Supplemental Information

Materials and Methods

Mice. Mice bearing the conditional oncogenic Nras (Lox-stop-Lox (LSL) Nras) mutation were crossed to Mx1-Cre mice to generate LSL $Nras^{G12D/+}$; Mx1-Cre mice. These lines were maintained in a pure C57BL/6 genetic background (>N10). $Erk2^{fl/+}$ [15] and $Nras^{G12D/+}$; $Erk2^{fl/+}$; Mx1-Cre mice were in a mixed CD1/B6 genetic background. CD45.1-positive congenic C57BL/6 recipient mice were purchased from NCI.

Flow cytometric analysis of hematopoietic tissues (Figs. 1A-1C, S2, S4, and S6D). For lineage analysis of peripheral blood, bone marrow and spleen, flow cytometric analyses were performed as previously described [1]. Myeloid progenitors and common lymphoid progenitors in bone marrow and spleen were analyzed as previously described [2,3]. Hematopoietic stem cells (HSCs) in bone marrow and spleen were analyzed as described in [4]. Because hind limb bone marrow represents ~25% of total bone marrow [5], the number of HSCs in total bone marrow is calculated as 4 X the number of HSCs in hind limb bone marrow. The stained cells were analyzed on a FACS Calibur or a LSRII (BD Biosciences).

Directly conjugated antibodies specific for the following surface antigens were purchased from eBioscience: CD45.2 (104), B220 (RA3-6B2), CD19 (eBio1D3), Thy1.2 (53-2.1), Mac-1 (M1/70), Gr-1 (RB6-8C5), CD4 (GK1.5), CD8 (53-6.7), CD3 (145-2C11), IgM (II/41), IL7Rα (A7R34), Sca-1 (D7), TER119(TER-119), CD34 (RAM34), cKit (2B8). FcγRII/III (2.4G2) was purchased from BD Biosciences. CD150 (TC15-12F12.2) was purchased from Biolegend. Following biotin-conjugated antibodies were purchase from eBioscience: B220 (RA3-6B2), Gr-1 (RB6-8C5), CD8 (53-6.7). Following biotin-conjugated antibodies were purchased from BD Biosciences: CD19 (1D3), CD4 (RM4-5), CD3 (145-2C11), IgM (R6-60.2), IL7Rα (B12-1), TER119 (TER-119).

Cell cycle analysis of HSCs (Figs 1D and S3A). Cell cycle analysis was performed essentially as described [6]. Cells were stained with PECy7-conjugated antibodies against CD41, CD48, B220, TER119 and Gr1 and were simultaneously stained for PE-CD150, APC-cKit, PerCP Cy5.5-Sca1, FITC-Ki67 (BD Biosciences), and DAPI (Invitrogen). The stained cells were analyzed on a LSRII (BD Biosciences).

EdU incorporation (Figs. 1E, 2F, and S3B). EdU (Invitrogen) was administered as a single dose of 1 mg by intraperitoneal injection. EdU incorporation in vivo was measured 16 hours later using the Click-It EdU Pacific Blue Flow Kit (Invitrogen). $Sca1^+$ cells were enriched using an AutoMACS (Miltenyi). Enriched cells were first stained with FITC-conjugated antibodies against CD41, CD48, B220, TER119 and Gr1 and APC-CD150. After Click-It reaction, cells were then stained with PE-cKit and PerCP Cy5.5-Sca1. The stained cells were analyzed on a LSRII (BD Biosciences).

Bone marrow transplantation of HSCs (Figs. 1F and 1G). HSCs were purified as $B220^-$ $Gr1^-$ $TER119^-$ $CD41^-$ $CD48^-$ $Sca1^+$ $cKit^+$ $CD150^+$ bone marrow cells using a FACS AriaII (BD Biosciences) as described [4]. Twenty purified HSCs ($CD45.2^+$) were transplanted with 2 X 10^5 whole bone marrow cells ($CD45.1^+$) into individual lethally

irradiated mice as described [1]. Sixteen weeks after transplantation, 2 X 10^6 whole bone marrow cells were isolated from primary recipients and transplanted into individual lethally irradiated mice.

Gene expression profiling (Fig. S6A). Five hundred HSCs were sorted using a FACS AriaII (BD Biosciences) and used in each biological replica. Sorting purity was routinely >96%. Microarray analysis was performed by Miltenyi Biotech using Agilent Mouse Whole Genome 4X44K array chips. Heat maps were generated using the dChip software.

Gene set enrichment analysis (Figs. S6B and S6C). Gene set enrichment analysis was performed using Gene Set Enrichment Analysis software (GSEA V2.07) [7,8]. The following running parameters were used: gene set as permutation type, and 1,000 permutations and values of normalized density of classes as metric for ranking genes. Gene sets related to myeloid and lymphoid differentiation were from the MSig database of the Broad Institute, Cambridge, MA.

Flow cytometric analysis of phospho-ERK1/2 and –Akt in HSCs and MPPs (Figs. 2A, 2B, S5, and S8). Flow cytometric analysis of phospho-ERK1/2 and –Akt in HSCs and MPPs were performed as previous described [9]. Briefly, $Sca1^+$ cells were enriched from the bone marrow cells using an AutoMACS (Miltenyi Biotec, Bergisch Gladbach, Germany). $CD150^+$ $CD41^-$ (enriched for HSCs) and $CD150^-$ $CD41^-$ cells (enriched for MPPs) were sorted using a FACS AriaII (BD Biosciences). Sorted cells were subjected to phospho-flow analysis. Surface proteins were detected with FITC-conjugated antibodies (BD Biosciences unless specified) against B220 (6B2), Gr-1 (RB6-8C5), TER119, and CD48, and PE-conjugated anti-CD117/c-Kit antibody (eBiosciences, San Diego, CA). p-ERK1/2 or p-Akt was detected by a primary antibody against pERK (Thr202/Tyr204; Cell signaling Technology) or pAkt (Ser473; Cell Signaling Technology) followed by APC conjugated donkey anti-rabbit F(ab')2 fragment (Jackson ImmunoResearch).

Administration of AZD6244 (Figs. 2D, 2E, and S7). AZD6244 (Sequocia and ChemieTek) was administered at 50 mg/kg by oral gavage twice a day as described [10,11]. The treatment started on Day 5 and lasted for 7 days.

Flow cytometric analysis of pERK1/2 and pSTAT5 in progenitor cells (Fig. 2C). Nras G12D/+ bone marrow cells were serum- and cytokine-starved and incubated with vehicle (Veh) or 5 μM of AZD6244 for 30 minutes. Cells were then stimulated with or without 10 ng /ml of GM-CSF for 10 minutes at 37°C. Phosphorylated ERK1/2 and STAT5 were analyzed in defined $Lin^{-/low}$ $c\text{-}Kit^+$ cells essentially as previously described [2].

Table S1. Genetically altered HSCs are required to initiate and maintain CMML-like phenotypes

Donor cell types	Genotypes	Donors	Number of cells	Number of recipients	Observation time (Months)	CMML
BM	$Nras^{G12D/+}$	Primary mice	$2.5X10^5$	54	24	51
MP	$Nras^{G12D/+}$	Primary mice	$1\text{-}2X10^4$	26	18	1
HSC (BM)	$Nras^{G12D/+}$	Primary mice	10-50	33	18-24	17

BM	$Nras^{G12D/+}$	CMML mice	$2\text{-}10X10^6$	26	17	12
MP	$Nras^{G12D/+}$	CMML mice	$2X10^4$	12	17	0
BM without HSC	$Nras^{G12D/+}$	CMML mice	$1\text{-}5X10^6$	8	17	0
SP	$Nras^{G12D/+}$	CMML mice	$2\text{-}5X10^6$	23	17	9
HSC (SP)	$Nras^{G12D/+}$	CMML mice	10	13	17	2
SP without HSC	$Nras^{G12D/+}$	CMML mice	$2\text{-}5X10^6$	9	17	0

In the first round of transplantation, various numbers of cells were isolated from primary Nras G12D/+ mice and transplanted with $2.5X10^5$ whole bone marrow cells (CD45.1+) into individual lethally irradiated mice. Once the recipient mice developed a lethal CMML, various cell types were isolated from moribund mice and further transplanted into sublethally irradiated 2nd recipient mice as described [1]. BM, bone marrow; MP, myeloid progenitor; SP, spleen.

Supplementary Figure Legends

Supplementary Figure 1. Evaluation of recombination efficiency of the $Nras^{G12D/+}$ allele in HSCs from Mx1-Cre; $Nras^{G12D/+}$ mice treated with or without pI-pC. Individual HSCs were sorted into 96-well plates and cultured for 14 days. Genomic DNA was extracted from individual colonies and analyzed by PCR. Control DNA (C) was extracted from a $Nras^{G12D/+}$ mouse to show the wild-type and 2LoxP alleles. More than 50 HSC colonies per animal were analyzed.

Supplementary Figure 2. Evaluation of pI-pC treatment on Sca1 expression in total bone marrow cells and defined HSCs.

Supplementary Figure 3. Representative plots of HSC cell cycle and proliferation analyses.

Supplementary Figure 4. Analysis of multi-lineage reconstitution in recipient mice. Donor-derived myeloid cells ($Mac1^+$), B cells ($CD19^+$), and T cells ($Thy1.2^+$) were analyzed in the peripheral blood of primary (A) and secondary (B) recipient mice using flow cytometry. Data are presented as mean $\pm$ s.d.. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Supplementary Figure 5. A MEK inhibitor (AZD6244) blocks constitutive hyperactivation of ERK1/2 in $Nras^{G12D/+}$ HSCs.

Supplementary Figure 6. Gene set enrichment analysis (GSEA) of HSC microarray results identifies a gene signature of myeloid differentiation in $Nras^{G12D/+}$ HSCs. (A, B, C) 500 HSCs were purified from control or $Nras^{G12D/+}$ mice for microarray analysis. (A) Heat-map analysis of known genes associated with HSC self-renewal. (B) GSEA analysis of the Wnt and Notch pathways. (C) GSEA analysis of myeloid versus lymphoid differentiation. FDR, false discovery rate; NES, normalized enrichment score. (D) Quantification of bone marrow (BM) common lymphoid progenitors (CLPs) from control and $Nras^{G12D/+}$ mice. Data are presented as mean $\pm$ s.d.. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Supplementary Figure 7. A MEK inhibitor reduces the enlarged spleen in $Nras^{G12D/+}$ mice.

Supplementary Figure 8. $ERK2^{+/-}$ partially downregulates ERK signaling in $Nras^{G12D/+}$ HSCs.

Supplementary Figure 9. $ERK2^{+/-}$ does not affect slightly enlarged spleen in $Nras^{G12D/+}$ mice.

Supplementary References

1. Zhang J, Wang J, Liu Y, et al. Oncogenic Kras-induced leukemogeneis: hematopoietic stem cells as the initial target and lineage-specific progenitors as the potential targets for final leukemic transformation. Blood. 2009;113:1304-1314.
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9. Du J, Wang J, Kong G, et al. Signaling profiling at the single cell level identifies a distinct signaling signature in murine hematopoietic stem cells
. Stem Cells. 2012;30:1447-1454.
10. Yeh TC, Marsh V, Bernat BA, et al. Biological characterization of ARRY-142886 (AZD6244), a potent, highly selective mitogen-activated protein kinase kinase 1/2 inhibitor. Clin Cancer Res. 2007;13:1576-1583.
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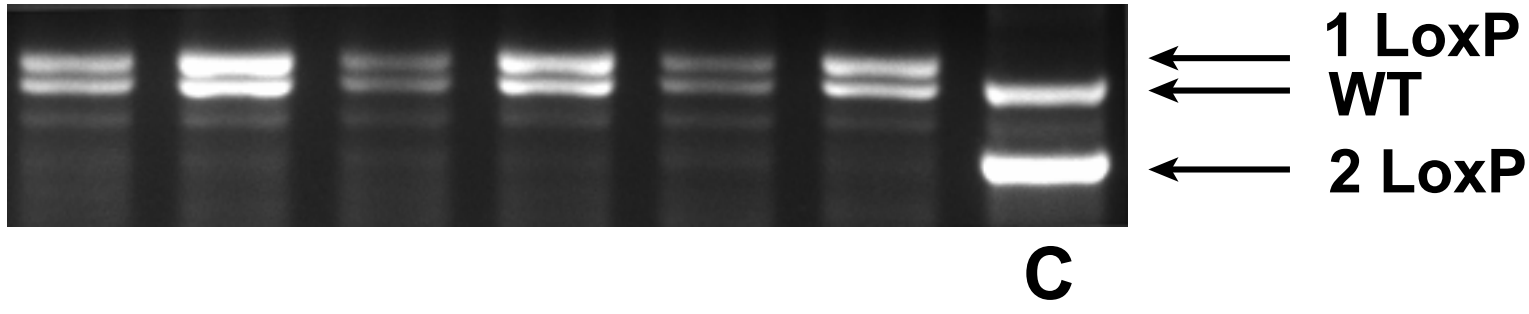
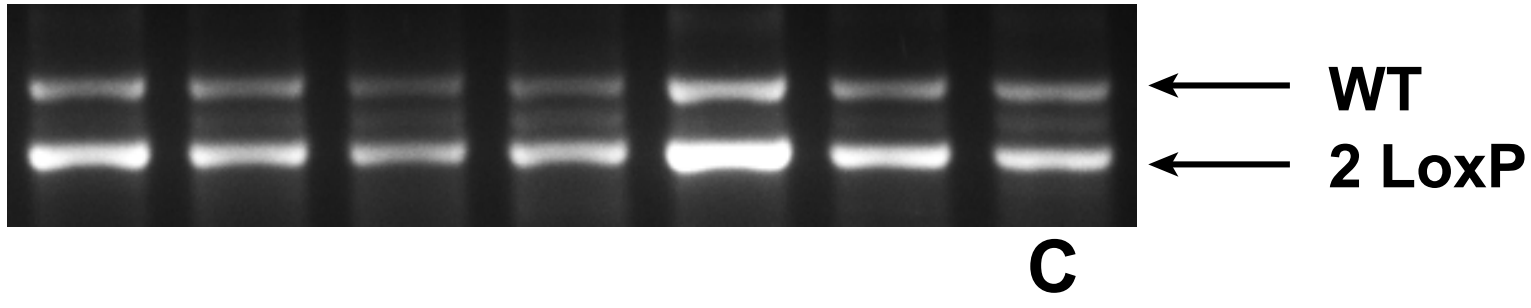
Fig S1-Zhang

Nras G12D/+ with pI-pC



Nras G12D/+ without pI-pC #1



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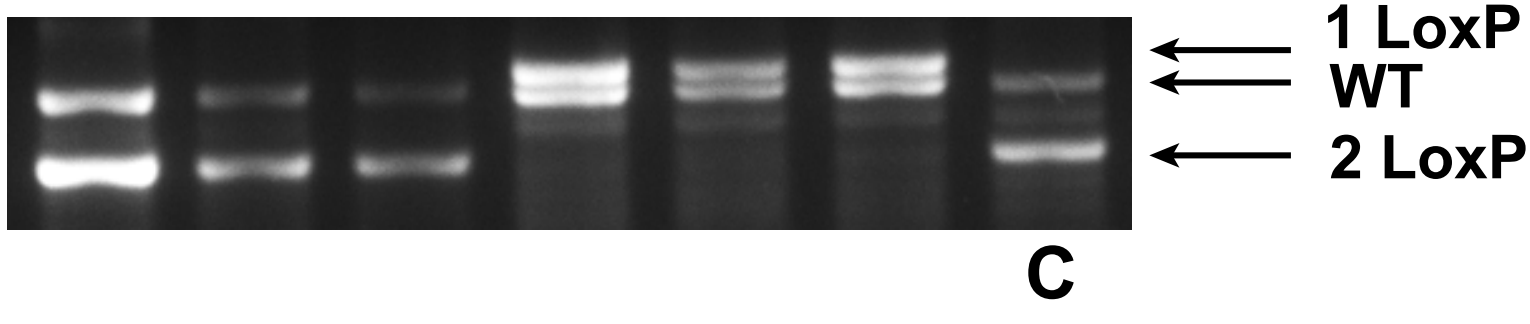


Fig S2-Zhang

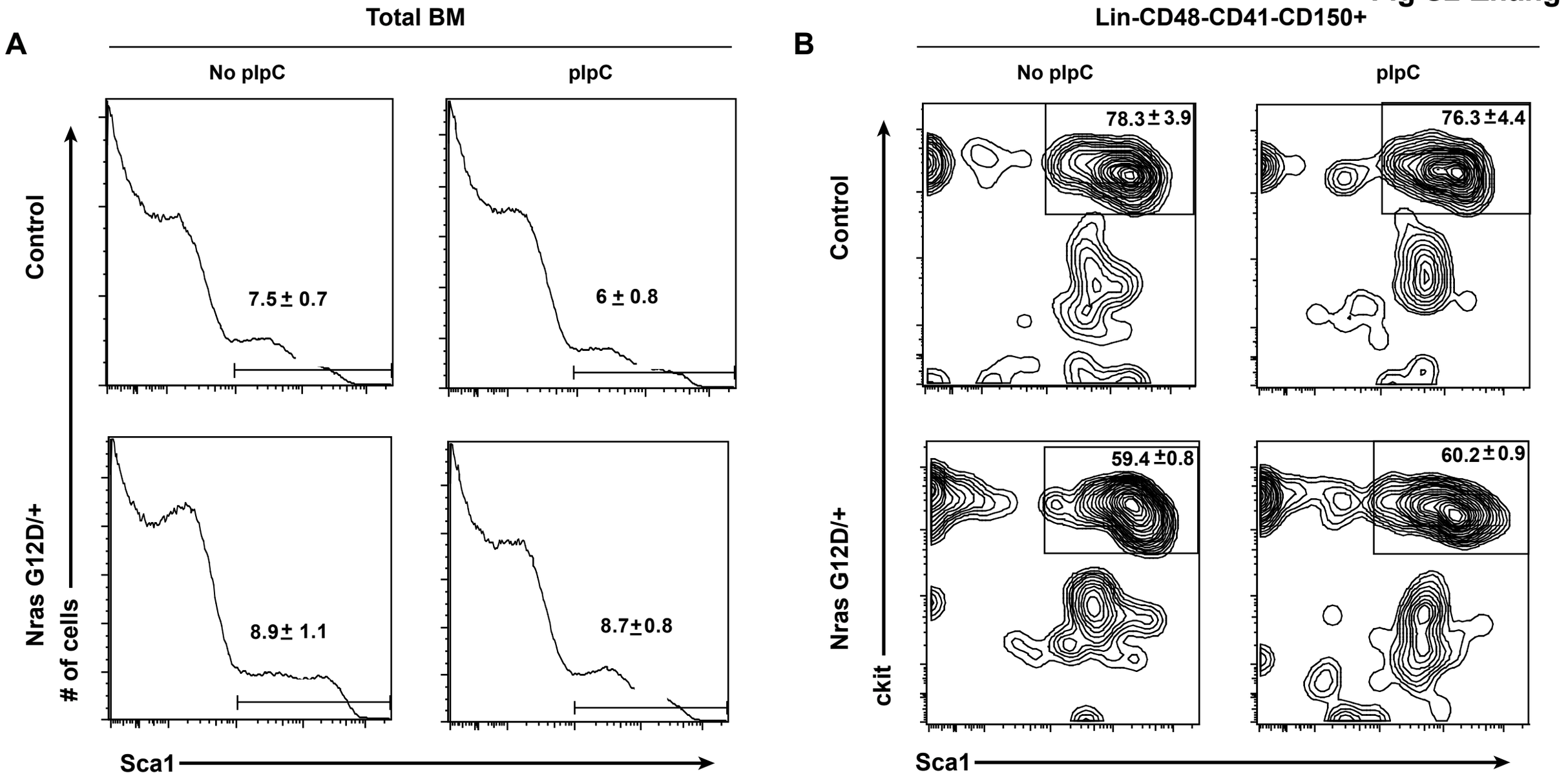

A
Total BM
No plpC
plpC
Control
Nras G12D/+
# of cells
7.5 ± 0.7
6 ± 0.8
8.9± 1.1
8.7±0.8
Sca1
B
Lin-CD48-CD41-CD150+
No plpC
plpC
Control
Nras G12D/+
ckit
78.3 ± 3.9
76.3 ±4.4
59.4 ±0.8
60.2 ± 0.9
Sca1

Fig S3-Zhang

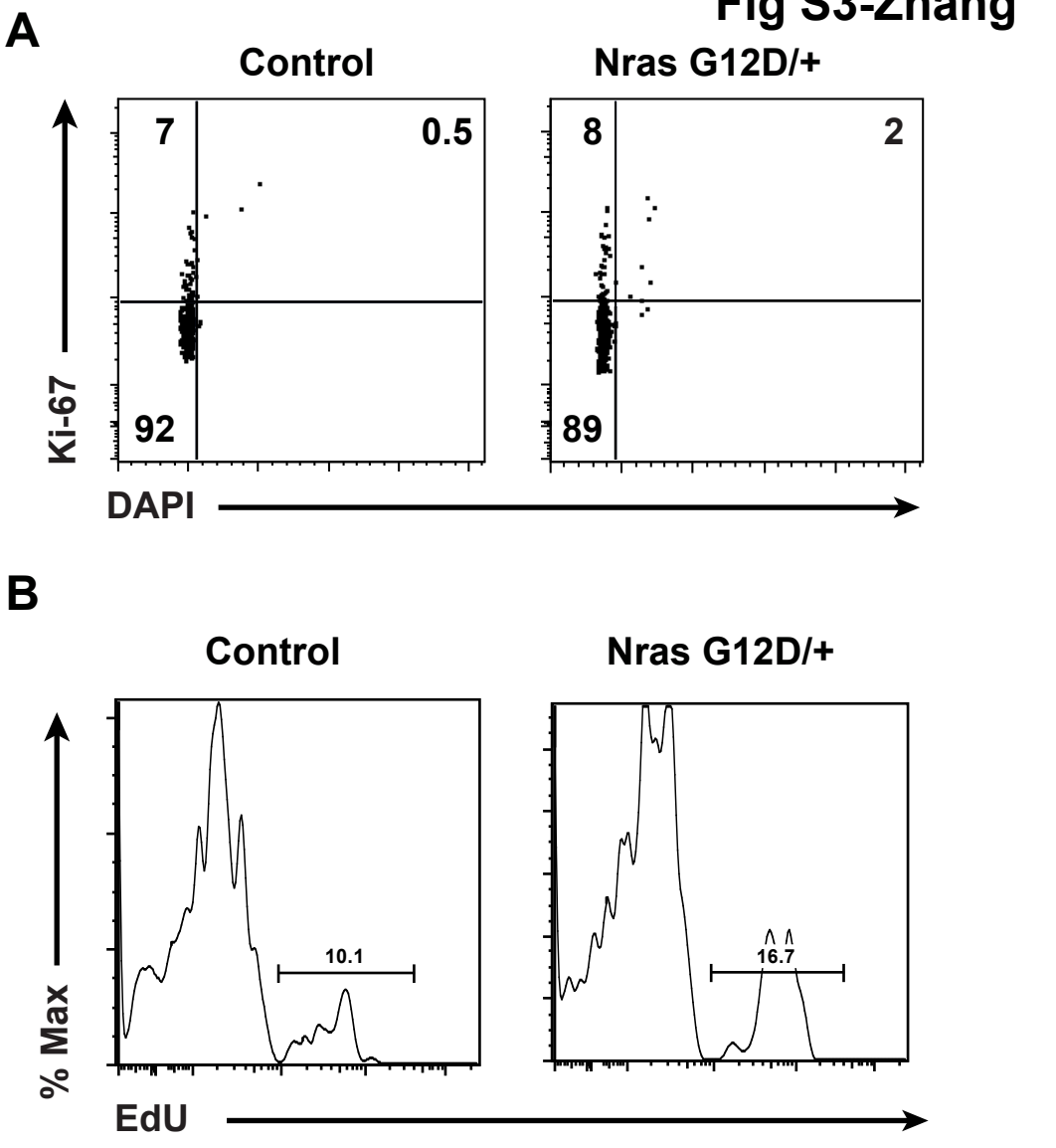

A
Control
Nras G12D/+
7
0.5
92
8
2
89
Ki-67
DAPI
B
Control
Nras G12D/+
10.1
16.7
% Max
EdU

Fig S4-Zhang

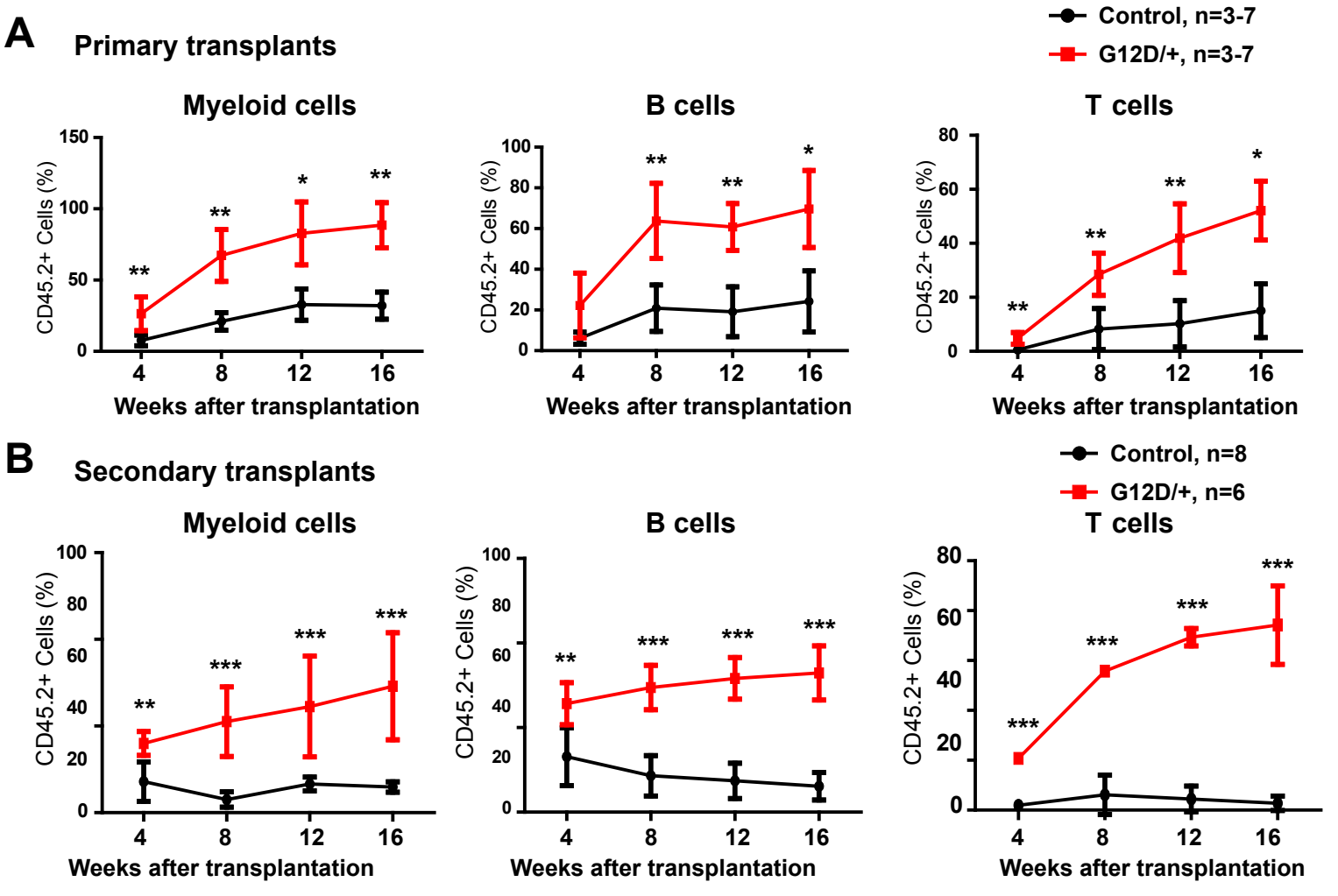

A
Primary transplants
Control, n=3-7
G12D/+, n=3-7
Myeloid cells
B cells
T cells
CD45.2+ Cells (%)
Weeks after transplantation
B
Secondary transplants
Control, n=8
G12D/+, n=6
Myeloid cells
B cells
T cells
CD45.2+ Cells (%)
Weeks after transplantation

Fig S5-Zhang

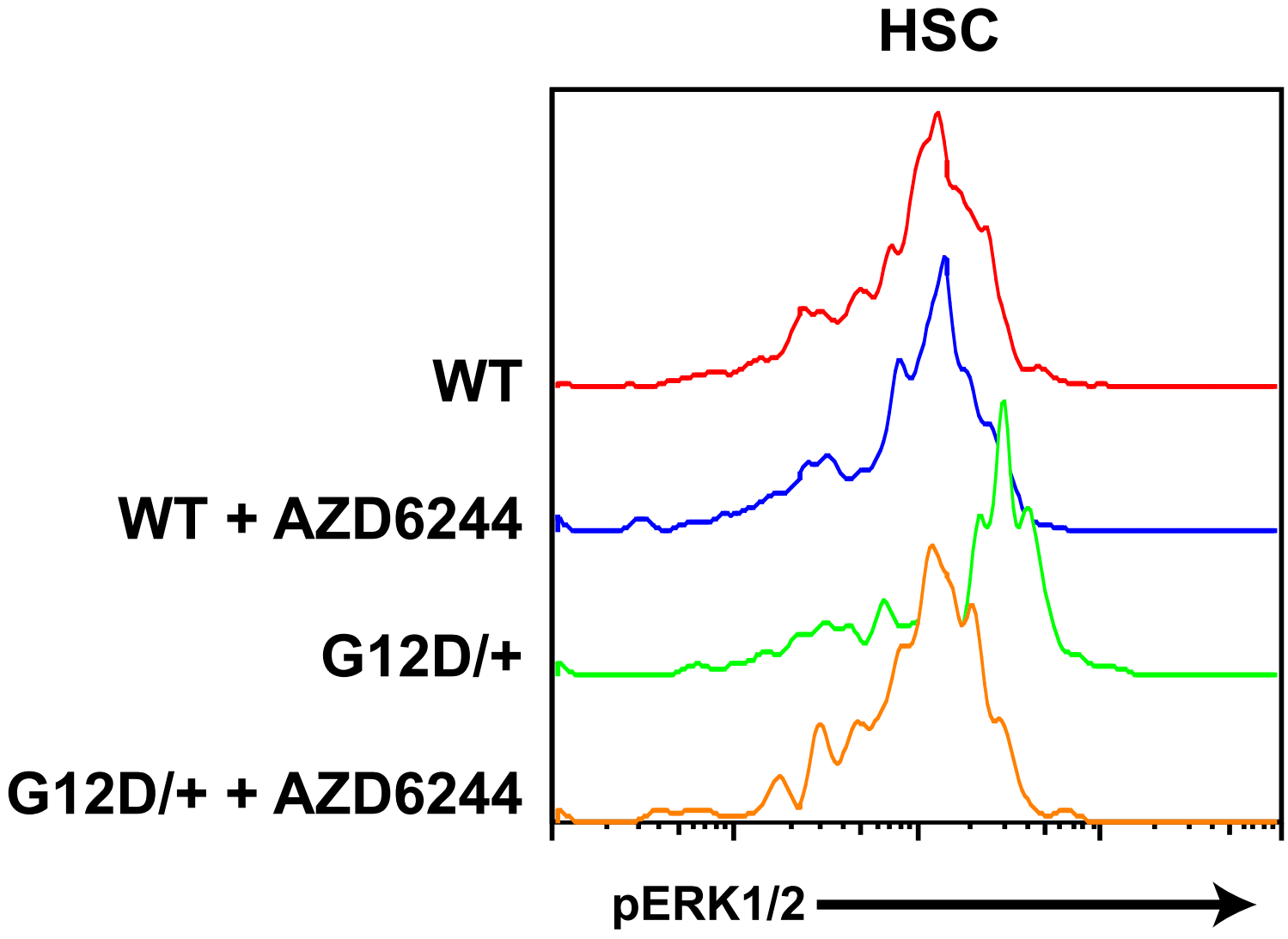


Fig S6-Zhang

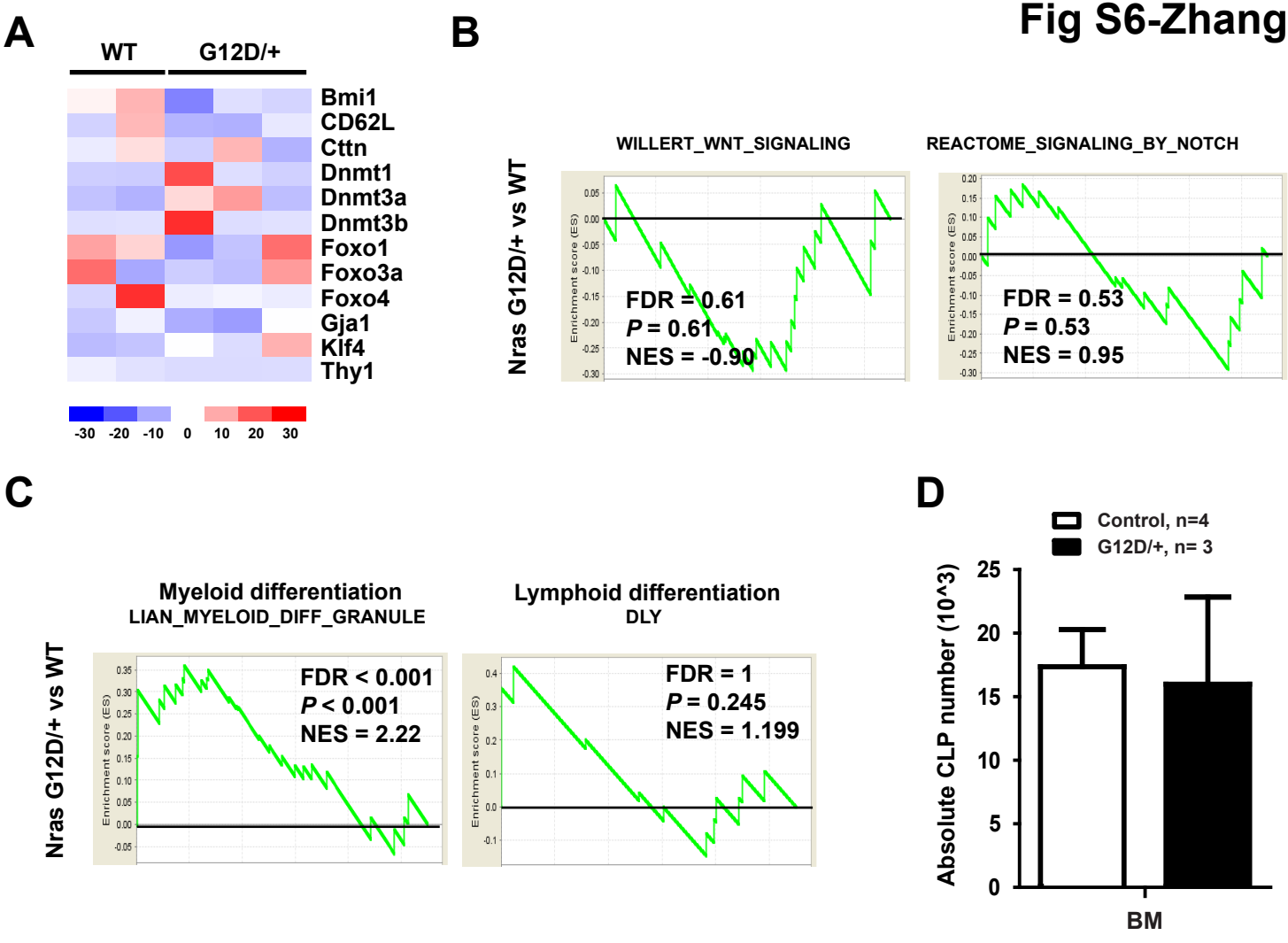

A
WT
G12D/+
Bmi1
CD62L
Cttn
Dnmt1
Dnmt3a
Dnmt3b
Foxo1
Foxo3a
Foxo4
Gja1
Klf4
Thy1
-30 -20 -10 0 10 20 30
B
Nras G12D/+ vs WT
WILLERT_WNT_SIGNALING
FDR = 0.61
P = 0.61
NES = -0.90
REACTOME_SIGNALING_BY_NOTCH
FDR = 0.53
P = 0.53
NES = 0.95
Enrichment score (ES)
C
Nras G12D/+ vs WT
Myeloid differentiation
LIAN_MYELOID_DIFF_GRANULE
FDR < 0.001
P < 0.001
NES = 2.22
Lymphoid differentiation
DLY
FDR = 1
P = 0.245
NES = 1.199
Enrichment score (ES)
D
Control, n=4
G12D/+, n= 3
Absolute CLP number (10^3)
0
5
10
15
20
25
BM

Fig S7-Zhang

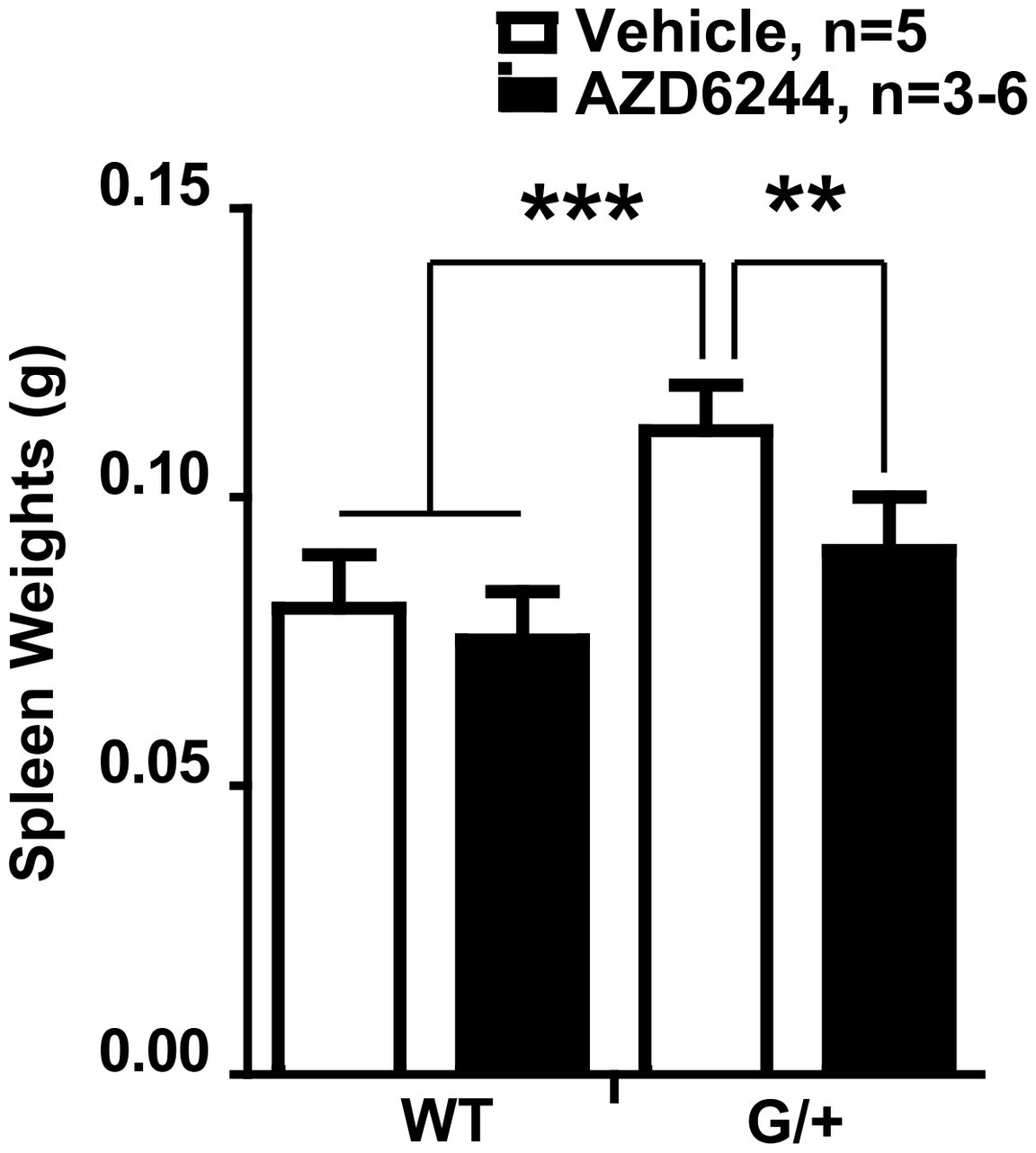


Fig S8-Zhang

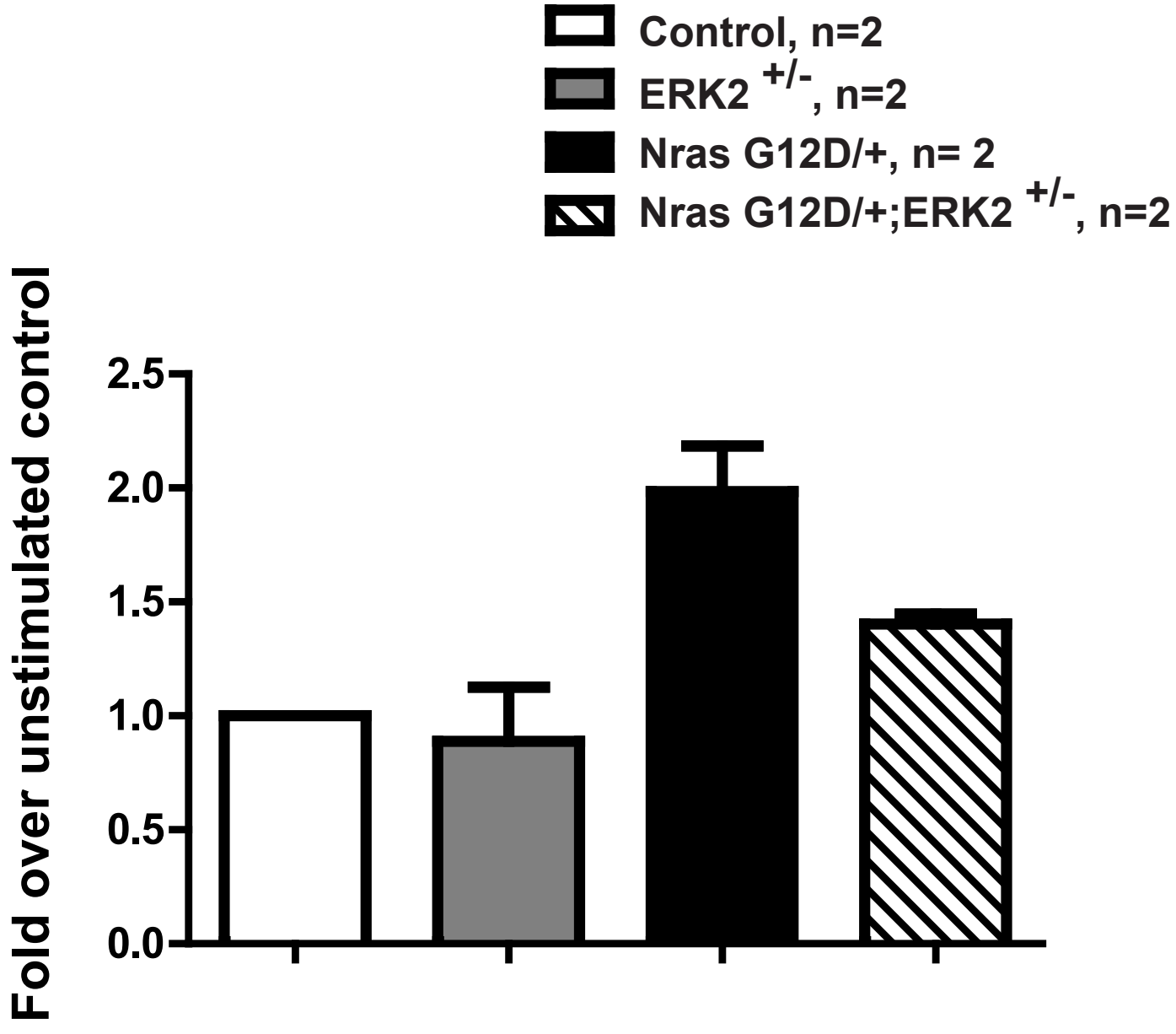
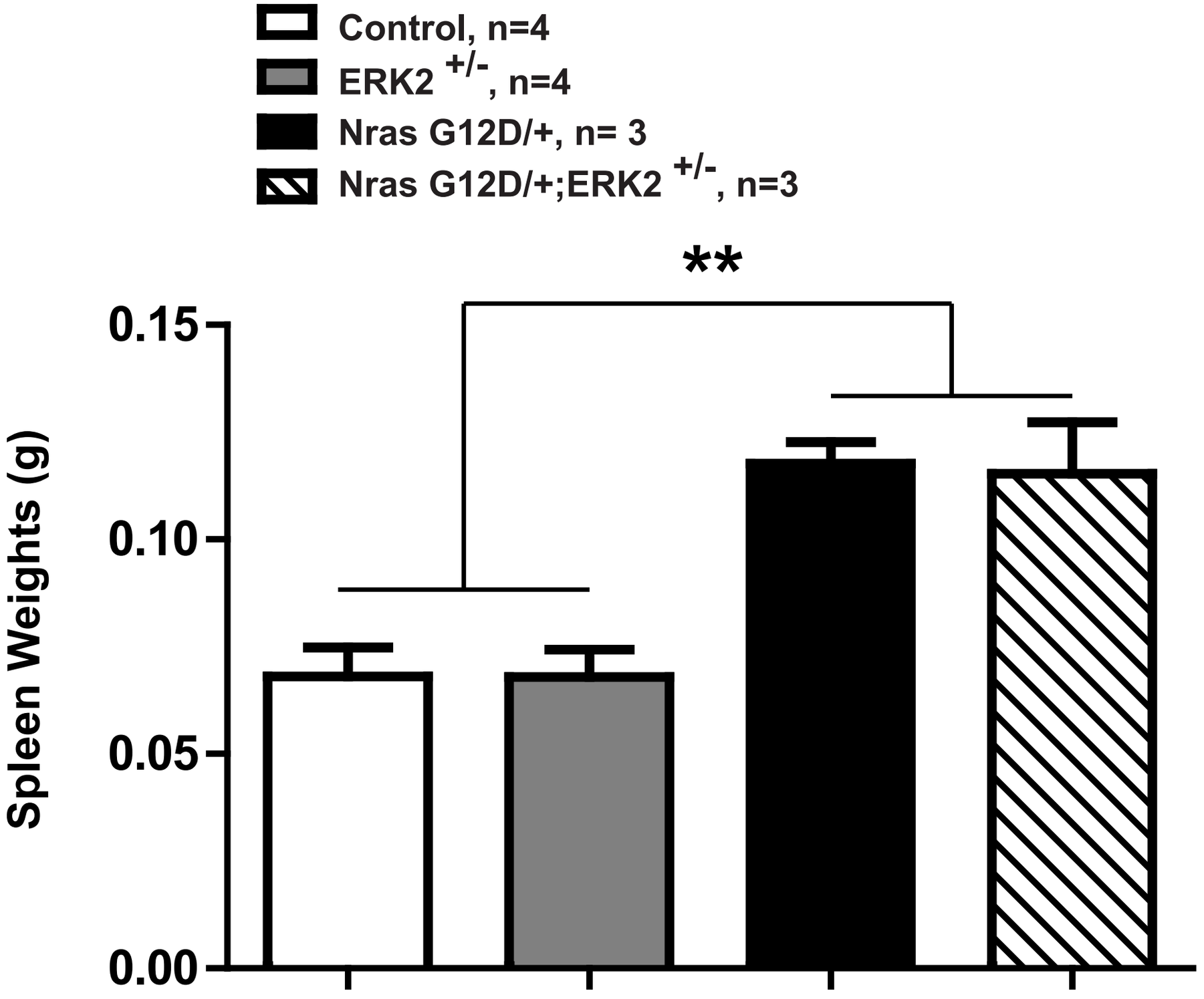

Control, n=2
ERK2 +/-, n=2
Nras G12D/+, n= 2
Nras G12D/+;ERK2 +/-, n=2
Fold over unstimulated control
2.5
2.0
1.5
1.0
0.5
0.0

Fig S9-Zhang


Control, n=4
ERK2 +/-, n=4
Nras G12D/+, n= 3
Nras G12D/+;ERK2 +/-, n=3
**
Spleen Weights (g)
0.15
0.10
0.05
0.00