SUPPLEMENTARY APPENDIX

BMP signaling is required for postnatal murine hematopoietic stem cell self-renewal

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Supplementary methods

Cell preparation

Peripheral blood was collected from the tail vein and analyzed on a Boule Medonice CA 530-16 blood analyzer to determine blood counts. Single-cell suspension of bone marrow (BM) and spleen were obtained by crushing and passage through a 40-μm nylon mesh cell strainer (Fisher Scientific). Cells were kept in phosphate buffered saline (PBS; Thermo Scientific) containing 2% fetal calf serum (FCS; HyClone Thermo Scientific). When necessary, red blood cells were lysed with ammonium chloride (NH_4Cl; Stem Cell Technologies). For c-kit enrichment, cells were incubated with anti-CD117 micro beads (Miltenyi Biotec). C-kit$^+$ cells were enriched using magnetic separation columns (MACS 25 LS; Miltenyi Biotec) according to the manufacturer's instructions.

Flow cytometry

Cells were stained with fluorescently labeled rat antibodies (conjugated with either PE, FITC, APC, PE-Cy5, PE-Cy7, Brilliant Violet 421, or eFlour 780) against mouse B220, CD3, Gr1, Mac1, Ter119, Sca1, CD9, CD48, CD150, CD49, CD45.1, CD45.2, CD41, CD105, CD16/32, CD42 (all from BioLegend), c-kit (eBioscience), CD31, CD41, CD61, CD34 and Flt3 (BD Pharmingen, BD Biosciences). Dead cells were excluded by 7-aminoactinomycin D (7-AAD; Sigma-Aldrich). For apoptosis assays, PE Annexin V was used according to the manufacturer's protocol (BD Pharmingen/BD Biosciences). Cells were sorted by fluorescence-activated cell sorting (FACS) on a FACS Aria or analyzed on a FACS Canto (BD). Data was analyzed using FlowJo software (Treestar).

Cell cycle analysis

Freshly isolated BM was enriched for c-kit$^+$ cells and stained with antibodies against lineage markers, Sca-1, c-kit, CD48, CD150, prior to fixation in 0.4 % formaldehyde (VWR). Following permeabilization with 0.2% Triton-X (Sigma-Aldrich), cells were incubated with fluorescently conjugated anti-Ki67 antibody (BD Biosciences). DNA was stained using DAPI (Molecular Probes, Invitrogen). Cells were analyzed as described for flow cytometry above.

CFU assay and in vitro cell culture

For analysis of CFU-GM/E/mix, BM was seeded in methylcellulose (M3434 or M3234; Stem Cell Technologies) at 30 000 cells/ml in 35 mm Petri dishes. To verify recombination at the *BMPR-II* locus, DNA from individual colonies was analyzed by PCR. For megakaryocytic CFU-Mk assay the MegaCult™-C kit (StemCell Technologies) was used according to manufacturer's protocol. For liquid cultures, c-kit$^+$ cells were seeded at a concentration of 0.25 x 10^6 cells/ml in serum-free media (Stemspan SFEM; Stemcell Technologies) containing 50 ng/ml mSCF, 10 ng/ml mIL-3 (Peprotech), 10 ng/ml hIL-6 (Peprotech), 100 U/ml penicillin, and 100 μg/ml streptomycin (Hyclone, Thermo Scientific). Cells were counted and re-plated every third day until day 15.

Serial replating assay

BM of *BMPR-II$^{fl/fl}$;Vav-Cre* and WT littermate mice was harvested and seeded at a density of 300 000 cells per replicate into cytokine-supplemented methylcellulose medium (Methocult M3434; STEMCELL Technologies). Colonies were scored and replated at day 7-10, except for the fourth replating, which was scored at day 12 and could not be replated due to lack of *BMPRII$^{-/-}$* colonies. For replating, cells were resuspended and counted and 40 000 cells per replicate were taken for replating.

Hematopoietic stress assay using 5-fluorouracil

Mice (*BMPR-II$^{fl/fl}$;Vav-Cre* and WT littermates) were give one intravenous bolus injection of 0.15 mg/g 5-fluorouracil. On day 12 after 5-fluorouracil injection blood, BM, and spleen was harvested and analyzed for blood counts and using flow cytometry as described above.

BRE-GFP reporter assay

Canonical BMP reporter mice (BRE) are previously described[1-3]. To detect BMP-responsive hematopoietic cells, BM was stained with antibodies as above. For BMP stimulation, c-kit$^+$ cells were incubated for 16 hours in serum-free SFEM containing 50 ng/ml BMP4 (RnD Systems) supplemented with 50 ng/ml mSCF

and 100 U/ml penicillin, 100 μg/ml streptomycin. Cells were analyzed on a FACS Canto II.

Cell stimulation

BM cells (c-kit enriched or FACS sorted) were isolated and subsequently cultured for 16 hours in serum-free SFEM containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 ng/ml mouse stem cell factor. Subsequently, cells were stimulated with recombinant mouse BMP4 (RnD Systems) at 50-100 ng/ml for 5 to 45 minutes. Cells were then used for western blot analysis, flow cytometry, FACS, or qPCR.

Western blot analyses

Lysates were prepared using Laemli buffer (Bio-Rad Laboratories). Samples were loaded on pre-casted gels (Novex NuPage 4-12% Bis Tris gel; Life Technologies) and run according to the manufacturer's instruction. Transfer was performed using iBlot gel transfer device (Invitrogen/Life Technologies). The following antibodies were used: anti-phosphoSMAD1/5 (Cell Signaling Technology), anti-Actin (BD Transduction Laboratories), anti-β-tubulin (CST), anti-phospho-p38 (CST), anti-total p38 (CST), anti-phospho-Limk (Biorbyt), anti-phospho-Cofilin (CST), Horseradish peroxidase (HPR)-conjugated secondary antibodies against mouse and rabbit IgG (GE Healthcare). Detection was performed using ECL select WB detection reagent (GE Healthcare/Amersham). Data was collected using Chemidoc XRS^{+} Molecular Imager (Bio-Rad Laboratories) and data was analyzed and quantified using Image Lab software (Bio-Rad Laboratories).

Microarray

For microarray analysis, 5000–10000 LSK CD150^{+}CD48^{-}CD9^{hi} BM cells[4] were sorted into 350 μl RLT buffer (Qiagen) and immediately frozen on dry ice. Samples were then shipped on dry ice to the authorized Affymetrix service provider Kompetenzzentrum Fluoreszente Bioanalytik (KFB) Center of Excellence for Fluorescent Bioanalytics (Regensburg, Germany) and subsequent processing was performed at KFB. RNA isolation was done using the RNeasy

Micro Kit (Qiagen) according to manufacturer's protocol. Purity and integrity of the RNA was assessed on the Agilent 2100 Bioanalyzer with the RNA 6000 Pico LabChip reagent set (Agilent). Sample preparation for microarray (Affymetrix Gene Chip; Mouse Gene 1.0 ST array) was done as described in the NuGEN Ovation PicoSL WTA System V2 and NUGEN Encore Biotin Module manuals (NuGEN Technologies). Summarized probe set signals in log2 scale were calculated by using the RMA algorithm[5] with the Affymetrix GeneChip Expression Console v1.4 Software. After exporting into Microsoft Excel, average signal values, comparison fold changes and significance P-values were calculated. Probe sets with a fold change above 2.0 fold and a t-test P-value lower than 0.05 were considered significantly regulated.
Validation of microarray results was done for selected genes through qRT-PCR as above. The gene list generated by KFB was also further analyzed by gene set enrichment analysis (GSEA) using an online GSEA resource (Broad Institute)[6,7] and the Molecular Signatures Database (MSigDB) hallmark gene sets collection[6,8].

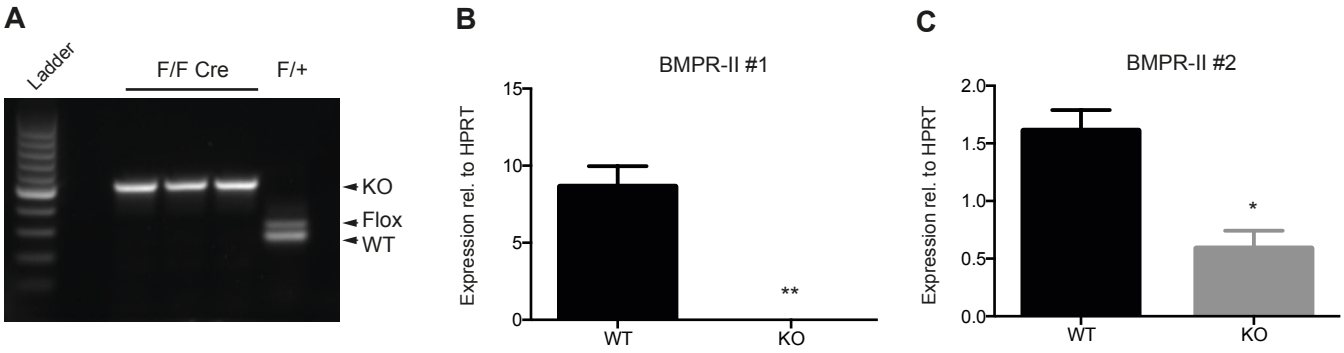
Quantitative RT-PCR
Cells were sorted into RLT buffer as described above. RNA isolation, cDNA synthesis, and qRT-PCR were performed as previously described[9]. Gene-specific primers (Taqman probes) were from Applied Biosystems.

Statistical analyses
Statistical analyses were performed in Prism (v 6-8, GraphPad) or Excel (Microsoft). Unless otherwise stated unpaired *t*-tests (parametric) or Wilcoxon-Mann-Whitney tests (non-parametric) were used, or for grouped analyses ANOVA or Kruskal-Wallis tests with *post hoc* multiple comparisons tests. Assumption of normality was tested on representative data using the D'Agostino & Pearson test where applicable, or the Shapiro-Wilk test as a secondary alternative. Similarly, assumption of homoscedasticity was evaluated using the F-test and appropriate statistical corrections applied where necessary. *P*-values less than 0.05 were considered statistically significant. Data are shown as mean ± standard error of the mean (SEM).

References (supplementary methods)

1. Monteiro RM, de Sousa Lopes SM, Bialecka M, de Boer S, Zwijsen A, Mummery CL. Real time monitoring of BMP Smads transcriptional activity during mouse development. *Genesis.* 2008;46(7):335-346.
2. Crisan M, Kartalaei PS, Vink CS*, et al.* BMP signalling differentially regulates distinct haematopoietic stem cell types. *Nature communications.* 2015;6:8040.
3. Crisan M, Solaimani Kartalaei P, Neagu A*, et al.* BMP and Hedgehog Regulate Distinct AGM Hematopoietic Stem Cells Ex Vivo. *Stem cell reports.* 2016;6(3):383-395.
4. Karlsson G, Rorby E, Pina C*, et al.* The tetraspanin CD9 affords high-purity capture of all murine hematopoietic stem cells. *Cell Rep.* 2013;4(4):642-648.
5. Irizarry RA, Hobbs B, Collin F*, et al.* Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics.* 2003;4(2):249-264.
6. Subramanian A, Tamayo P, Mootha VK*, et al.* Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A.* 2005;102(43):15545-15550.
7. Mootha VK, Lindgren CM, Eriksson KF*, et al.* PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nat Genet.* 2003;34(3):267-273.
8. Liberzon A, Birger C, Thorvaldsdottir H, Ghandi M, Mesirov JP, Tamayo P. The Molecular Signatures Database (MSigDB) hallmark gene set collection. *Cell Syst.* 2015;1(6):417-425.
9. Utsugisawa T, Moody JL, Aspling M, Nilsson E, Carlsson L, Karlsson S. A road map toward defining the role of Smad signaling in hematopoietic stem cells. *Stem Cells.* 2006;24(4):1128-1136.

Supplement 1

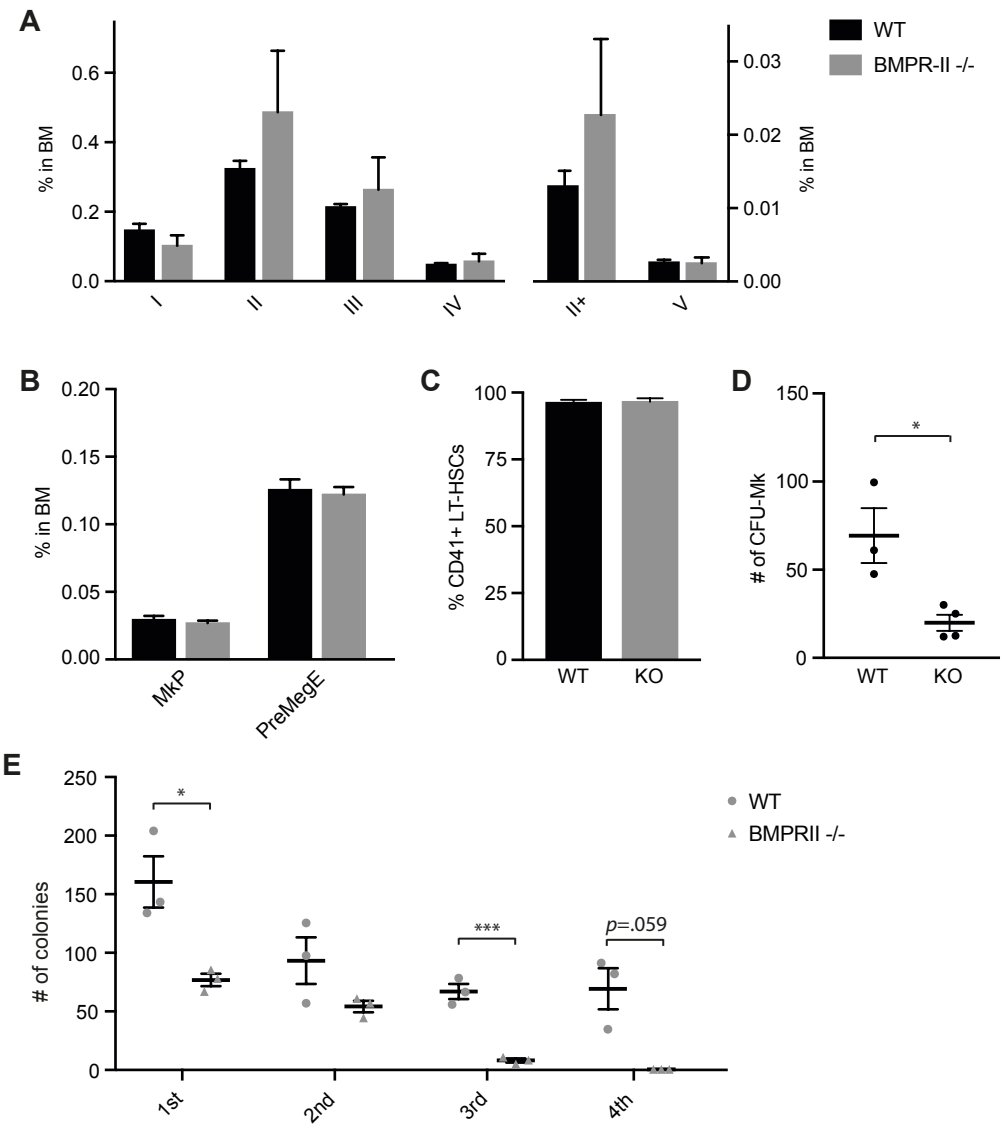


Validation of knockout.

A. Representative image from PCR analysis of individual colonies from bone marrow, confirming deletion of exon 4-5 of the *BMPR-II* gene.

B-C. qPCR analysis using two different probe sets, targeting exon 6 (B) and exon 1 (C), showing reduction of *BMPR-II* mRNA in purified LT-HSCs (n=3). A small amount of truncated mRNA was detected using the latter probe set (C).
** $P < .01$ and * $P < .05$

Supplement 2



Megakaryocytic lineage distribution, Mk progenitors, and CFU-Mk. Assessment of phenotypic aging of LT-HSCs. Serial replating assay.

A. Percentage of various megakaryocytic lineages in bone marrow (n=3).

B. Percentage of megakaryocyte progenitors in bone marrow (n=3).

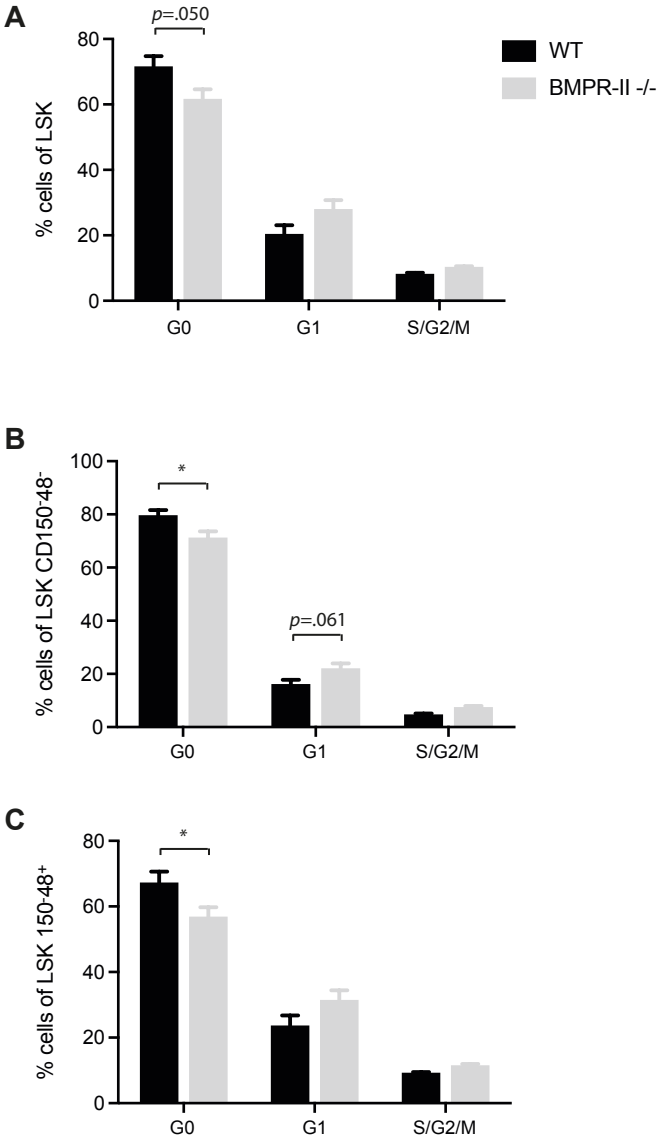
C. CD41+ LT-HSCs as assessed by flow cytometry (n=6-7).

D. Number of megakaryocytic colonies in CFU-Mk assay.

D. Serial replating of BMPRII deficient and WT littermate bone marrow cells. X-axis indicatesthe replating at which colony number was counted. Y-axis indicates average number of colonies counted per plate.

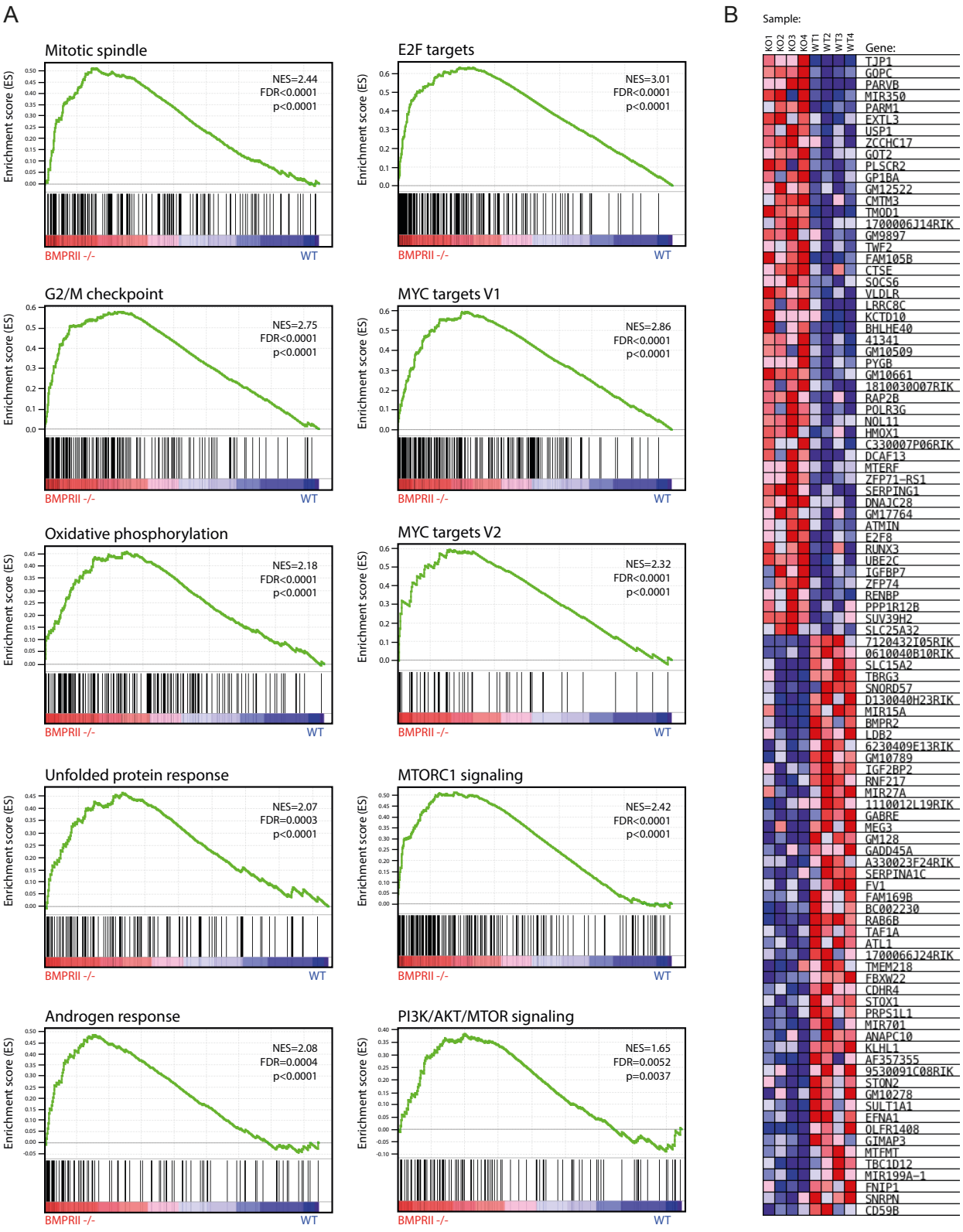
* $P < .05$; *** $P < .001$

Supplement 3



Cell cycle assay results in LSK cells (A), LSK CD48-CD150- cells (B), and LSK CD48-CD150+ cells (C). (n=9-10) * $P < 0.05$

Supplement 4



Analysis of microarray data.

A. GSEA on microarray data to identify gene sets enriched or depleted in BMPRII -/- HSCs compared to WT controls. NES, normalized enrichment score; FDR, false discovery rate.

B. Heat map of top differentially expressed genes in BMPRII -/- HSCs (samples KO1-4) compared to WT controls (samples WT1-4).

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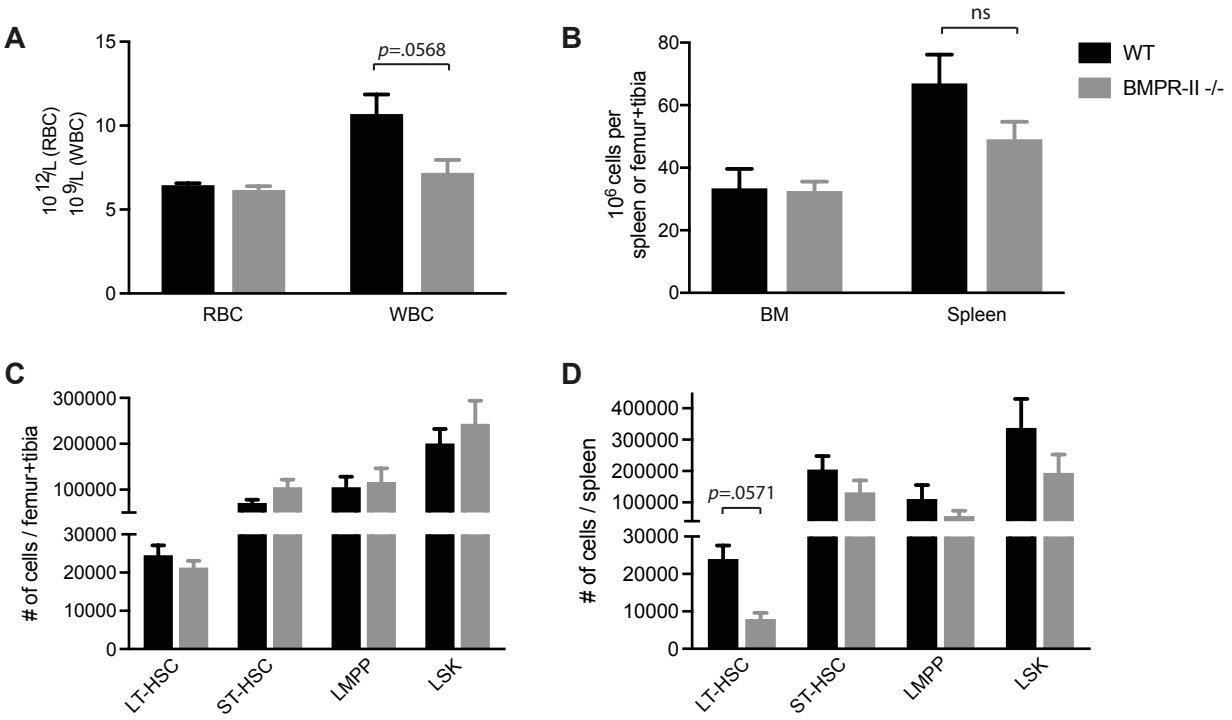

Analysis of hematopoietic stress response following 5-fluorouracil (5-FU) injections *in vivo*.
Analyses done on day 12 after intravenous injection of 5-FU (n=3-4).

A. Sysmex analysis of red blood cells (RBC) and white blood cells (WBC) in peripheral blood of mice.

B. Cellularity, i.e. total number of cells, in bone marrow and spleen.

C-D. Number of primitive hematopoietic cells in bone marrow (C) and spleen (D).

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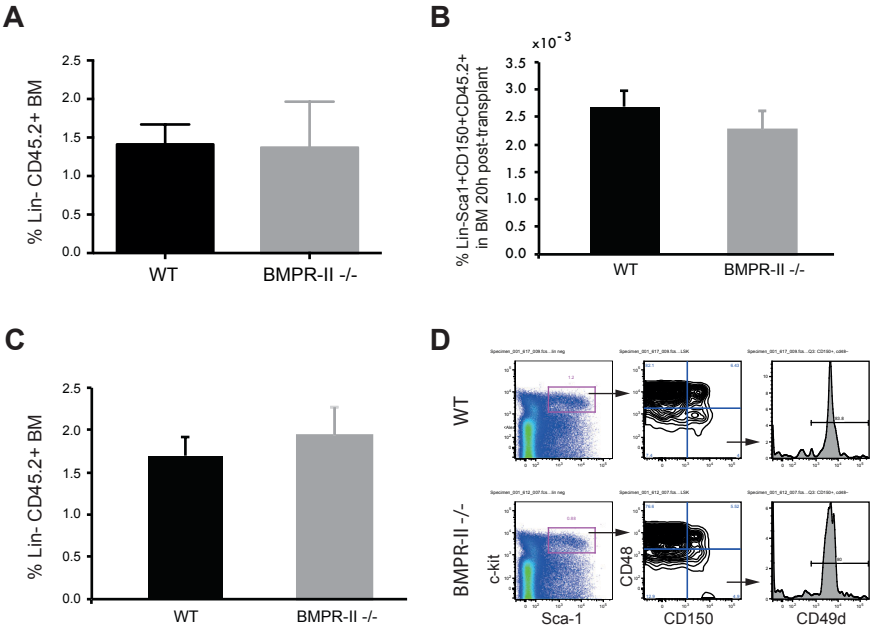


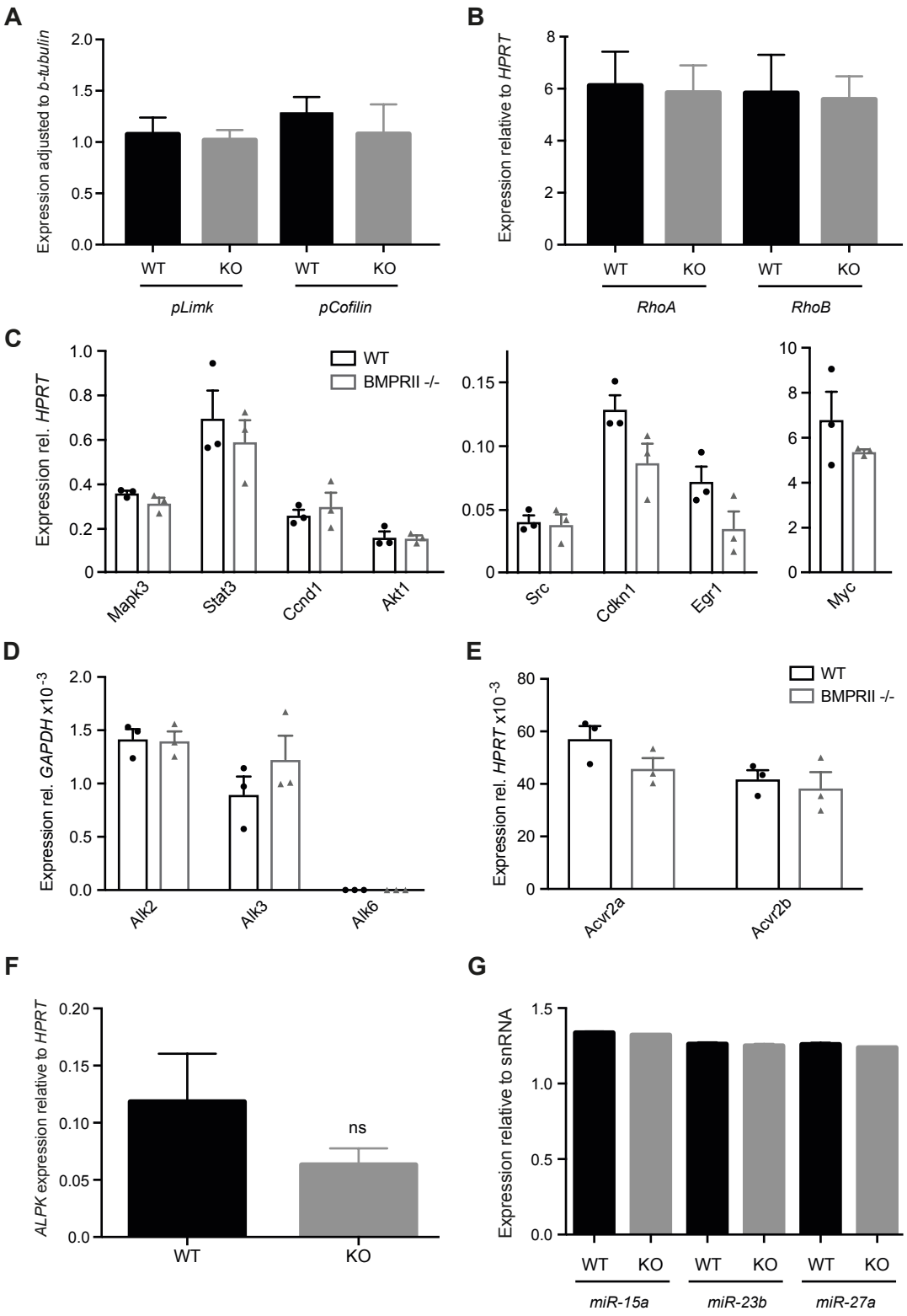
Homing assays and analysis of integrin-a4 (CD49d) expression.

A. Percentage of Lin-/CD45.2+ cells in BM of transplanted recipients in the homing assay (n=5).

B-C. Percentage of Lin-/Sca1+/CD150+/CD45.2+ cells and Lin-/CD45.2+ cells in BM of transplanted recipients in the competitive homing assay (n=3).

D. Representative plot of CD49d expression on LT-HSCs as measured by flow cytometry, showing no differences between BMPR-II knockout and control cells.

Supplement 7



Several known signaling factors associated with TJP1 or canonical BMP signaling are not affected by BMPR-II deficiency. Array hits Alpk and microRNA levels are also not affected.

A. Western blot of Limk & Cofilin phosphorylation in WT and BMPR-II deficient c-kit+ cells (n=3).

B. qPCR of RhoA and RhoB expression in WT and BMPR-II deficient LT-HSCs (n=3).

C. Gene expression relative *HPRT* in LSK cells (no singificant differences). Evaluation of factors known to associate with TJP1 (Stat3 and Src) as well as known downstream BMP mediators.

D-E. Gene expression of BMP type I (D) and type II (E) receptors in primitive hematopoietic (LSK CD48-) cells (no significant differences).

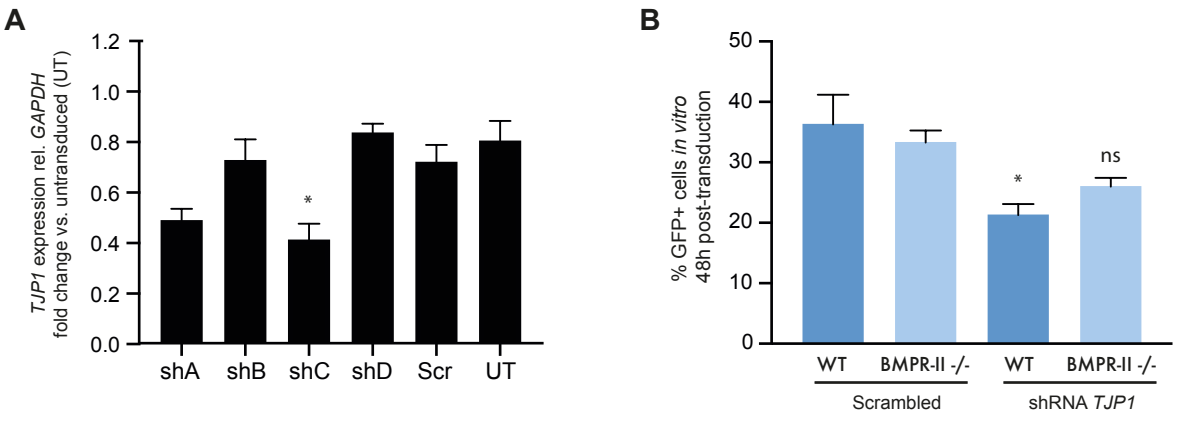
F-G. qPCR of Alpk (D) and microRNA miR-15a, miR-23b, and miR-27a (E) expression in WT and BMPR-II deficient LT-HSCs (n=3). ns = not significant.

Supplement 8

Microarray results from KFB Center of Excellence for Fluorescent Bioanalytics.
Genes up- or down-regulated >1.75-fold in *BMPR2* knock-out LT-HSCs in comparison to wild type control LT-HSCs.

Gene symbol	Gene description	Fold change	p-value (t-test)
Mir99a	microRNA 99a	-2,59	0,1415
Mir32	microRNA 32	-2,58	0,0683
Scn2a1	sodium channel, voltage-gated, type II, alpha 1	-2,44	0,2891
Alpk1	alpha-kinase 1	-2,35	0,0528
Mir15a	microRNA 15a	-2,29	0,0226
Scarna8	small Cajal body-specific RNA 8	-2,12	0,2590
Rbm33	RNA binding motif protein 33	-2,12	0,1128
Tbc1d5	TBC1 domain family, member 5	-2,07	0,0098
Scn2a1	sodium channel, voltage-gated, type II, alpha 1	-2,04	0,4087
Snord57	small nucleolar RNA, C/D box 57	-2,02	0,0272
Mir27b	microRNA 27b	-1,98	0,0975
Mir23b	microRNA 23b	-1,97	0,2874
Slc15a2	solute carrier family 15 (H+/peptide transporter), member 2	-1,95	0,0065
Meg3	maternally expressed 3	-1,94	0,0286
Hspb1	heat shock protein 1	-1,94	0,1271
Nlrc5	NLR family, CARD domain containing 5	-1,93	0,1463
Scn2a1	sodium channel, voltage-gated, type II, alpha 1	-1,92	0,3681
Mir340	microRNA 340	-1,90	0,1755
Snora69	small nucleolar RNA, H/ACA box 69	-1,89	0,2001
Adamts3	a disintegrin-like and metallopeptidase, thrombospondin type 1 motif, 3	-1,86	0,1420
Rtn2	reticulon 2 (Z-band associated protein)	-1,86	0,1714
Syngap1	synaptic Ras GTPase activating protein 1 homolog (rat)	-1,85	0,1989
Gimap3	GTPase, IMAP family member 3	-1,82	0,0601
Mir27a	microRNA 27a	-1,81	0,0321
Tbrg3	transforming growth factor beta regulated gene 3	-1,81	0,0072
Igf2bp2	insulin-like growth factor 2 mRNA binding protein 2	-1,78	0,0300
Hspb1	heat shock protein 1	-1,76	0,1222
Taf1a	TATA box binding protein (Tbp)-associated factor, RNA polymerase I, A	-1,76	0,0471
Plk2	polo-like kinase 2	-1,75	0,0816
Ldb2	LIM domain binding 2	-1,75	0,0210
Mir701	microRNA 701	-1,75	0,0426
Gm10509	zinc finger protein 51 pseudogene	1,75	0,0587
Rnf213	ring finger protein 213	1,79	0,0115
Gm10509	zinc finger protein 51 pseudogene	1,81	0,1167
Ear1	eosinophil-associated, ribonuclease A family, member 1	1,82	0,2062
Plscr2	phospholipid scramblase 2	1,83	0,0800
Dock6	dedicator of cytokinesis 6	1,85	0,0057
Adamts3	a disintegrin-like and metallopeptidase, thrombospondin type 1 motif, 3	1,86	0,0482
Xlr	X-linked lymphocyte-regulated complex	1,92	0,4001
Mir350	microRNA 350	1,96	0,0509
Xist	inactive X specific transcripts	2,81	0,5341
Tjp1	tight junction protein 1	3,06	0,0009

Supplement 9



TJP1 knockdown using shRNA vectors and transduction efficiency.

A. *TJP1* gene knockdown seen as fold change compare to untransduced cells (UT). Four different *TJP1*-shRNA lentiviral vectors (sh A-D) and one scrambled control shRNA (Scr) were used to transduce ckit+ wild type bone marrow cells, which were grown in vitro for 48 hours before sorting into RLT buffer using a FACS Aria III. Sorting purity of experiments were 50-75 % on average. Sorted and lysed cells were subsequently prepared for and analysed by qPCR. (n=6) * $P < .05$ (in comparison to UT) shRNA-C was chosen for the following transductions in transplantation experiments.

B. Transduction efficiency (using shRNA-C) for TJP1 knockdown transplantation experiments, analyzed by flow cytometry 48 hours post-transduction. (n=6) * $P < .05$ (compared to both Scrambled groups). ns = not significant.