Bone marrow niches of germline FANCC/FANCG deficient mice enable efficient and durable engraftment of hematopoietic stem cells after transplantation

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Supplemental Methods

Animals and *in vivo* treatment

Mouse colonies were maintained under pathogen-free conditions in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines. All experiments were performed according to protocols approved by the IACUC at The Children's Hospital of Philadelphia. Double heterozygous *Fancc*$^{+/-}$;*Fancg*$^{+/-}$ mice were generously provided by Dr. Feng-Chun Yang and Dr. Wade Clapp (Indiana University School of Medicine, Indianapolis, IN) and were used to generate single mutant *Fancc*$^{-/-}$, single mutant *Fancg*$^{-/-}$ and double mutant *Fancc*$^{-/-}$;*Fancg*$^{-/-}$ mice. To genotype the Fanconi Anemia (FA) mice, PCR was used to detect wildtype alleles, *Fancc* mutant alleles and *Fancg* mutant alleles as previously described[1]. For the primary bone marrow transplantation (BMT), transgenic C57BL/6 mice expressing green fluorescent protein (GFP) under control of the H2K promoter (H2K-GFP) were used as donors[2]. For the secondary competitive transplantation assay, eight to eleven-week-old C57BL/6 wildtype (WT) mice were used as recipients and were purchased from Jackson Laboratories (Bar Harbor, ME, http://www.jax.org). For polyinosinic-polycytidylic ribonucleic acid (pIpC) treatment, pIpC were purchased from Sigma and injected intraperitoneally (300 µg per mouse) twice a week for eight weeks.

Peripheral blood counts

100 microliter peripheral blood was collected from retro-orbital plexus of the mice anesthetized with isoflurane, and stored in micro centrifuge tubes containing EDTA. Complete blood counts were analyzed using Hemavat 950FS (Drew Scientific Group).

Bone marrow (BM) cell isolation

To prepare single BM cell suspension, BM were vigorously flushed from the femurs and tibias of mice. Prior to cell counting and flow cytometry analysis, red blood cells were lysed with ammonium chloride buffer. The number of live BM cells were determined using a hematocytometer after stained with Trypan Blue Solution (0.4% w/v, Corning).

Colony-forming unit (CFU) assay

To determine the CFU number in the BM of WT and FA mice, CFU assays were performed in duplicate for each mouse. Briefly, BM cells were plated at $1x10^4$/mL with duplicate for each mouse in methylcellulose-based medium with recombinant cytokines for mouse cells (Stem Cell Technology) and incubated at 37°C, 5% CO_2. The total number of colonies were scored after 7 days of incubation.

Total body irradiation

TBI was performed with a radiograph source (X-RAD 320). Recipients for the primary BMT received a single 800 or 1125 cGy dose. Recipients for the secondary competitive transplantation assay received 1100 cGy in 2 split dose to decrease late non-hematologic toxicity.

Quantitive PCR (qPCR) analysis

To measure the expression of CXCL12 transcripts in the BM of irradiated mice, total RNA was isolated from flushed BM using RNeasy Mini Kit (Qiagen). First-strand cDNA was synthesized by SuperScript™ III First-Strand Synthesis System (Thermo Fisher Scientific) according to manufacture's protocol. qPCR was performed on 7500 Fast Real-Time PCR System (Applied Biosystems) with SYBR Green PCR Master Mix (Applied Biosystems). The following target-specific primers were used:

CXCL12_F: 5'-AGTAAGCACAACAGCCCAAAG-3'

CXCL12_R: 5'-CTTGCATCTCCCACGGATGT-3'

Primary and secondary transplantation assays

To study the efficiency of donor engraftment, primary WT or FA recipients with 1125cGy TBI received 5×10^6 whole BM cells from H2K-GFP donor mice via a tail vein injection. After 3 weeks, BM were collected from the bilateral femurs and tibias and pooled from >=3 recipient mice per group. A 5% BME dose (one fifth of all marrow collected from a single primary recipient) were injected into irradiated (1100 cGy) WT secondary recipients together with 10^5 whole BM cells from un-irradiated WT mice. Secondary recipients were assessed at 3-27 weeks after secondary transplantation for GFP^+ cell reconstitution in peripheral blood lineages, including RBCs, platelets, $Gr1^+$ myeloid cells, $B220^+$ B-cells and $CD3^+$ T-cells.

To assess the durability of donor engraftment, irradiated (800cGy) WT or FA primary recipients received TBI received 5×10^6 whole BM cells from H2K-GFP donor mice via a tail vein injection. BM was collected at 5

months after primary BMT. 10^6 BM from each primary recipient together with 5 x 10^5 whole BM cells from unirradiated WT mice were injected into irradiated (1100 cGy) secondary recipients. The rest of primary recipient BM were counted after RBC lysis. Flow cytometry was used to assess the absolute number of engrafted GFP^+ HSC and hematopoietic progenitors within primary recipients. Secondary recipients were assessed at 3 to 27 weeks after secondary transplantation for GFP^+ cell reconstitution in peripheral blood lineages, including RBCs, platelets, $Gr1^+$ myeloid cells, $B220^+$ B-cells and $CD3^+$ T-cells.

Flow cytometric analysis

Flow cytometry analysis was performed on BD FACSCalibur. RBC and platelets in peripheral blood were gated using FSC and SSC by flow cytometry. The other cell populations were identified using the following antibodies and dyes: PE-anti-CD3e (eBioscience, clone 145-145-2C11), PE-Cy7-anti-B220 (eBioscience, clone RA3-6B2), APC-anti-Gr1 (BD Pharmingen, clone RB6-8C5), PE-Cy7-anti-CD4 (BD Pharmingen, clone RM4-5), PE-Cy7-anti-CD8a (eBioscience, clone 53-6.7),), PE-Cy7-anti-TER-119 (eBioscience, clone TER-119), PE-Cy7-anti-CD11b (eBioscience, clone M1/70), PE-Cy7-anti-Gr1 (eBioscience, clone RB6-8C5), PE-anti-Sca-1(BD Pharmingen, clone D7), APC-eFlour780-anti-c-Kit (eBioscience, clone ACK2), APC-anti-CD48 (eBioscience, clone HM48-1), PerCP-eFlour710-anti-CD150 (eBioscience, clone mShad150), eFlour660- or FITC-anti-CD34 (eBioscience clone RAM34), PE-Cy5-anti-CD135 (eBioscience, clone A2F10), PE-Cy5-anti-CD127 (eBioscience clone A7R34), Fixable Viability Dye eFluo 660 (eBioscience), APC-Annexin V (Biolegend), 7-AAD Viability Staining Solution (eBioscience), PE-eflour310-Ki-67 (Invitrogen, clone SolA15) and DAPI (Sigma-Aldrich). Data were analyzed using FlowJo V10 (Tree Star, Inc.).

Statistical analysis

We used the Student 2-tailed t test to determine statistical significance of two-group comparisons. One-way analysis of variance (ANOVA) with Student-Newman-Keuls (SNK) post hoc test analysis was used for comparing multiple groups. The statistical analysis was performed using GraphPad Prism 7.00 (Graphpad Software, Inc.) and SNK test in R package agricolae v1.2-8.

Reference

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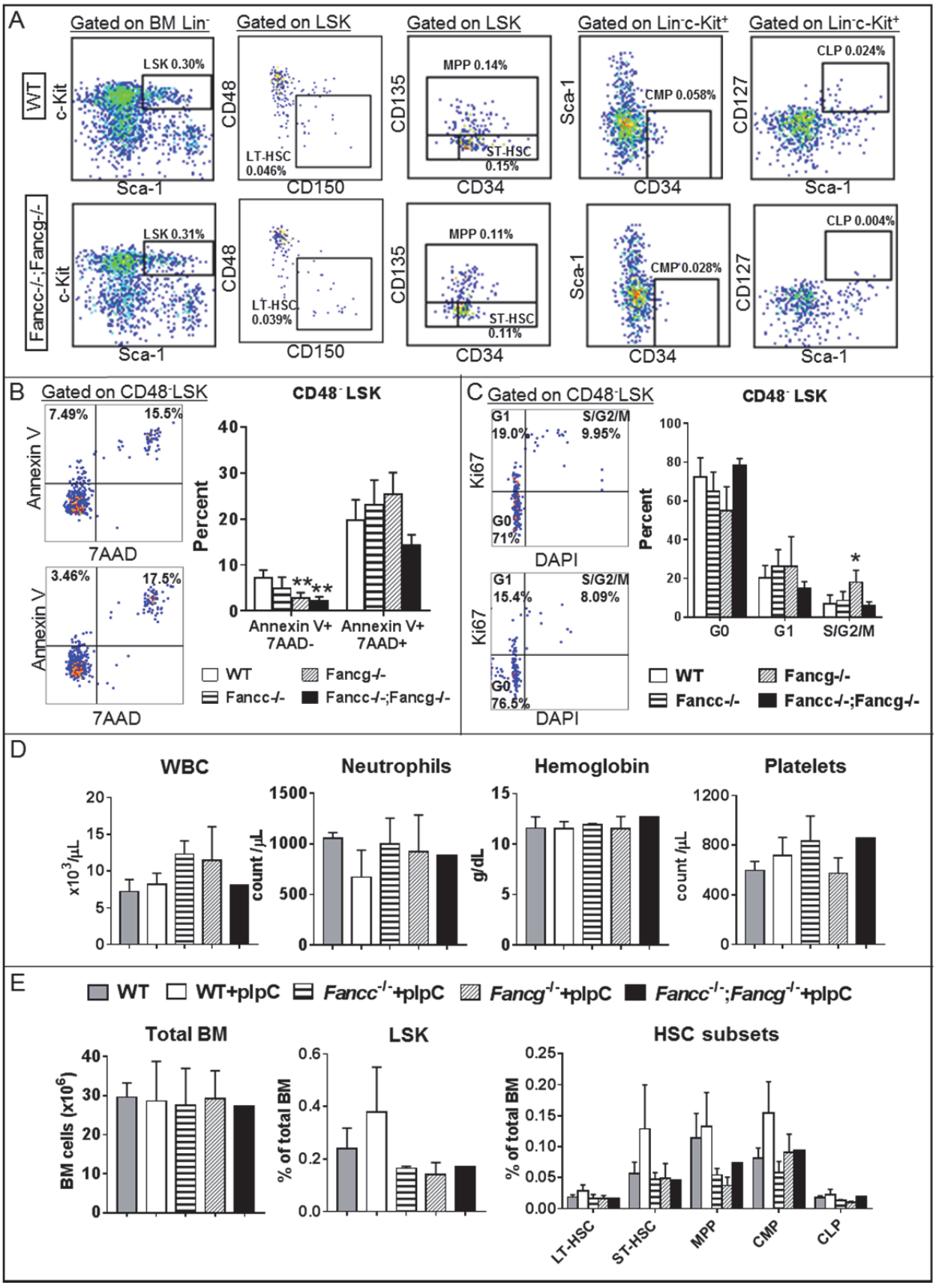
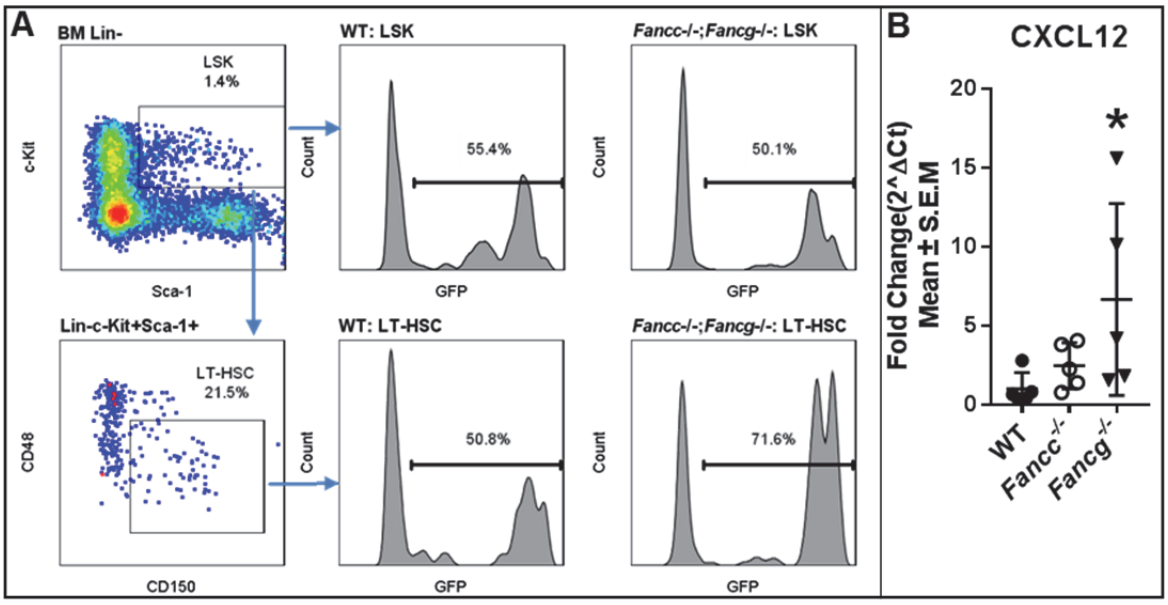
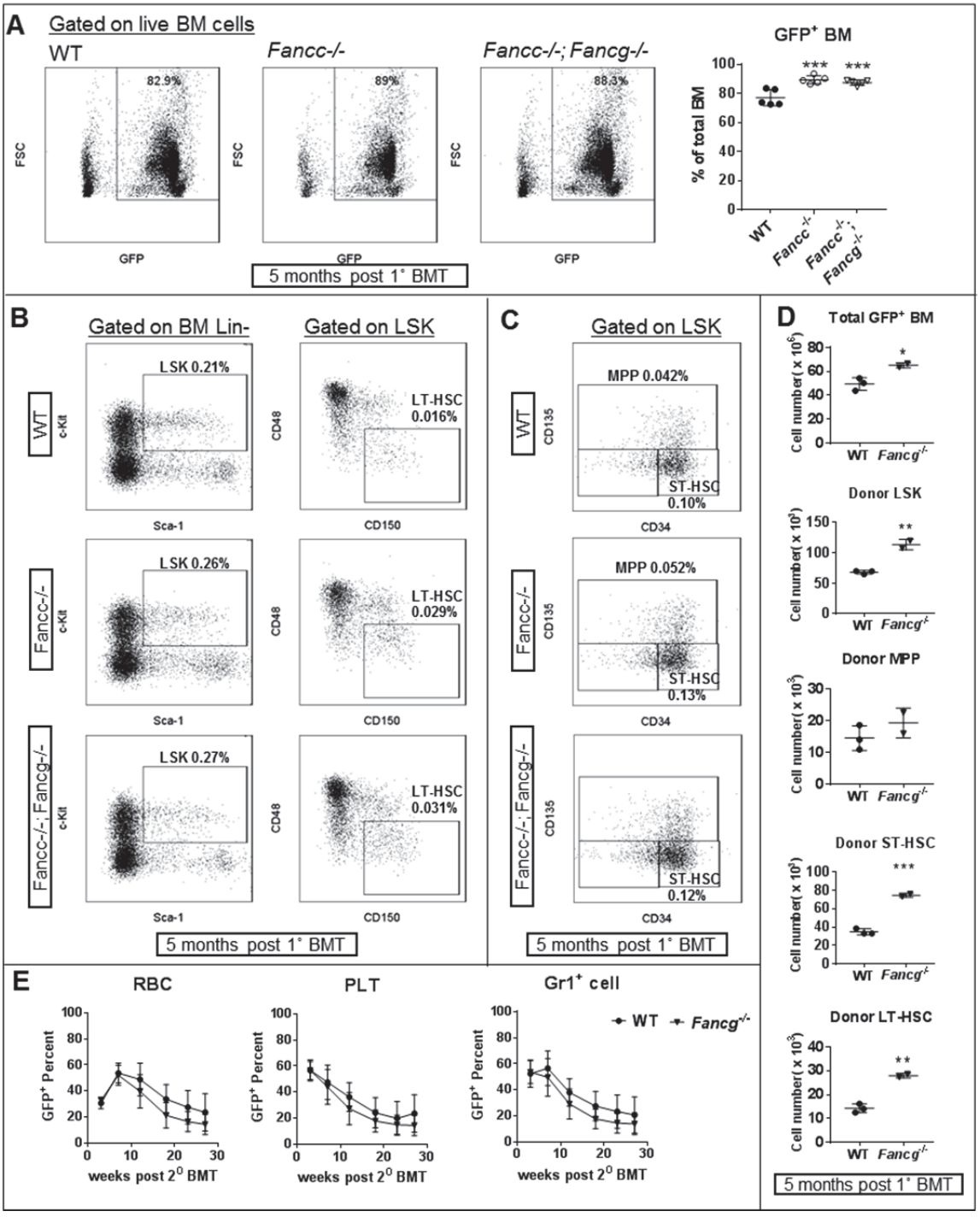

A
Gated on BM Lin-
Gated on LSK
Gated on LSK
Gated on Lin-c-Kit+
Gated on Lin-c-Kit+
WT
Fancc-/-;Fancg-/-
c-Kit
Sca-1
LSK 0.30%
LSK 0.31%
CD48
CD150
LT-HSC 0.046%
LT-HSC 0.039%
CD135
CD34
MPP 0.14%
MPP 0.11%
ST-HSC 0.15%
ST-HSC 0.11%
Sca-1
CD34
CMP 0.058%
CMP 0.028%
CD127
Sca-1
CLP 0.024%
CLP 0.004%
B
Gated on CD48-LSK
CD48- LSK
Annexin V
7AAD
7.49%
15.5%
3.46%
17.5%
Percent
Annexin V+ 7AAD-
Annexin V+ 7AAD+
WT
Fancc-/-
Fancg-/-
Fancc-/-;Fancg-/-
C
Gated on CD48-LSK
CD48- LSK
Ki67
DAPI
G1 19.0%
S/G2/M 9.95%
G0 71%
G1 15.4%
S/G2/M 8.09%
G0 76.5%
Percent
G0
G1
S/G2/M
WT
Fancc-/-
Fancg-/-
Fancc-/-;Fancg-/-
D
WBC
Neutrophils
Hemoglobin
Platelets
x10^3/μL
count /μL
g/dL
count /μL
E
WT
WT+pIpC
Fancc-/-+pIpC
Fancg-/-+pIpC
Fancc-/-;Fancg-/-+pIpC
Total BM
LSK
HSC subsets
BM cells (x10^6)
% of total BM
% of total BM
LT-HSC
ST-HSC
MPP
CMP
CLP

Supplemental Figure S1. Mice with germline deficiency of FANCC and/or FANCG display no reduction in blood counts or BM cellularity after pIpC-induced cell cycling. (A) Representative dot plots show the gating strategies of HSC/progenitor cell population including LSK, LT-HSC, ST-HSC, MMP, CMP and CLP in WT and *Fancc*$^{-/-}$;*Fancg*$^{-/-}$ mice. (B) Representative dot plots and bar graph show the percentages of CD48^{-}LSK cells at early apoptosis (Annexin V^{+}7AAD^{-}) and late apoptosis (Annexin V^{+}7AAD^{+}) stage (mean ± SD, n=3-4 mice per group). (C) Representative dot plots and bar graph show the percentage of CD48^{-}LSK cells at G0 (Ki67^{-}DAPI^{-}), G1 (Ki67^{+}DAPI^{-}) and S/G2/M (Ki67^{+}DAPI^{+}) phases of cell cycle (mean ± SD, n=4 mice per group). (D) Complete blood count (CBC) analysis of WBC, neutrophils, hemoglobin and platelets in untreated WT versus pIpC-treated WT, *Fancc*$^{-/-}$, *Fancg*$^{-/-}$ and *Fancc*$^{-/-}$; *Fancg*$^{-/-}$ mice. (E) Total BM cells (left), as well as frequencies of LSK (middle), LT-HSC, ST-HSC, MMP, CMP and CLP (right) subsets in untreated WT controls versus pIpC-treated WT, *Fancc*$^{-/-}$, *Fancg*$^{-/-}$ and *Fancc*$^{-/-}$; *Fancg*$^{-/-}$ mice, expressed as the percentage of total BM cells (mean ± SD, n=1-3 mice per group). *p<0.05, **p<0.01 compared to WT.



Supplemental Figure S2. (A) Representative dotplots and histogram demonstrating the gating method to determine the GFP^+ percentage in the BM subsets of LSK and LT-HSC in 2° recipients at 27 weeks after 2° BMT. (B) qPCR analysis of CXCL12 relative mRNA expression (normalized to GAPDH, mean ± SD) in BM of WT, *Fancc*$^{-/-}$ and *Fancg*$^{-/-}$ mice at 48 hours after 1125cGy total body irradiation. *p<0.05 compared to WT, Kruskal–Wallis one-way analysis of variance with Dunn's multiple comparisons test.


A
Gated on live BM cells
WT
Fancc-/-
Fancc-/-; Fancg-/-
82.9%
89%
88.3%
FSC
GFP
5 months post 1° BMT
GFP+ BM
% of total BM
***
***
WT
Fancc-/-
Fancc-/-; Fancg-/-
B
Gated on BM Lin-
Gated on LSK
LSK 0.21%
LT-HSC 0.016%
LSK 0.26%
LT-HSC 0.029%
LSK 0.27%
LT-HSC 0.031%
c-Kit
Sca-1
CD48
CD150
5 months post 1° BMT
C
Gated on LSK
MPP 0.042%
ST-HSC 0.10%
MPP 0.052%
ST-HSC 0.13%
ST-HSC 0.12%
CD135
CD34
5 months post 1° BMT
D
Total GFP+ BM
Donor LSK
Donor MPP
Donor ST-HSC
Donor LT-HSC
Cell number (x 10^6)
Cell number (x 10^3)
WT
Fancg-/-
5 months post 1° BMT
E
RBC
PLT
Gr1+ cell
WT
Fancg-/-
GFP+ Percent
weeks post 2° BMT

Supplemental Figure S3. Mice with germline deficiency of FANCC and/or FANCG demonstrate no reduction in the durability of donor HSC engraftment after HSCT. (A) Flow cytometry dot plots and scatterplots show the percentage of donor GFP^+ cells in the total BM of 1˚ WT, *Fancc*$^{-/-}$ and *Fancc*$^{-/-}$;*Fancg*$^{-/-}$ recipients at 5 months after 1˚ BMT. (B) Representative dot plots show the gating methods to determine the percentage of LSK and LT-HSC in total donor GFP+ BM cells. (C) Representative dot plots demonstrate the gating methods to determine the MPP, ST-HSC and LT-HSC in total donor GFP^+ BM cells. (D) The number of total GFP^+ donor BM, LSK, MPP, ST-HSC and LT-HSC in WT and *Fancg*$^{-/-}$ 1˚ recipients at 5 months after 1˚ HSCT. (E) Donor engraftment in WT and *Fancg*$^{-/-}$ 1˚ recipients was measured by GFP^+ cell percentage in multiple mature blood lineage including RBC, PLT and $Gr1^+$ myeloid cells in 2˚ recipient mice at 3-27 weeks after 2˚ BMT. **p*<0.05, ***p*<0.01, ****p*<0.001 compared to WT.