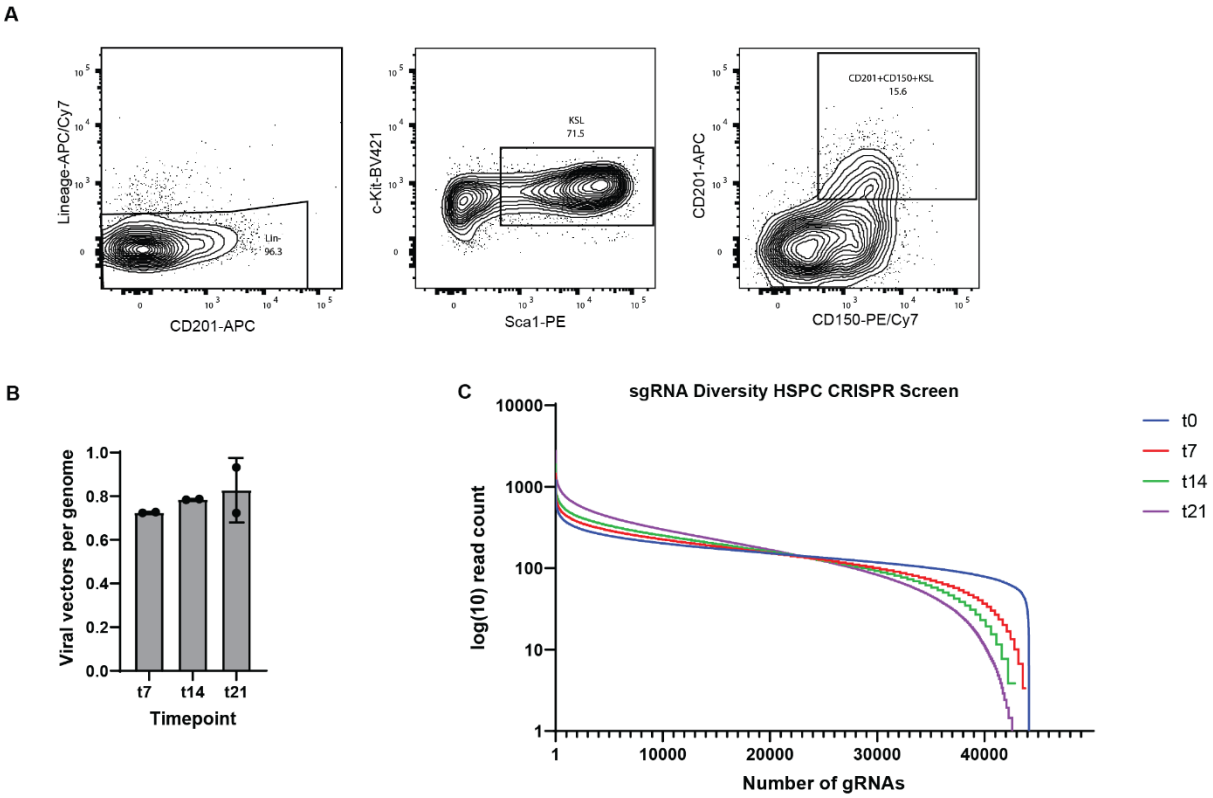
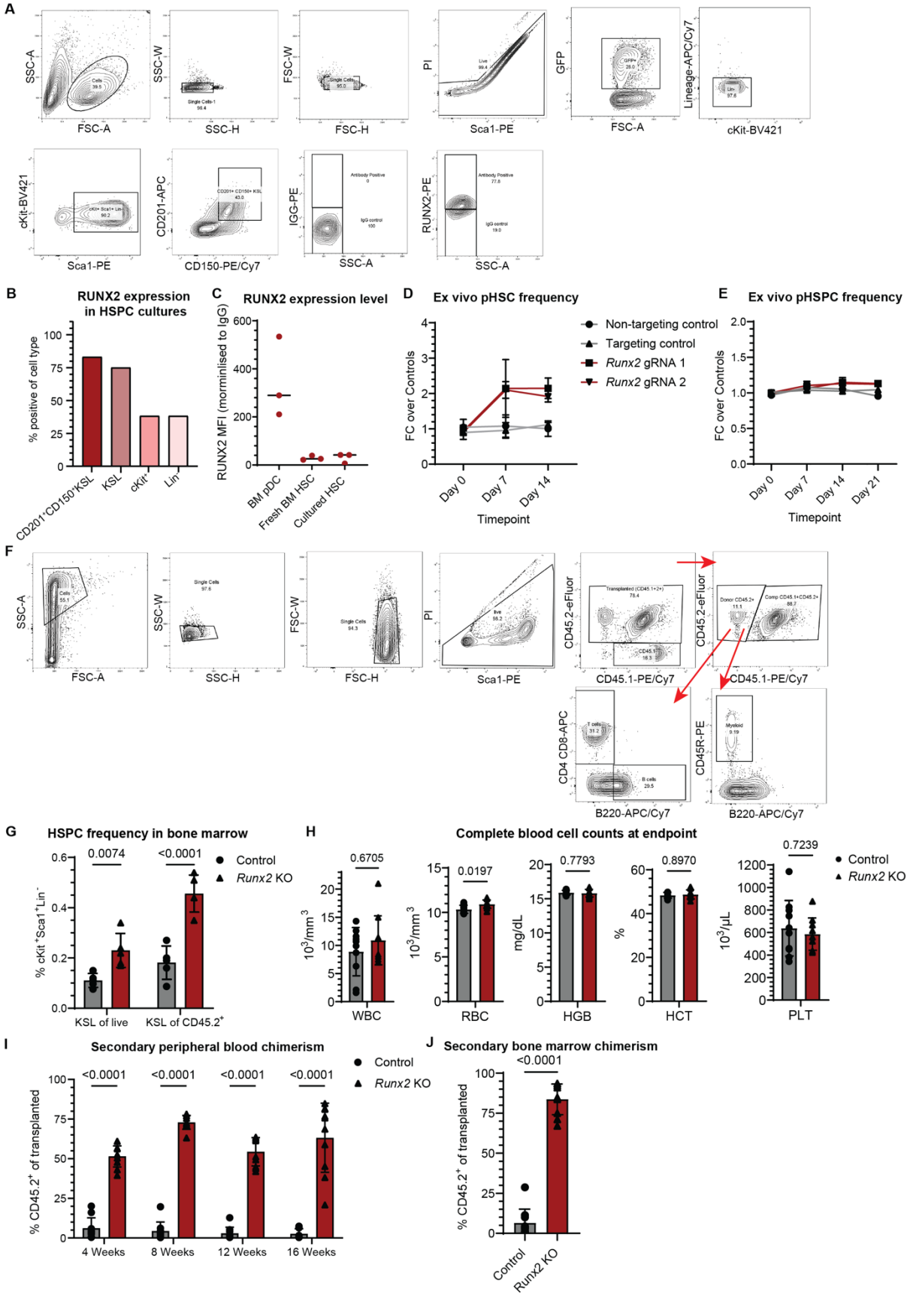
Supplementary Figures and Tables

Figure S1: Related to Figure 1



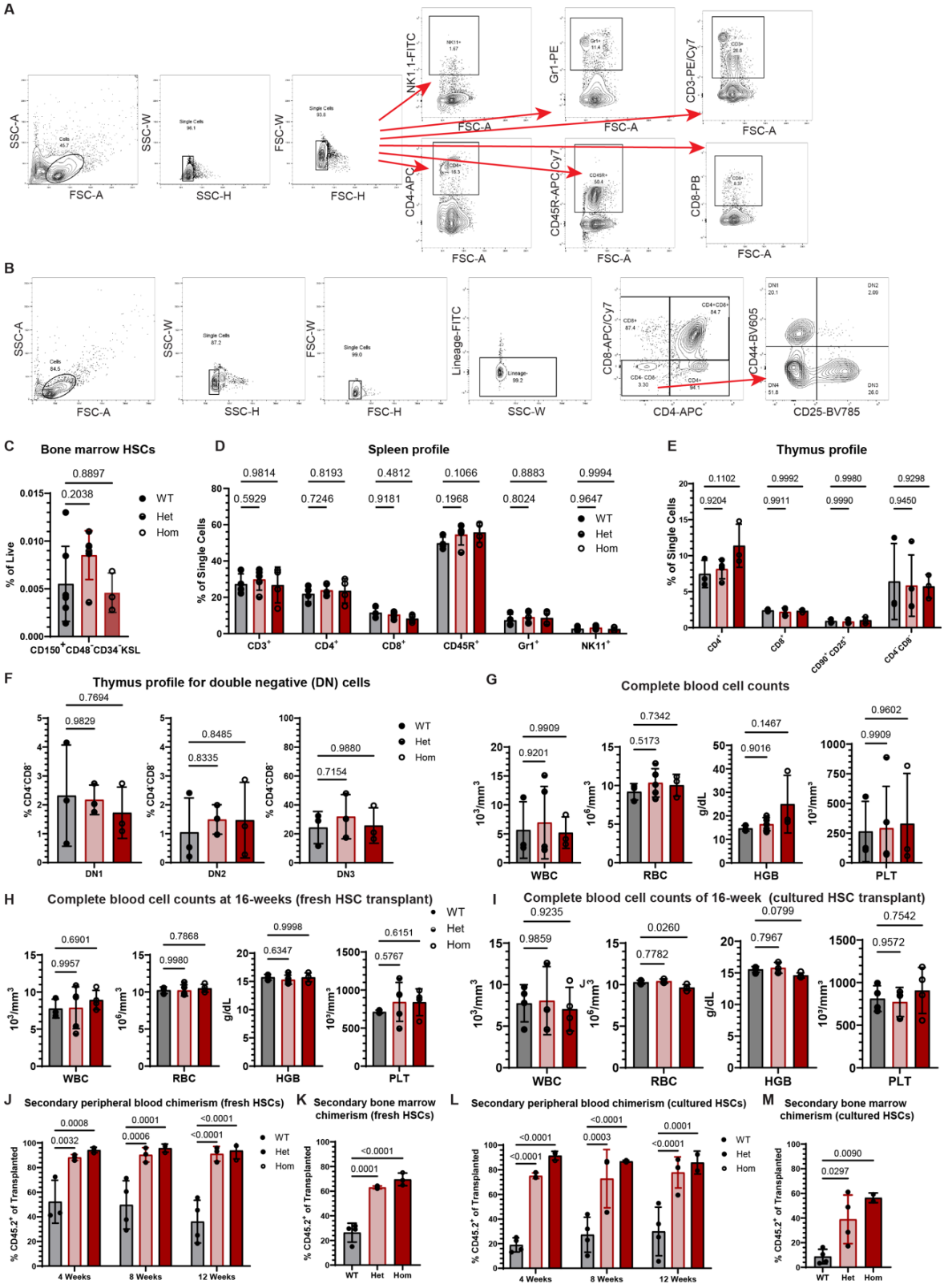
(A) *Ex vivo* HSPC culture immunophenotype pre-transduction of the CRISPR screen lentivirus library.
(B) Viral vector copies per genome at indicated screen timepoints, determined by droplet digital PCR.
(C) sgRNA diversity over time from *ex vivo* HSPC CRISPR screen.

Figure S2: Related to Figure 2



(A) Representative flow cytometric gating for pHSCs, including GFP gate for CRISPR-based KOs. The final two gates demonstrate intracellular flow gating using IgG control antibody.
(B) RUNX2 protein expression in HSPC cultures quantified by intracellular flow cytometry.
(C) Mean fluorescence intensity of RUNX2 expression, normalised to IgG control for fres bone marrow (BM) plasmacytoid dendritic cells (pDCs), fresh BM $CD150^{+}CD48^{-}$KSL HSCs and ex vivo cultured $CD201^{+}CD150^{+}$KSL pHSCs.
(D) *Ex vivo* pHSC frequency over days 0-14. See Figure 2B for experimental details.
(E) *Ex vivo* c-$Kit^{+}Sca1^{+}Lineage^{-}$ HSPC frequency over days 0-21. See Figure 2B for experimental details.
(F) Representative flow cytometric gating for peripheral blood analysis. Red arrows indicate order of gating.
(G) Donor BM frequency of c-$Kit^{+}Sca1^{+}Lineage^{-}$ in CRISPR transplants (see Figure 2F for experimental details). n = 5. 2-way ANOVA was performed.
(H) Complete blood cell counts at 20-weeks post-transplantation in CRISPR transplantation assay recipients (see Figure 2F for experimental details). WBC = white blood cells, RBC = red blood cells, HGB = haemoglobin, HCT = haematocrit, PLT = platelet counts. n = 10. Unpaired T test was performed.
(I) Donor peripheral blood chimerism for secondary transplant, with 5 million whole bone marrow cells from primary recipients injected into irradiated secondary recipients. 2-way ANOVA was performed.
(J) Bone marrow chimerism for secondary transplant at 16-week endpoint. 2-way ANOVA was performed.

Figure S3: Related to Figure 3

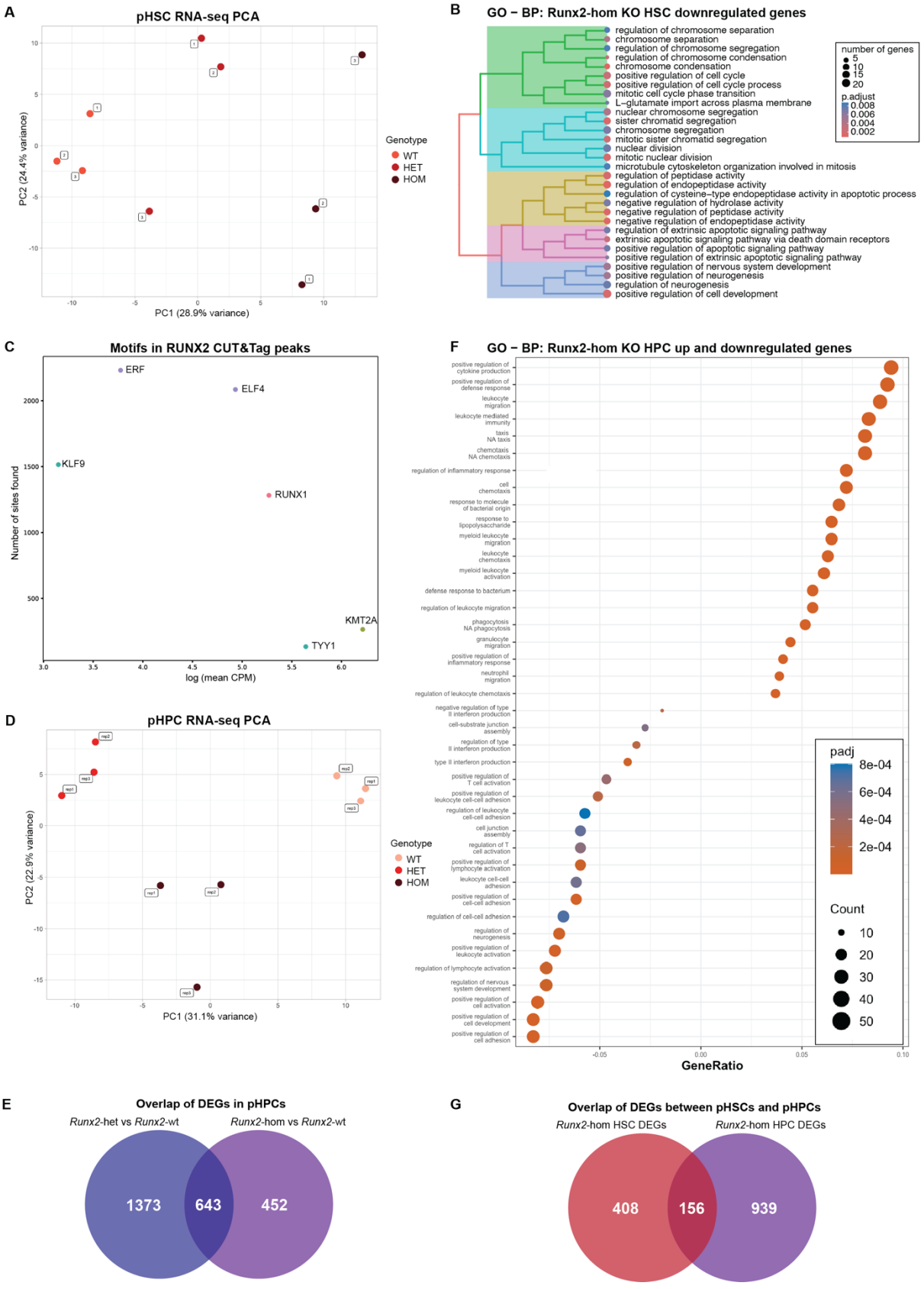


(A) Representative flow cytometric gating of spleen cells. Red arrows indicate order of gating.

(B) Representative flow cytometric gating of thymic cells. Red arrows indicate order of gating.

(C) Bone marrow cell frequencies in *Runx2*$^{flox/flox}$ mice. n = 3-5. 1-way ANOVA was performed.
(D) Spleen cell frequencies in *Runx2*$^{flox/flox}$ mice. n = 3-5. 2-way ANOVA was performed.
(E) Thymus cell frequencies in *Runx2*$^{flox/flox}$ mice. n = 3-5. 2-way ANOVA was performed.
(F) Double Negative (DN) cell proportions in the thymus of *Runx2*$^{flox/flox}$ mice. n = 3-5. 2-way ANOVA was performed.
(G) Complete blood cell counts for *Runx2*$^{flox/flox}$ mice. n = 3-5. WBC = white blood cells, RBC = red blood cells, HGB = haemoglobin, PLT = platelet counts. n = 10. Unpaired T test was used.
(H) Complete blood cell counts at 16-week post-transplantation in fresh HSC transplantation assays (described in Figure 3B-C). n = 3-5. WBC = white blood cells, RBC = red blood cells, HGB = haemoglobin, PLT = platelet counts.
(I) Complete blood cell counts of 16-week post-transplantation in expanded HSC transplantation assays (described in Figure 3F-G). n = 3-4. WBC = white blood cells, RBC = red blood cells, HGB = haemoglobin, PLT = platelet counts. n = 10.
(J) Donor peripheral blood chimerism for fresh HSC secondary transplant, with 5 million whole bone marrow cells from primary recipients injected into irradiated secondary recipients. 2-way ANOVA was performed.
(K) Bone marrow chimerism for fresh HSC secondary transplant at 12-week endpoint. 2-way ANOVA was performed.
(L) Donor peripheral blood chimerism for cultured HSC secondary transplant, with 5 million whole bone marrow cells from primary recipients injected into irradiated secondary recipients. 2-way ANOVA was performed.
(M) Bone marrow chimerism for cultured HSC secondary transplant at 12-week endpoint. 2-way ANOVA was performed.

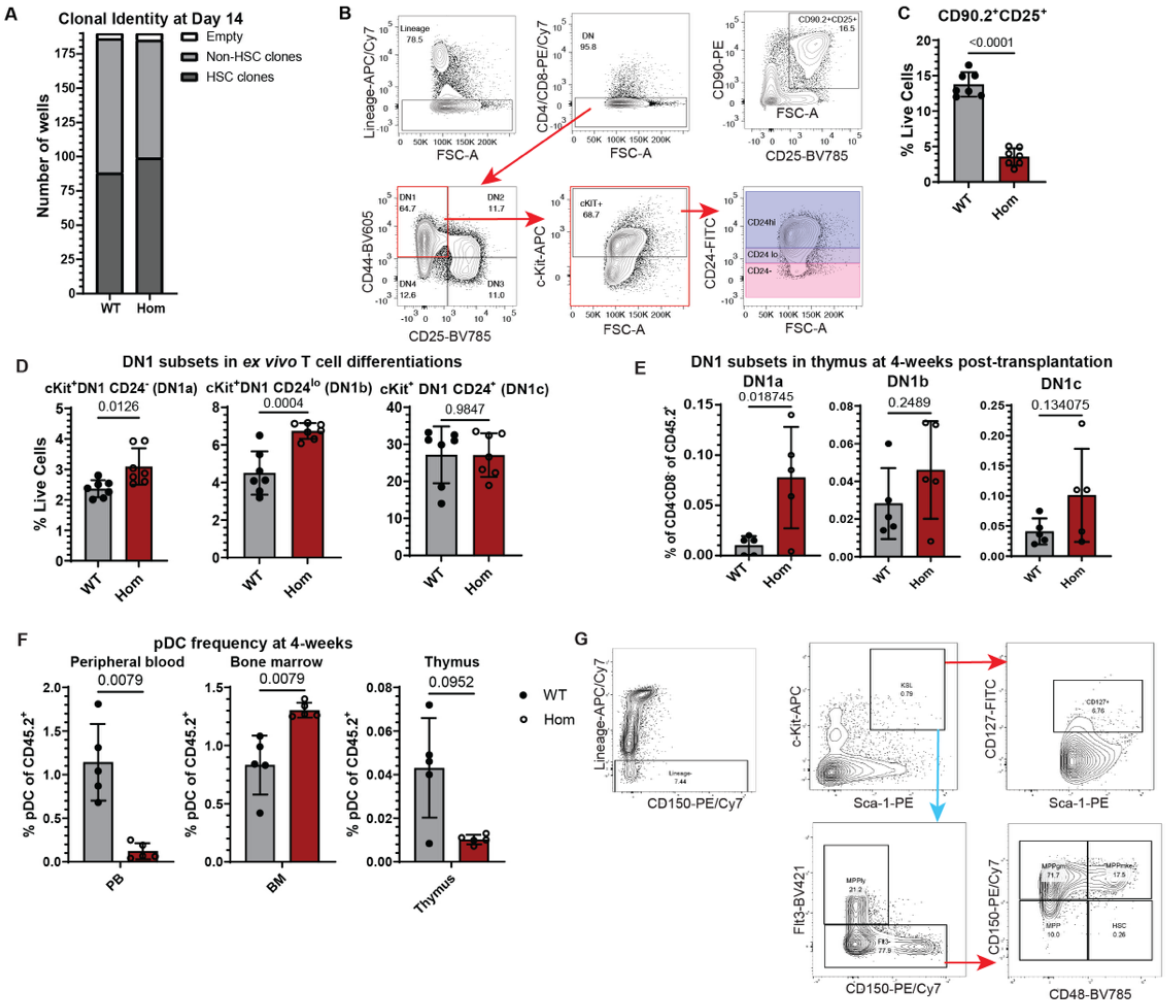
Figure S4: Related to Figure 4



(A) Principal component analysis of CD201^{+}CD150^{+}KSL pHSC RNA-seq samples from *Runx2*-hom, *Runx2*-het and *Runx2*-wt HSPC cultures.

(B) Top 30 downregulated Gene Ontology Biological Process for *Runx2*-hom KO vs *Runx2*-wt pHSC RNA-seq.
(C) Motif analysis of RUNX2-bound promoter peaks after filtering by genes expressed in *Runx2*-wt HSC RNA-seq.
(D) Principal component analysis of $CD201^{-}CD150^{-}$KSL pHPC RNA-seq samples from *Runx2*-hom, *Runx2*-het and *Runx2*-wt HSPC cultures.
(E) Gene overlap for *Runx2*-hom vs *Runx2*-wt DEGs and *Runx2*-het vs Runx2-wt DEGs in HPC RNA-seq.
(F) Top 20 up and downregulated Gene Ontology Biological Process for *Runx2*-hom KO vs *Runx2*-wt pHPC RNA-seq.
(G) Gene overlap for *Runx2*-hom vs *Runx2*-WT HSC DEGs vs *Runx2*-hom vs *Runx2*-wt HPC HPC DEGs.

Figure S5: Related to Figure 5



(A) Clonal identities at 14 days after single HSC plating and number of wells with this identity. Empty = no live cells, HSC clones = clones containing CD201$^+$KSL cells, non-HSC clones = clones not containing CD201$^+$KSL cells. See Figure 5A for details.

(B) Representative flow cytometric gating for *ex vivo* T cell gating. Red arrows indicate order of gating.

(C) Frequency of CD90.2$^+$CD25$^+$CD4$^-$CD8$^-$Lin$^-$ T cell precursor cells generated in *ex vivo* T-cell differentiation assays. n = 7, unpaired T-test was performed. See Figure 5E for details.

(D) Frequency of CD24$^-$c-Kit$^+$ CD44$^+$CD25$^-$CD4$^-$CD8$^-$Lin$^-$ (DN1a), CD24^{lo}c-Kit$^+$CD44$^+$CD25$^-$CD4$^-$CD8$^-$Lin$^-$ (DN1b), and CD24^{hi}c-Kit$^+$CD44$^+$CD25$^-$CD4$^-$CD8$^-$Lin$^-$ (DN1c) cell subsets generated in *ex vivo* T-cell differentiation assays. n = 7, unpaired T-test was performed. See Figure 5E for details.

(E) Frequency of CD24$^-$c-Kit$^+$ CD44$^+$CD25$^-$CD4$^-$CD8$^-$Lin$^-$ (DN1a), CD24^{lo}c-Kit$^+$CD44$^+$CD25$^-$CD4$^-$CD8$^-$Lin$^-$ (DN1b), and CD24^{hi}c-Kit$^+$CD44$^+$CD25$^-$CD4$^-$CD8$^-$Lin$^-$ (DN1c) cell subsets in thymic donor cells at 4-weeks post-transplantation. n = 5, unpaired T-test was performed. See Figure 5H for details.

(F) Frequency of BST2$^+$B220$^+$CD11b$^-$ pDCs in the donor cells from the peripheral blood, bone marrow, and thymus at 4-weeks post-transplantation. n = 5, unpaired T-test was performed. See Figure 5H for details.

(G) Representative flow cytometric gating for lymphoid progenitor panel in bone marrow of transplant endpoint. Red arrows indicate order of gating.

Table S1: Reagents referenced in the methods, item code and supplier

Reagent	Code	Supplier
APC-cKit antibody	17-1171-83	Thermo Fisher
anti-APC magnetic beads	130-090-855	Miltenyi Biotec
CELLBIND plates	3337	Corning
F12 base media	15172529	Gibco
Penicillin/Streptomycin/Glutamine	10378-016	Gibco
N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)	15630-080	Gibco
PVA	P8136	Sigma
ITS-X	51500056	Gibco
TPO	130-108-958	Miltenyi Biotec
SCF	AF-250-03	Peprotech
DMEM	15630080	Thermo Fisher
Sodium pyruvate	15630080	Thermo Fisher
GlutaMax	35050061	Thermo Fisher
Sodium propionate	A8960	Alfa Aesar
Sodium butyrate	A11079	Alfa Aesar
Caffeine	A10431	Alfa Aesar
FBS	12303C	Sigma-Aldrich
Fibronectin-coated plates	354409	BD Biosciences
Genome-wide lentiviral sgRNA KO library	136987	Addgene
Puromycin	A1113803	Thermo Fisher
pMCB306 lentiviral plasmid	89360	Addgene
ddPCR™ Supermix for Probes	186-3010	BioRad
Doxycycline	J60422.06	Thermo Fisher
BD Pharmigen Transcription Factor Buffer Set	562574	BD Biosciences
RUNX2-PE Conjugate Ab	98059	Cell Signaling
IGG-PE control Ab	02-6102	Cell Signaling
Direct-zol RNA Microprep kit	R2063	Zymo
Biotinylated Recombinant Human DLL4	10171-H49H-B	Sino Biological
Streptavidin Polystyrene Particles	SVP-200-4	Spherotech
IMDM with GlutaMAX Supplement	31980030	Thermo Fisher
BIT 15 9500 Serum Substitute	09500	Stem Cell Technologies
Penicillin/Streptomycin	15140122	Thermo Fisher
Recombinant Murine FLT3 Ligand	250-31L-50UG	Peprotech
Recombinant Murine IL-7	217-17-50UG	Peprotech
Mouse CXCL12 (SDF-1α) Recombinant Protein	PMC1354	Peprotech
c-Kit BV421	105828	Biolegend
Sca1 PE	108108	Biolegend
CD150 PE/Cy7	115914	Biolegend
CD4 APC/Cy7	100526	Biolegend
CD8 APC/Cy7	100714	Biolegend
Ter119 APC/Cy7	116223	Biolegend
CD127 APC/Cy7	135040	Biolegend
CD45R APC/Cy7	103224	Biolegend
Gr1/Ly6G APC/Cy7	108424	Biolegend

CD201 APC	17-2012-82	Invitrogen
CD11b PE	101208	Biolegend
Gr1/Ly6G PE	108408	Biolegend
CD4 APC	100516	Biolegend
CD8 APC	100712	Biolegend
CD45.1 PE/Cy7	110730	Biolegend
CD45.2 eFluor450	48-0454-82	Invitrogen
Gr1/Ly6C – biotin	108404	Biolegend
Ter119 – biotin	116204	Biolegend
CD4 – biotin	100508	Biolegend
CD8a – biotin	100704	Biolegend
CD45R – biotin	103204	Biolegend
CD127 – biotin	135006	Biolegend
CD34 FITC	11-0341-85	Invitrogen
Streptavidin APC/Cy7	405208	Biolegend
CD48 BV421	103428	Biolegend
CD11b FITC	101205	Biolegend
Gr1/Ly6G FITC	108406	Biolegend
NK1.1 FITC	156508	Biolegend
CD19 FITC	152403	Biolegend
CD44 BV605	103047	Biolegend
CD25 BV785	102051	Biolegend
CD90 PE	140307	Biolegend
CD3 PE/Cy7	300310	Biolegend
CD8a PE/Cy7	25-0081-82	Biolegend
Viability Dye eFluor	65-0863-14	ThermoFisher
CD45.2 BUV395	564616	BDBiosciences
CD45.1 AF700	110723	Biolegend
BST2 FITC	127007	Biolegend
CD127 FITC	135007	Biolegend
c-Kit APC	105811	Biolegend
Sca-1 FITC	108105	Biolegend
CD48 BV785	103449	Biolegend
Flt3 BV421	135313	Biolegend
CD127 FITC	135007	Biolegend
CD24 FITC	101805	Biolegend
H3K27me3	07-449	Millipore
H3K4me3	39159	Active Motif
Anti-RUNX2	ab236639	Abcam

Table S2: Primers and probes used in this paper (purchased from IDT)

Name	**Sequence**	**Purpose**
Genome-fwd	GGATGGGGAATGCAGCTCTT	Genome reference ddPCR
Genome-rev	AGTGCGGCAGAATACAGCA	Genome reference ddPCR
Genome-probe (Hex)	TGATGGGTTGTGAAGGCAGCTGCACCT	Genome reference ddPCR
PuroR-fwd	AGCAACAGATGGAAGGCCTC	Lentivirus ddPCR
PuroR-rev	GACGGTGGCCAGGAACCA	Lentivirus ddPCR
PuroR-probe (Fam)	CACCGGCCCAAGGAGCCCG	Lentivirus ddPCR
vav1-cre_fwd	AGATGCCAGGACATCAGGAACCTG	Vav1 Cre genotyping

vav1-cre_rev	ATCAGCCACACCAGACACAGAGATC	Vav1 Cre genotyping
runx2_geno_fwd	ACTTCGGTTGGTCAGAATAAACAG	*Runx2*^{flox} mice genotyping
runx2_geno_rev	CAAGCTAACGGGACTTGGAAGAG	*Runx2*^{flox} mice

Table S3: Reagents for 1 ml of HSC *ex vivo* culture media

Reagent	**Final concentration in F-12**	**Volume (µL)**
Ham's F-12 media	N/A	958
100X Penicillin-Streptomycin-Glutamine	1X	10
1 M HEPES	10 mM	10
100 mg/mL PVA stock	1 mg/mL	10
100X ITSX	1X	10
100 µg/mL TPO stock	100 ng/mL	1
10 µg/mL SCF stock	10 ng/mL	1

Table S4: sgRNA sequences used in this paper

Guide Name	**Target Gene/Region**	**Sequence**
Runx2 gRNA 1	Runx2 (exon 3)	GTCGGTGCGGACCAGTT
Runx2 gRNA 2	Runx2 (exon 3)	GTGCGGCGGCCTCAATCG
Rosa26	Safe harbour locus	GTGCCATGGTCCCCTGCCTA
Non-targeting control	N/A	GCACTACCAGAGCTAACTCA

Table S5: Cultured HSC antibody cocktail (50 samples)

Antibody	**Volume (µL)**
c-Kit BV421	12.5
Sca1 PE	12.5
CD150 PE/Cy7	12.5
CD4 APC/Cy7	5
CD8 APC/Cy7	5
Ter119 APC/Cy7	5
CD127 APC/Cy7	5
CD45R APC/Cy7	5
Ly6C/Ly6G APC/Cy7	5
CD201 APC	5
PBS	27.5

Table S6: Cultured HSC antibody cocktail for intracellular flow (50 samples)

Antibody	**Volume (µL)**
c-Kit BV421	12.5
Sca1 FITC	12.5
CD150 PE/Cy7	12.5
CD4 APC/Cy7	5
CD8 APC/Cy7	5
Ter119 APC/Cy7	5

CD127 APC/Cy7	5
CD45R APC/Cy7	5
Ly6C/Ly6G APC/Cy7	5
CD201 APC	5
PBS	27.5

Table S7: Whole bone marrow HSC antibody cocktail for intracellular flow (3 samples)

Antibody	Volume (µL)
c-Kit APC	3
CD4 APC/Cy7	3
CD8a APC/Cy7	3
Gr-1 APC/Cy7	3
CD11b APC/Cy7	3
B220 APC/Cy7	3
CD127 APC/Cy7	3
TER119 APC/Cy7	3
CD150 PE/Cy7	3
CD48 BV421	3
Sca-1 FITC	5
PBS	265

Table S8: Plasmacytoid dendritic cell panel for intracellular flow (3 samples)

Antibody	Volume (µL)
B220 APC/Cy7	2.5
CD11b APC	2.5
BST2 FITC	1.25
c-Kit BV421	6.25
PBS	287.5

Table S9: Peripheral blood analysis cocktail (40 samples)

Stain	Volume (µL)
CD11b PE	2
Gr1/Ly6G PE	2
CD4 APC	2
CD8 APC	2
CD45R/B220 APC/Cy7	4
CD45.1 PE/Cy7	10
CD45.2 eBioscience	12
PBS	3972

Table S10: Biotin antibody cocktail (100 samples)

Antibody	Volume (µL)
Ly6G/Ly6C – biotin	100

Ter119 – biotin	100
CD4 – biotin	25
CD8a – biotin	25
CD45R – biotin	50
CD127 – biotin	50
Sterile PBS	350

Table S11: Fresh HSC antibody cocktail (3 samples)

Antibody	**Volume (µL)**
CD34 FITC	4
c-Kit APC	1
Sca1 PE	1
Streptavidin APC/Cy7	1
CD150 PE/Cy7	1
CD48 BV421	1
Sterile PBS	291

Table S12: **Thymus antibody cocktail (8 samples)**

Antibody	**Volume (µL)**
CD11b FITC	1
Gr1/ly6G FITC	1
NK1.1 FITC	2
CD19 FITC	2
CD4 APC	2
CD8 APC/Cy7	2
CD44 BV605	4
CD25 BV785	1
CD90 PE	4
PBS	781

Table S13: Spleen antibody cocktail (4 samples)

Antibody	**Volume (µL)**
CD11b PE	1
Gr1/Ly6G PE	1
CD4 APC	1
CD8 PB	2
CD45R/B220 APC/Cy7	2
NK1.1 FITC	2
CD3 PE/Cy7	2
PBS	389

Table S14: T cell differentiation antibody cocktail (8 samples)

Antibody	**Volume (µL)**
CD44 BV605	8

CD25 BV785	2
CD90.2 PE	8
CD8a PE/Cy7	4
CD4 APC	4
Gr-1/Ly6C APC/Cy7	4
CD45R/B220 APC/Cy7	2
TER119 APC/Cy7	2
c-Kit BV421	2
CD24 FITC	2
PBS	766

Table S15: Thymus antibody cocktail for transplant endpoint (15 samples)

Antibody	**Volume (µL)**
B220 APC/Cy7	4
Gr-1/Ly6G APC/Cy7	4
TER119 APC/Cy7	4
CD24 FITC	4
c-Kit APC	4
CD8a PE/Cy7	4
CD44 BV605	8
CD25 BV785	4
CD90 PE	8
CD4 BV421	4
CD45.1 AF700	10
PBS	1558

Table S16: Bone marrow HSPC antibody cocktail for transplant endpoint (15 samples)

Antibody	**Volume (µL)**
CD45.2 BUV395	45
CD45.1 AF700	45
Streptavidin APC/Cy7	15
CD127 FITC	45
c-Kit APC	15
Sca-1 PE	15
CD150 PE/Cy7	15
CD48 BV785	30
Flt3 BV421	45
PBS	1230

Table S17: Plasmacytoid dendritic cell (pDC) antibody cocktail for transplant endpoint (60 samples)

Antibody	**Volume (µL)**
B220 APC/Cy7	33
CD11b PE	33
BST2 FITC	16

CD45.2 eFluor450	79
CD45.1 PE/Cy7	66
PBS	3773

Table S18: Motif analysis output from RUNX2-bound peaks in pHSC CUT&Tag data. See methods for data analysis pipeline. See Excel file.

Table S19: Differentially expressed genes from *Runx2*-hom vs *Runx2*-wt pHSC RNA-seq. See methods for data analysis pipeline. See Excel file.

Table S20: Differentially expressed genes from *Runx2*-het vs *Runx2*-wt pHSC RNA-seq. See methods for data analysis pipeline. See Excel file.

Table S21: Differentially expressed genes from *Runx2*-hom vs *Runx2*-wt pHPC RNA-seq. See methods for data analysis pipeline. See Excel file.

Table S22: Differentially expressed genes from *Runx2*-het vs *Runx2*-wt pHPC RNA-seq. See methods for data analysis pipeline. See Excel file.

Table S23: Lymphoid media for T cell differentiation

Reagent	**Final concentration in media**
IMDM with GlutaMAX Supplement	N/A
BIT 15 9500 Serum Substitute	20%
Penicillin/Streptomycin	1%
mFLT3-L	100 ng/mL
mSCF	100 ng/mL
mIL-7	100 ng/mL
Recombinant Human CXCL12/SDF-1α	25 ng/mL

Table S24: Genes identified in genome-wide CRISPR screen. See Excel file.