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## Loss of Cbl and Cbl-b ubiquitin ligases abrogates hematopoietic stem cell quiescence and sensitizes leukemic disease to chemotherapy.

Wei An

*University of Nebraska Medical Center*

Scott A. Nadeau

*University of Nebraska Medical Center*

Bhopal C. Mohapatra

*University of Nebraska Medical Center, bmohapat@unmc.edu*

Dan Feng

*University of Nebraska Medical Center, dan.feng@unmc.edu*

Neha Zutshi

*University of Nebraska Medical Center*

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## Authors

Wei An, Scott A. Nadeau, Bhopal C. Mohapatra, Dan Feng, Neha Zutshi, Matthew D. Storck, Priyanka Arya, James E. Talmadge, Jane L. Meza, Vimla Band, and Hamid Band

## SUPPLEMENTAL DATA

### SUPPLEMENTARY EXPERIMENTAL PROCEDURES

#### Reagents and antibodies

The murine recombinant cytokines SCF, TPO, IL-3, IL-6 and FLT3L were obtained from PeproTech. MethoCult M3234 Methylcellulose medium was obtained from Stem Cell Technologies. The following antibodies for flow cytometry were obtained from eBioscience: CD45.1 (A20); CD45.2 (104); Sca-1 (D7); c-Kit (2B8); CD16 (93), CD48(HM48-1), Ki-67(SolA15) and ILR7(A7R34). CD34 (RAM34) and FLT3 (A2F10.1) antibodies were obtained from BD Biosciences. CD150 (TC15-12F12.2) antibody was obtained from Biolegend. The following antibodies were obtained from Cell Signaling Technology: p-Erk 1/2 (D13.14.4E), p-Akt (D9E) and p-S6 (D57.2.2E). Fetal bovine serum (FBS) was from Hyclone. Imatinib and AC220 were from LC Laboratory. Biochemicals were from Sigma or Life Technologies unless indicated. OP-9 stromal cell line was obtained from ATCC and maintained by culture in  $\alpha$ MEM (Gibco) with 10% FBS.

#### BM preparation and FACS analysis

Whole bone marrow cell suspensions were prepared from femurs and tibiae. For stem and progenitor cell analysis and sorting, mature hematopoietic cells (lineage-positive cells) were labeled with antibodies against CD5, B220, CD11b, Gr-1, and 7-4 (mouse lineage depletion kit; Miltenyi Biotechnology) and magnetically depleted using the autoMACS (Miltenyi Biotechnology). Lineage-negative cells were then stained with antibodies followed by cell analysis or sorting. Flow cytometry was performed on a BD LSR II or Aria II at the UNMC Flow Cytometry Research Facility. Data were analyzed using FlowJo software (Tree Star). Cell populations were defined as follows<sup>1</sup>: LT-HSC: CD34<sup>-</sup> FLT3<sup>-</sup> Lin<sup>-</sup> Sca-1<sup>+</sup> c-Kit<sup>+</sup>; ST-HSC: CD34<sup>+</sup> FLT3<sup>-</sup> Lin<sup>-</sup> Sca-1<sup>+</sup> c-Kit<sup>+</sup>; MPP: CD34<sup>+</sup> FLT3<sup>+</sup> Lin<sup>-</sup> Sca-1<sup>+</sup> c-Kit<sup>+</sup>; LSK: Lin<sup>-</sup> Sca-1<sup>+</sup> c-Kit<sup>+</sup>; CMP: CD16<sup>-</sup> CD34<sup>+</sup> Lin<sup>-</sup> Sca-1<sup>-</sup> c-Kit<sup>+</sup>; GMP: CD16<sup>+</sup> CD34<sup>+</sup> Lin<sup>-</sup> Sca-1<sup>-</sup> c-Kit<sup>+</sup>; MEP: CD16<sup>-</sup> CD34<sup>-</sup> Lin<sup>-</sup> Sca-1<sup>-</sup> c-Kit<sup>+</sup>; CLP: IL-7R<sup>+</sup> FLT3<sup>+</sup> Lin<sup>-</sup> Sca-1<sup>low</sup> c-Kit<sup>low</sup>.

#### Cell cycle analysis

Bone marrow-derived lineage-negative cells were isolated using autoMACS and stained with the cell surface markers Sca-1 and c-Kit. After staining, cells were fixed in 2% paraformaldehyde, washed and permeabilized in PBS containing 0.5% saponin, 0.25% Triton X-100, 2% FBS, and 2% BSA for intracellular staining. Cells were then stained with Hoechst 33342 at 40ug/ml (Sigma) and

Ki-67 antibody. After washing, cells were analyzed on BD LSR II. Data were analyzed using FlowJo software (Tree Star).

#### FACS-based phosphorylation (phospho-Flow) analysis

For phospho-Flow analyses, lineage-negative cells were stained with anti-Sca-1 antibody and incubated for one hour in serum-free IMDM medium at 37°C before stimulation with mouse SCF or FLT3 ligand. At the indicated times, cells were fixed and permeabilized, as described in methods followed by staining for c-Kit together with anti-phospho-Erk and anti-phospho-Akt antibodies. Cells were washed and analyzed on BD LSR II. To assess surface c-kit or FLT3 levels after SCF or FLT3L stimulation respectively, lineage-negative cells were incubated in growth factor-free IMDM at 37°C for 1 hour and stained with anti-Sca-1 antibody followed by ligand stimulation for the indicated times. Cells were then fixed with 2% paraformaldehyde, washed and labeled with antibodies against surface c-Kit or FLT3 before analyses. Data are shown as the mean fluorescence intensity expressed as a % of unstimulated control.

#### In vitro assays

For the long-term culture-initiating cell (LTC-IC) assay,  $2 \times 10^5$  bone marrow-derived mononuclear cells were co-cultured with pre-irradiated OP-9 stromal cells in IMDM medium (Gibco) supplemented with 12.5% fetal bovine serum (FBS) (Hyclone), 12.5% horse serum (Lonza) and  $10^{-8}$  M dexamethasone (Sigma). After 2 weeks, total hematopoietic cells were counted with a hemocytometer and the CD45.2<sup>+</sup> cells were FACS-sorted. In the subsequent colony-forming assays,  $3 \times 10^4$  sorted cells were mixed with 1ml of MethoCult M3234 medium supplemented with cytokines (SCF 50 ng/ml, TPO 20 ng/ml, IL-3 10 ng/ml, IL-6 10 ng/ml and FLT3-L 10 ng/ml, referred as 5-cytokines combination) and plated in 35 mm tissue culture plates (Falcon). Colonies that have more than 50 cells were visually counted on day 7. For the serial plating assay, FACS-sorted LSK cells were used for colony-forming assays. 50 LSK cells per 35 mm plate were cultured in 1 ml of MethoCult M3234 medium with 5-factor combination for 7 days. Colonies were counted as above, cells were collected and  $1 \times 10^4$  cells were plated for a second round of colony forming assays.

#### Bone marrow transplantation assays

For all transplantation experiments, 8-week old B6.SJL-Ptprca Pepcb/BoyJ mice (CD45.1) were used as

recipients. Recipients were lethally-irradiated (1100 rad in two split doses with 3 hours interval) 24 hours before transplantation. Donor cells were derived from 8-week old gender-matched mice (CD45.2) of the genotypes indicated in various experiments. For whole BM transplantation,  $2 \times 10^6$  donor BM cells were transplanted. For purified BM population transplants, the indicated numbers of LSK, LKS<sup>+</sup> and Lin<sup>+</sup> c-kit<sup>+</sup> cells isolated by FACS-sorting were transplanted together with  $2 \times 10^5$  helper BM cells (heterozygous at CD45.1 and CD45.2, radio-protection). For HSC serial diluting transplantation (non-responder assays) to assess functional HSC frequency, the indicated numbers of FACS-sorted LSK cells were transplanted together with  $2 \times 10^5$  helper cells. The chimerism of donor cells in peripheral blood was calculated using L-Calc™ Software (STEMCELL Technologies) to assess the percentage of non-responder mice. For serial transplantation, 2000 donor-derived LSK cells were sorted from primary recipients at around 20 weeks after primary transplantation and transplanted into secondary recipients. Peripheral blood was obtained by submandibular vein

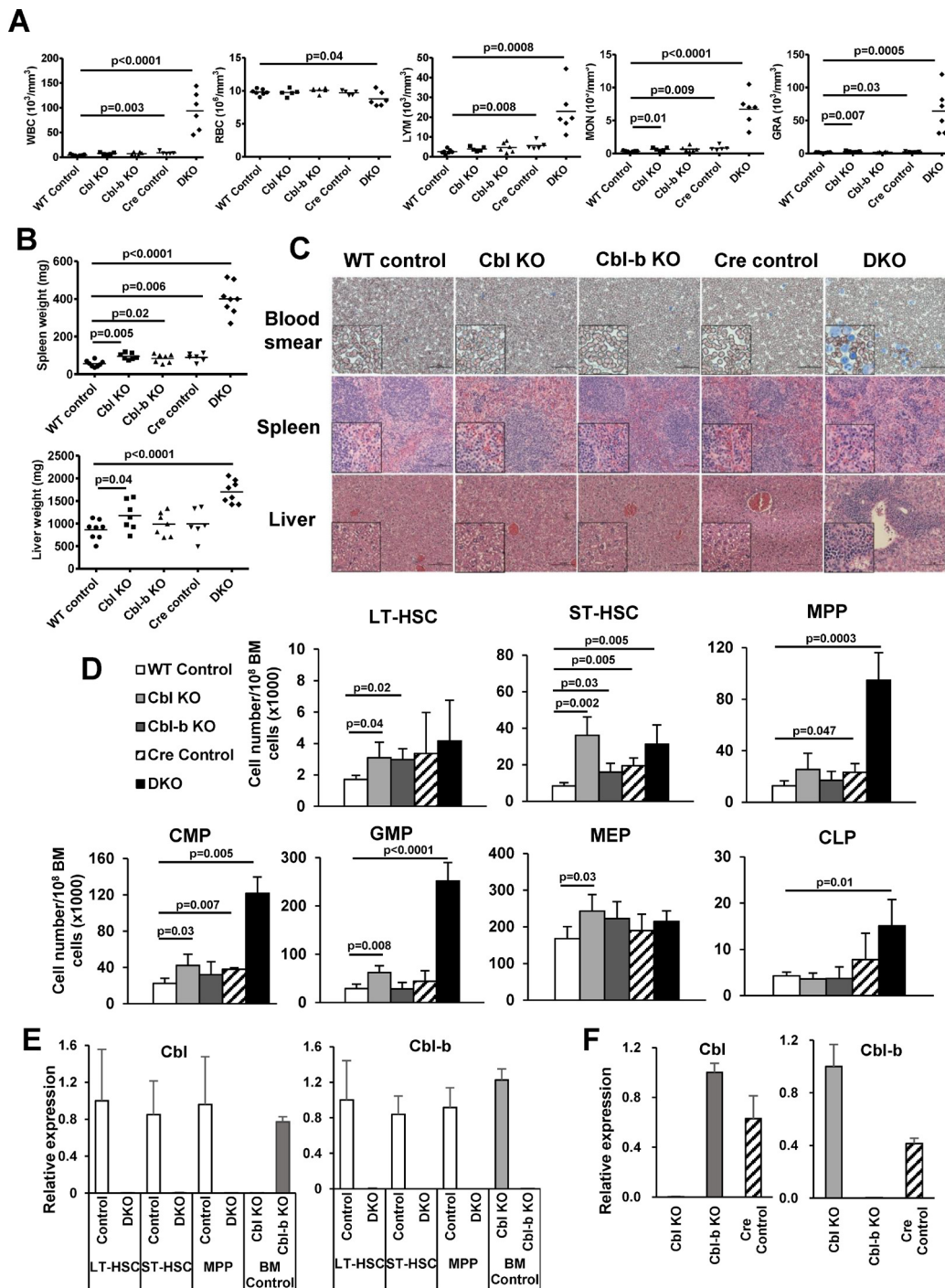
bleeding at 4, 8 and/or 16 weeks post-transplantation and engraftment efficiency in recipients was monitored based on the chimerism of CD45.2<sup>+</sup> cells in peripheral blood. 0.5% or higher donor-derived cells in the peripheral blood were scored as responders.

#### **Imatinib and 5-Fluorouracil *in vivo* treatment**

For Imatinib treatment, mice were treated with placebo (PBS) or Imatinib in a volume of 100  $\mu$ l PBS (100 mg/kg). Treatment was administered by oral gavage injection twice daily until the end of the experiment. For 5-Fu treatment, 5-Fu was prepared as 50 mg/ml solution in DMSO and diluted in PBS. Treatment was administered by i.p. injection at indicated time at the dose of 125 mg/kg.

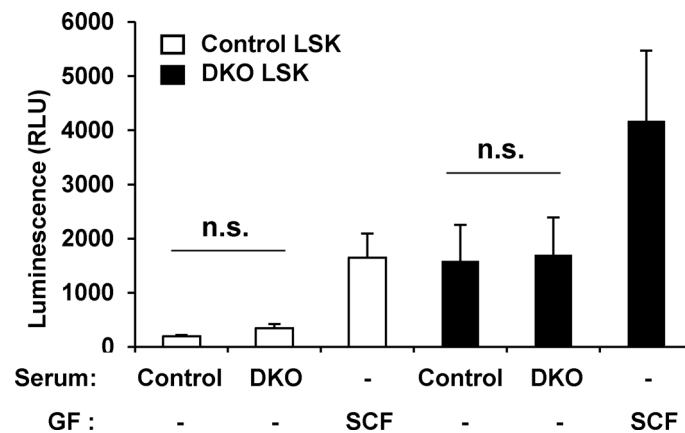
#### **SUPPLEMENTARY REFERENCES**

1. Seita J, Weissman IL. Hematopoietic stem cell: self-renewal versus differentiation. Wiley Interdiscip Rev Syst Biol Med. 2010; 2:640–653.

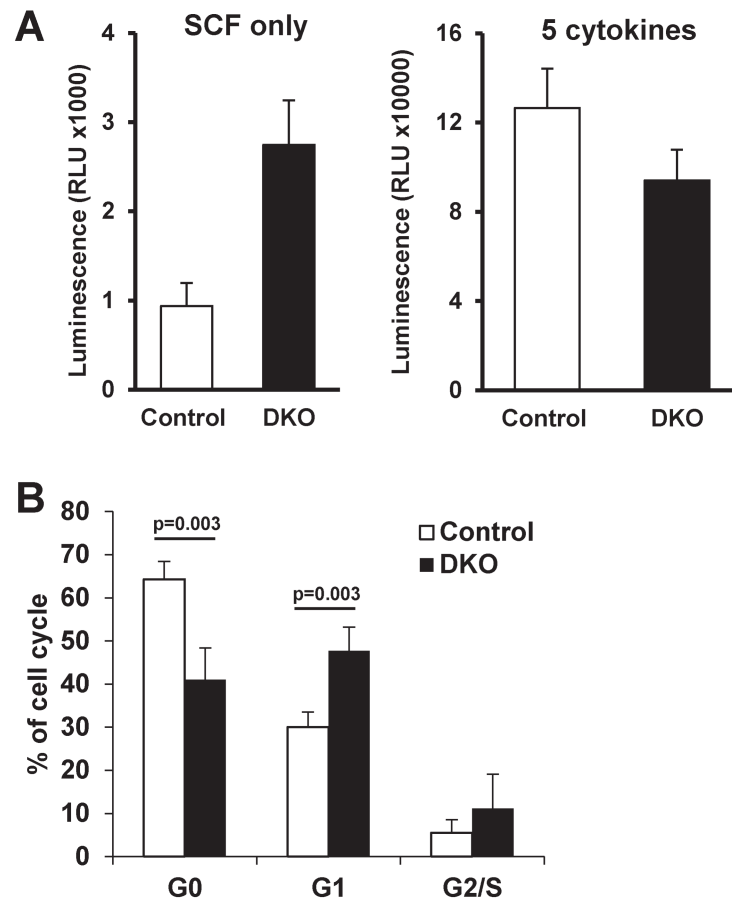


### Supplementary Figure S1: Myeloproliferative disease (MPD) is uniquely observed in *Cbl/Cbl-b* DKO mice.

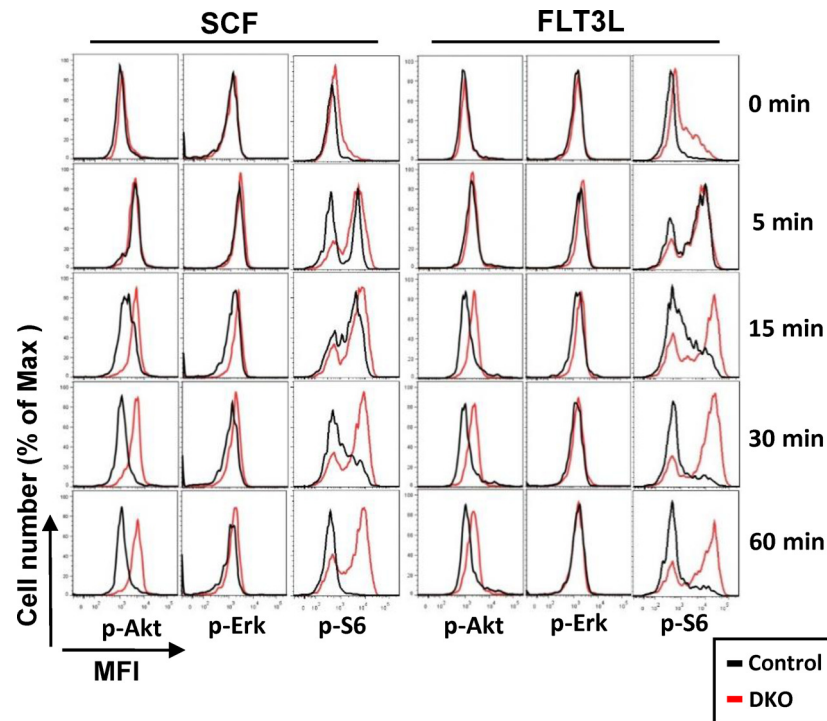
(A) Peripheral blood cell counts on mice of the indicated genotypes. WBC, white blood cell; LYM, lymphocyte; MON, monocyte; GRA, granulocyte; RBC, red blood cell. (B) Spleen and liver weights. Dots represent individual mice. (C) Representative Wright-Giemsa stained peripheral blood smears, and H&E stained spleen and liver sections. Higher magnification insets highlight leukocytosis in peripheral blood, loss of splenic architecture and perivascular leukocytes in liver. Bars, 0.1 mm. (D) FACS analysis of bone marrow cells from mice of the indicated genotypes for HSCs (LT-HSC, ST-HSC and MPP) and progenitors (CMP, GMP, MEP and CLP). Data show mean  $\pm$  SD of at least 3 mice for each genotype (\* $p < 0.05$ ). long-term HSCs (LT-HSC, CD34<sup>+</sup> FLT3<sup>-</sup> Lin<sup>-</sup> Sca-1<sup>+</sup> c-Kit<sup>+</sup>); short-term HSCs (ST-HSC, CD34<sup>+</sup> FLT3<sup>-</sup> Lin<sup>-</sup> Sca-1<sup>+</sup> c-Kit<sup>+</sup>) and multipotent progenitors (MPP, CD34<sup>+</sup> FLT3<sup>+</sup> Lin<sup>-</sup> Sca-1<sup>+</sup> c-Kit<sup>+</sup>). (E) mRNA expression levels of *Cbl* (left) and *Cbl-b* (right) were analyzed in FACS-sorted LT-HSC, ST-HSC and MPP populations of WT control or *Cbl/Cbl-b* DKO by quantitative real-time PCR. (F) mRNA levels of *Cbl* (left) and *Cbl-b* (right) in bone marrow cells from Cre Control mice (MMTV-Cre, *Cbl<sup>flx/flx</sup>*, *Cbl-b<sup>-/-</sup>*) were analyzed by quantitative RT-PCR. *Cbl*-KO and *Cbl-b*-KO bone marrow cells was used as internal controls. mRNA level was normalized using GAPDH. Data are pooled from two independent experiments and shown as mean  $\pm$  SD.



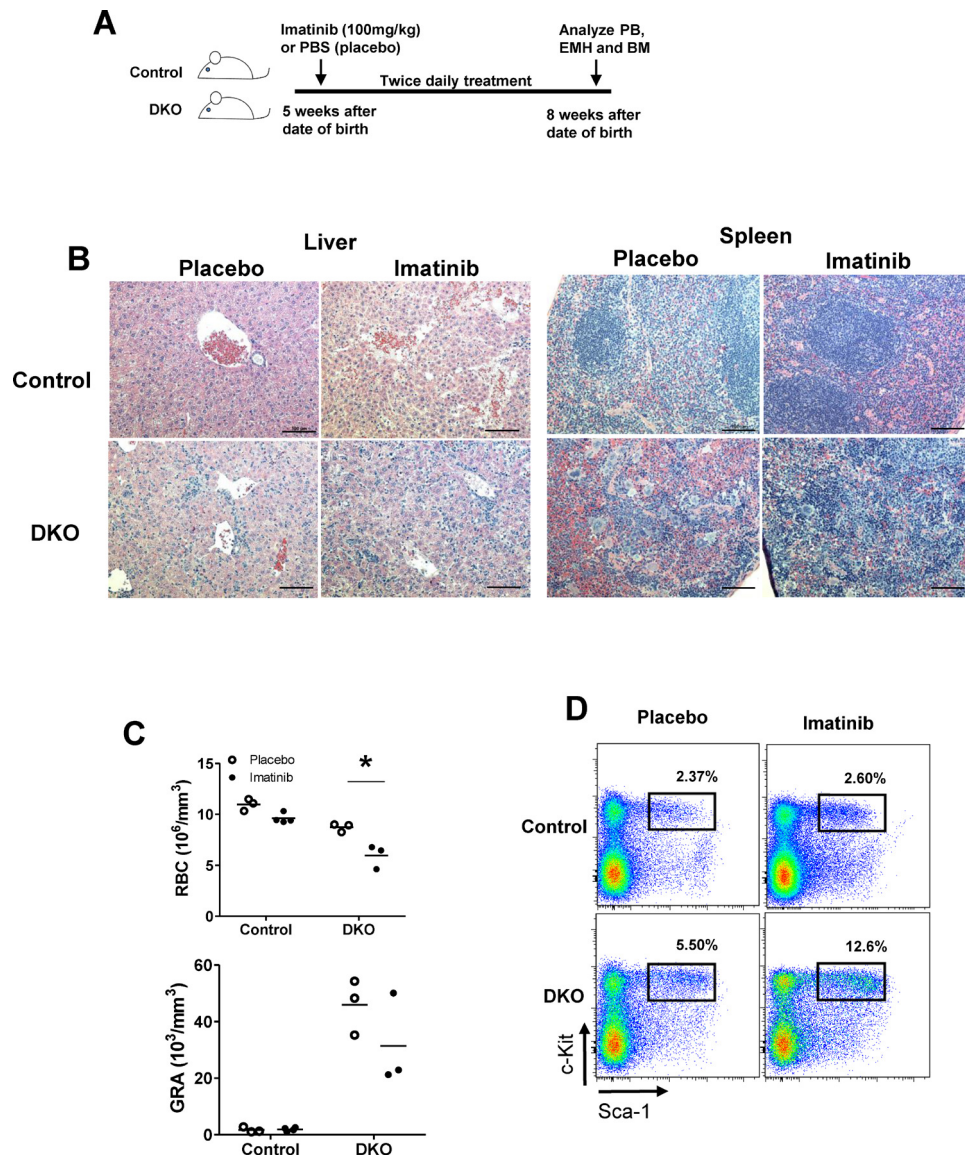
**Supplementary Figure S2: WT or DKO LSK was unaltered upon adding DKO or control mouse sera.** FACS-sorted LSK cells from control or Cbl/Cbl-b DKO mice were cultured in the presence of 10% serum derived from control or DKO mice. Cell proliferation was assayed using TiterGlo assay 3 days later. A representative experiment of three independent experiments is shown; 5 replicates in each experiment.



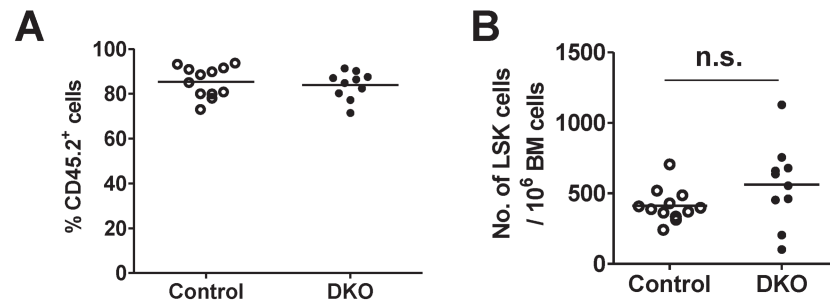
**Supplementary Figure S3: Reduced quiescent HSC in DKO mice.** (A) FACS-sorted LSK cells were cultured for 3 days at 50 cells per well in 96-well plate with SCF (50 ng/ml) only (left) or 5 cytokines (right) including 50 ng/ml SCF, 20 ng/ml TPO, 10 ng/ml FLT3L, 10 ng/ml IL-3 and 10 ng/ml IL-6. Cells proliferation was evaluated by TiterGlo assay. A representative experiment of two independent experiments is shown; at least 3 replicates in each experiment. (B) Cell cycle analysis of CD48<sup>-</sup> CD150<sup>+</sup> LSKs using Ki-67 and Hoechst staining. Data from at least three experiments are pooled and shown as mean  $\pm$  SD. \* $p < 0.05$ .



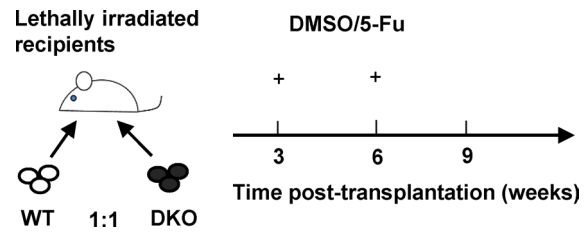
**Supplementary Figure S4: Loss of Cbl and Cbl-b enhances c-Kit and FLT3 signaling in HSCs.** Levels of p-Akt, p-Erk and p-S6 in unstimulated vs. ligand-stimulated LSK cells over time were analyzed by FACS (Phospho-FLOW). A representative FACS plot is shown.



**Supplementary Figure S5: Imatinib *in vivo* treatment.** (A) Experimental design. (B) Representative H&E stained spleen and liver sections. Bars, 0.1 mm. (C) Peripheral blood cell counts (RBC and GRA) on mice with indicated treatment. (D) Bone marrow analysis. Representative FACS plots are shown.



**Supplementary Figure S6: Cbl/Cbl-b DKO HSCs display impaired reconstitution ability.** (A) Peripheral blood chimerism of recipient mice with >70% donor cell reconstitution. (B) Donor LSK cells counts in BM of recipients shown in Fig 5C and 5D. Data are pooled from three independent experiments.



**Supplementary Figure S7: 5-FU promote DKO HSC exhaustion.** Experimental design.

**Supplementary Table S1. Genotypes of mice used in this paper**

Strain designation	Genotype
WT Control	Cbl <sup>WT/WT</sup> Cbl-b <sup>WT/WT</sup> or Cbl <sup>flox/flox</sup> Cbl-b <sup>WT/del</sup>
Cbl KO	Cbl <sup>del/del</sup>
Cbl-b KO	Cbl <sup>flox/flox</sup> , Cbl-b <sup>del/del</sup>
Cre Control	MMTV-Cre <sup>Tg/0</sup> Cbl <sup>flox/flox</sup> , Cbl-b <sup>WT/del</sup>
Cbl/Cbl-b DKO	MMTV-Cre <sup>Tg/0</sup> Cbl <sup>flox/flox</sup> , Cbl-b <sup>del/del</sup>

**Supplementary Table S2. Primers used for genotyping**

Target allele	Forward primer 5'-3'	Reverse primer 5'-3'
<b>Cbl WT</b>	AAGTTCCAAGCCTAGCCAGATATGTGTGTG	TCCCCTCCCCTTCCCATGTTTT TAATAGACTC
<b>Cbl Deletion</b>	TGGCTGGACGTAAACTCCTCTTCAGACCTAATAAC	TCCCCTCCCCTTCCCATGTTTT TAATAGACTC
<b>Cbl-b</b>	CCCAGCAAAAAGTAGCCAATG	CTTGCAAAAAGGACTAAGATTC
<b>MMTV-Cre</b>	GCGGTCTGGCAGTAAAACTATC	GTGAAACAGCATTGCTGTCACTT

**Supplementary Table S3. Primers used for quantitative real-time PCR**

Target	Forward 5'-3'	Reverse 5'-3'
<b>Cbl</b>	AGCTGATGCTGCCGAATT	TTGCAGGTCAGATCAATAGTGG
<b>Cbl-b</b>	GGAGCTTTTTGCACGGACTA	TGCATCCTGAATAGCATCAA
<b>p57</b>	GCGCAAACGTCTGAGATGAGT	AGAGTTCTTCCATCGTCCGCT
<b>p27</b>	AGTGTCCAGGGATGAGGAAGCGAC	TTCTTGGGCGTCTGCTCCACAGTG
<b>p21</b>	CCGCTGGAGGGCAACTTCGT	TTTCGGCCCTGAGATGTTCC
<b>GAPDH</b>	CCTGGAGAAACCTGCCAAGTATG	AGAGTGGGAGTTGCTGTTGAAGT