D-U) Fluxes of each reaction determined using quantitative ${ }^{13} \mathrm{C}$-MFA in HSCs from mice treated with 5FU (blue bars) or PBS (red bars) (D-L), or in HSCs after treatment with vehicle (black bars) or oligomycin (orange bars) (M-U). The net flux was calculated when the glucose uptake was set at 100. The name of the enzyme catalyzing each reaction is listed above the graph. Each gray dot represents the estimated flux obtained from 100 mathematical simulations. (See "Quantitative ${ }^{13} \mathbf{C}$ MFA with OpenMebius" under "Supplemental Methods" for more information.)
Data are presented as mean $\pm$ SD. ${ }^{*} \mathrm{p} \leq 0.05,{ }^{* *} \mathrm{p} \leq 0.01,{ }^{* * *} \mathrm{p} \leq 0.001$ as determined using Student's $t$-test (D-U). Abbreviations: HK, hexokinase; PGI, glucose-6-phosphate isomerase; PFK, phosphofructokinase; TPI, triose phosphate isomerase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGM, phosphoglycerate mutase; PK, pyruvate kinase; LDH, lactate dehydrogenase; PC, pyruvate carboxylase; PDH, pyruvate dehydrogenase; CS; citrate synthase; IDH, isocitrate dehydrogenase; $\alpha \mathrm{KGDH}$, $\alpha$-ketoglutaric acid dehydrogenase; SDH , succinate dehydrogenase; G6PD, glucose-6-phosphate dehydrogenase; TAL, transaldolase.

Figure S4


Figure S4. Quantified metabolite pool in HSCs from PBS- or 5-FU-treated mice (A) Experimental schema. (B-D) Heat maps of the glycolytic system (B), TCA cycle (C), PPP and NAS and glutathione labeling rates (D). (E-I) Labeling rates of Asp M+2 (E), Glu M+2 (F), IMP $\mathrm{M}+5$ (G), ATP $\mathrm{M}+5(\mathrm{H})$, and reduced glutathione $\mathrm{M}+2$ (I) in PBS- (blue bars) or 5-FU-treated HSCs (red bars). (J) Percentage of total ${ }^{13} \mathrm{C}$ labeled body mass of glycolysis, TCA cycle, PPP, and NAS detected in PBS-treated or 5-FU-treated HSCs. HSCs derived from one or two mice in the PBS group and two or three mice in the 5-FU group were pooled. $\mathrm{n}=4$ biological replicates for each group. (See "Preparation and storage of in vivo $\mathbf{U}^{\mathbf{1 3}} \mathbf{C}_{\mathbf{6}}$-glucose tracer samples" in "Supplementary Methods" for details.) Data are presented as mean $\pm \mathrm{SD} . * \mathrm{p} \leq 0.05, * * \mathrm{p} \leq 0.01$, *** $\mathrm{p} \leq 0.001$ as determined using Student's $t$-test (E-J).
Abbreviations: G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; F1,6BP, fructose-1,6bisphosphate; G3P, glycerol-3-phosphate; DHAP, dihydroxyacetone phosphate; 3PG, 3phosphoglycerate; 2PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate; PYR, pyruvate; LAC, lactate; CIT, citrate; ISOCIT; isocitrate; 2OG, 2-oxoglutarate; SUC, succinate; FUM, fumarate; MAL, malate; 6PG, glucose-6-phosphate; Ru5P, ribulose-5-phosphate; Xu5P, xylulose-5-phosphate; R5P, ribose-5-phosphate; S7P, sedoheptulose-7-phosphate; PRPP, phosphoribosyl pyrophosphate; IMP, inosine monophosphate; ATP, adenosine triphosphate; Asp, Aspartic acid; Glu, Glutamate.

Figure S5




Figure S5. Establishment of a real-time ATP concentration analysis system using GO-ATeam2; related to Figure 4
(A) Representative plot of HSPC fractions from GO-ATeam2+ mice. The identified fractions are shown at the top of the graph and the upper gating of that fraction is shown in parentheses. (B-C) Number of BMMNCs derived from C57B6/J and GO-ATeam2 mice (B) and percentage of each fraction present (C). (D) Schematic diagram showing effects of key metabolic pathways and regulators (orange) and their inhibitors (red). (E) Transformation of time-course plot of ATP concentration in individual HSCs from GO-ATeam2 mice (left) to a planar curve after fitting (right).
(F) Time-course analysis of ATP concentration in HSCs from GO-ATeam 2 mice in basal medium (Ba-M) without pre-saturation plus various indicated additives. (G-H) Effects of indicated metabolites on ATP concentration of HSCs (G) or MyPs (H) in Ba-M. (I) Apoptosis assay results for HSCs exposed to 2-DG $(50 \mathrm{mM})$ or oligomycin $(1 \mu \mathrm{M})$ for $10 \mathrm{~min} . \mathrm{n}=3$ biological replicates. (J) Average amount of ATP per cell. The amount of ATP detected in the luciferase assay was divided by the number of HSCs used in the analysis. $\mathrm{n}=3$ biological replicates for each group. Data are presented as mean $\pm$ SD. ${ }^{*} \mathrm{p} \leq 0.05,{ }^{* *} \mathrm{p} \leq 0.01,{ }^{* * *} \mathrm{p} \leq 0.001$ as determined using Student's $t$-test (B, J), two-way ANOVA with Sidak's test (C), or one-way ANOVA followed by Tukey's test (I).

Figure S6


Figure S6. Steady-state Pfkfb3 activity defines HSC and HPC metabolic kinetics and cell cycle; related to Figure 5
(A-D) Evaluation of factors affecting ATP concentration in MPPs (A), MEPs (B), CMPs (C), and CLPs (D) based on the GO-ATeam2 system. GO-ATeam2-knock-in BMMNCs were incubated with glucose, oligomycin, 2-DG, or glucose plus oligomycin, and the FRET/EGFP ratio was calculated. (E) Effects of DMSO (Ctl, red line), oligomycin ( $1 \mu \mathrm{M}$, blue lines), $\mathrm{FCCP}(2 \mu \mathrm{M}$, green lines), and rotenone ( $1 \mu \mathrm{M}$, orange lines) on ATP in HSCs. Dashed lines are ATP concentrations with additional $\mathrm{ddH}_{2} \mathrm{O}$ and solid lines are ATP concentrations when $200 \mathrm{mg} / \mathrm{dL}$ glucose is added. (F) ATP concentration during the last 1 min in (E). Data are representative results for pooled samples of two biological replicates. (G) Effects of indicated concentrations of glucose ( $\mathrm{mg} / \mathrm{dL}$ ) on ATP concentration in oligomycin-treated or control HSCs (left panel), or in MyPs (right panel) in PLFA medium. (H-K) Effects of inhibitors of PKM2 or LKB1 (PKM2i or LKB1i, respectively) on ATP concentration of HSCs from GO-ATeam2 mice in BaM with either glucose (Glc) or glucose plus oligomycin. ATP concentrations for the last 2 min of analysis time are summarized in $(\mathrm{J})$ and $(\mathrm{K})$, respectively. Each group represents at least 50 cells. Data are the result of one experiment. (L) Composition of adenine phosphates (AMP, ADP, and ATP) in HSCs treated with oligomycin (Oligo), or HSCs from 5-FU-treated mice (5-FU) and control HSCs ( Ctl ; no treatment or DMSO-treated). Data show results of tracer experiments shown in Figure 1 and Figure 2. (M-P) Effects of a PFKFB3 inhibitor (PFKFB3i) on ATP concentration in GMPs (M), MEPs ( N ), CMPs ( O ), or CLPs (P) from GO-ATeam2 mice in Ba-M treated with glucose (Glc) plus vehicle (red lines), glucose plus PFKFB3i (blue lines), or glucose plus oligomycin plus PFKFB3i (green lines). (Q) ATP concentration in indicated progenitor fractions in Ba-M with vehicle (red bars) or PFKFB3 inhibitor (dark blue bars). ATP concentrations for the last 1 min of the analysis period are shown. Data is summarized from Figure S6 M-P. Each group represents at least 350 cells. Data are representative results of pooled samples from two biological replicates. (R-U) Effects of inhibitors on ATP concentration in Pfkfb3-overexpressing GO-ATeam2 ${ }^{+}$HSCs. Cells were exposed to vehicle ( Ctl ) (R), 2-DG (S), oligomycin (T), or glucose $12.5 \mathrm{mg} / \mathrm{dL}$ and oligomycin (U). Data are representative results of pooled samples from two biological replicates.
Data are presented as mean $\pm$ SD. ${ }^{*} \mathrm{p} \leq 0.05,{ }^{* *} \mathrm{p} \leq 0.01,{ }^{* * *} \mathrm{p} \leq 0.001$ as determined using Student's $t$-test (F, Q) or one-way ANOVA followed by Tukey's test (J-L).


Figure S7. Pfkfb3 contributes to HSC proliferation and differentiation in vitro; related to Figure 7
(A-D) Effects of in vitro PFKFB3 inhibition, KO, or overexpression on HSCs. Experimental design (A). Number of cells in an HSC-derived colony following exposure to a PFKFB3 inhibitor (PFKFB3i) at indicated concentrations (B) or after Pfkfb3 KO by CRISPR-Cas9 (C); $\mathrm{n}=4$ technical replicates for each group. Control groups were vehicle (DMSO)-treated (B) or CD45 KO (C). (D) In vitro effect of Pfkfb3-overexpression on HSCs. Number of cells in Pfkfb3-overexpressing or mock (pMY-IRES-GFP)-transduced HSC-derived colonies; $\mathrm{n}=4$ technical replicates for each group. (B-D) are representative results of two or three independent experiments. (E-F) KO efficiency evaluated by $\operatorname{sgRNA}(\mathrm{E})$ or triple gRNA (F). The indel spectrum (horizontal axis) and the percentage of each indel (vertical axis) are shown. (G) qPCR results for Pfkfb3 expression in validation experiments of mock(black bar) or PFKFB3CA- (red bar) overexpression. $\mathrm{n}=4$ technical replicates for each group. Data are presented as mean $\pm$ SD. ${ }^{*} \mathrm{p} \leq 0.05,{ }^{* *} \mathrm{p} \leq 0.01,{ }^{* * *} \mathrm{p} \leq 0.001$ as determined using Student's $t$-test (C, D, G) or one-way ANOVA followed by Tukey's test (B).

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