Supplemental Methods

Mice

Homozygous JAK2V617F (JAK HOM) knock-in mice[1] were crossed with *Tet2* KO (TET HOM) mice from Ko et al., 2011[2]. HSCs from single (JAK HOM or TET HOM) and double mutant (JAK HOM TET HOM) animals between 2 and 4 months of age were compared to wild-type littermate (for JAK HOM) or age-matched (for TET HOM and double mutant) controls. c-Kit mutant C57Bl6$^{W41/W41\text{-}Ly5.1}$ (W41) or C57Bl6 mice were used as recipients for transplantation experiments. All mice were bred and maintained at the University of Cambridge in microisolator cages and provided continuously with sterile food, water, and bedding. All mice were kept in specified pathogen-free conditions, and all procedures performed according to the United Kingdom Home Office regulations.

Isolation of E-SLAM HSCs

Suspensions of BM cells were isolated from the femora, tibiae, and ilia of mice. Red blood cell lysis was performed by treatment with ammonium chloride (STEMCELL Technologies, Vancouver, Canada (STEMCELL)). E-SLAM cells were isolated as described previously[3] using CD45 FITC (Clone 30-F1,1 BD Biosciences, San Jose, CA, USA (BD)), EPCR PE (Clone RMEPCR1560, STEMCELL), CD150 Pacific Blue (PB) or PE-Cy7 (Clone TC15-12F12.2, both from Biolegend, San Diego, USA (Biolegend)), CD48 APC (Clone HM48-1, Biolegend), and 7-Aminoactinomycin D (7AAD) (Life Technologies, Carlsbad, CA, USA (Life Technologies)). The cells were sorted on an Influx (BD) using the following filter sets 530/40 (for FITC), 585/29 (for PE), 670/30 (for APC), 460/50 (for Pacific Blue) and 670/30 (for 7AAD). When single E-SLAM HSCs were required, the single-cell deposition unit of the sorter was used to place 1 cell into the wells of round bottom 96-well plates, each well having been preloaded with 50uL medium (described below).

In vitro cultures and clone size calculations

E-SLAM HSCs were sorted and cultured in StemSpan media (STEMCELL) containing fetal calf serum (FCS, STEMCELL or Sigma-Aldrich, St. Louis, MO, USA), 300ng/mL SCF (STEMCELL or Bio-techne, Abingdon, UK (Bio-techne)) and 20ng/mL IL-11 (STEMCELL or Bio-techne). Daily cell counts were performed and cell cycle kinetics determined for the first and second division by scoring wells manually as having 1, 2, or 3-4 cells. At 10 days, clones were estimated to be very small (less than 50 cells), small (50–500 cells), medium (500–10,000 cells), or large (10,000 or more cells), clone size estimates were validated as described previously using fluorescent

counting beads[4]. Ten-day clones were stained with Sca-1 PB (Clone E13-161.7, Biolegend), Mac1 BV605 (Clone M1/70, Biolegend), Gr1 PE-Cy7 (Clone RB6-8C5, Biolegend), c-Kit APC-Cy7 (Clone 2B8, Biolegend), CD45.2 FITC (Clone 104, Biolegend), and 7AAD (Life Technologies). To enumerate cells, a defined number of fluorescent beads (Trucount Control Beads, BD) were added to each well and each sample was back-calculated to the proportion of the total that were run through the cytometer. Small clones were not assessed individually by flow cytometry due to low numbers of cells. Flow cytometry was performed on an LSRFortessa (BD) and all data were analyzed using FlowJo (Treestar, Ashland, OR, USA).

Cobblestone-area-forming cell assay

The CAFC assay was modified from de Haan et al.[5] and performed as described previously[6]. The FBMD stromal cell line (obtained from G. de Haan, originally from R. Ploemacher, Erasmus University, Rotterdam, Netherlands) was maintained in Quantum 333 complete fibroblast medium with L-glutamine (PAA Laboratories) plus β-mercaptoethanol, penicillin, and streptomycin. Stromal cell layers were established in the inner 60 wells of 96-well plates to avoid potential evaporation related effects and were incubated at 33°C. Prior to sorting, medium was replaced with 200 µL Iscove's modified Dulbecco's medium, 20% horse serum (STEMCELL), 10^{-5} mol/L hydrocortisone (STEMCELL), 10^{-4} mol/L β-mercaptoethanol plus penicillin and streptomycin (collectively, CAFC medium). Each well was seeded with a single HSC, cultured at 37°C, and wells were examined each week for 12 weeks for the presence or absence of cobblestone areas (identified as colonies of at least five flat non-refractile cells growing underneath the stromal layer). Medium was changed each week by removing most of the old medium using a multichannel pipette (without disturbing the stromal layer) and adding 180µL of fresh CAFC medium.

Gene-expression profiling

Single-cell gene expression analysis was performed as described previously[7]. Single-cell expression data were collected using the Fluidigm Data Collection software. ΔCt values were calculated as previously described[7] by cell-wise normalization to the mean expression level of two housekeeping genes (Ubc and Polr2a). All housekeepers, *Cdkn2a*, *Eif2b1*, *Mitf*, *Cbx7*, *Meis1*, *Tet2*, and *Egfl7* were removed from the dataset for downstream analysis. *Cdkn2a* was not expressed in any of the cell types, *Egfl7, Mitf, Cbx7,* and *Eif2b1* did not pass quality control in any genotype, *Meis1* was excluded in the TET2 single and double-mutant genotypes

due to aberrant signal in no template controls, and *Tet2* was excluded since the TET2 knock-out cells had no expression and clustering would have been biased. Hierarchical clustering was performed in R (http://www.r-project.org) using the hclust package and heatmap.2 from the gplots package using Spearman rank correlations and ward linkage. t-SNE was performed in R (http://www.r-project.org) using the Rtsne package. PCA was performed using the prcomp function.

Bone Marrow Transplantation Assays

Donor cells ($5x10^5$ for whole BM competitive transplantations and doses as indicated in legend for limiting dilution transplantations) were obtained from C57BL/6J mice (CD45.2). Frequencies of phenotypic HSCs (per 1000 lin- BM cells) were as follows: WT (3-12), JAK HOM (1-3), TET HOM (3-10), and double-mutant (0.5-5). All competitor cells were obtained from WT C57BL/6J (CD45.1/CD45.2) mice and $2\text{-}5x10^5$ whole BM cells were transplanted alongside the donor cell fractions. For competitive transplantation assays, recipients were adult $C57Bl6^{W41/W41}$ (W41) mice as described previously[8,9]. Mice were irradiated with a single dose (400 cGy) by Caesium irradiation and all transplants were performed by intravenous tail vein injection using a 29.5G insulin syringe. On average, host cell recovery accounted for 30% of the total cells (17-60%) but since a known number of genotypically distinct competitor cells were transplanted, host numbers were not required for calculations of relative chimerism between donor and competitor cells (donor/(donor+competitor)). For secondary transplants, whole BM was obtained from primary recipients and $1x10^7$ cells containing a mixture of recipient, competitor, and donor-derived cells were transplanted.

Peripheral Blood Analysis

For all transplantation assays, peripheral blood samples were collected from the tail vein of mice at 4, 8, 16, 20, and 24 weeks following transplantation and analyzed for repopulation levels as described previously[7,8]. Antibodies used were Ly6g PB or BV421 (Clone 1A8, Biolegend), Mac1 FITC or BV605 (Clone M1/70, both Biolegend), CD3e PE (Clone 17A2, Biolegend), B220 APC (Clone RS3-6B2, Biolegend), CD45.1 AlexaFluor700 (Clone A20, Biolegend), CD45.2 APC-Cy7 or FITC (Clone 104, both Biolegend), and 7-AAD (Life Technologies). Peripheral blood cell counts were performed using a Woodley ABC blood counter (Woodley Equipment, Bolton, UK).

Overexpression Assays

Small pools (1500-3600 cells) of $CD45^+Lin^-CD150^+CD48^-$ hematopoietic stem and progenitor cells (HSPCs) were isolated and split between wells of a 96 well plate coated with RetroNectin (Clontech, Mountain View, CA, USA). Following their isolation, cells were kept in 50ul of medium as described above and were supplemented with polybrene (Sigma-Aldrich) and a lentivirus equipped with the gene of interest in a pCCL-c-MNDUS-X2-PGK-EGFP backbone. Plates were centrifuged at 600xg for 30 minutes, 30°C, to promote infection, before being transferred to a 37°C incubator. The following day, a further 150µL of HSC medium was added to dilute polybrene and the virus and three days after infection, cells were resorted for GFP expression and viability (e.g., 7AAD-negative). GFP^+ cells (300-2000) were transplanted into sublethally irradiated CD45.1 W41 or lethally irradiated CD45.1 C57Bl6/J recipient mice and monitored for donor chimerism and disease phenotype as described above.

Isolation of patient stem and progenitor cells

Fresh venous blood samples (40-60ml collected in Lithium-Heparin tubes) were collected from MPN patients (n=12, 3 ET, 5 PV, 4 secondary MF) with JAK2 and TET2 mutations diagnosed according to British Committee for Standards in Haematology (BCSH) guidelines. The study was approved by the Cambridge and Eastern Region Ethics Committee, and was carried out in accordance with the principles of the Declaration of Helsinki. Informed written consent was obtained from all patients before participating. Mononuclear cells (MNCs) were isolated using Lymphoprep (Axis Shield PLC, Dundee, UK) according to the manufacturer's instructions. MNCs were washed with and stored at 4°C overnight in PBS supplemented with 10% FCS and 5mM EDTA. In the morning, cells were spun at 300xg for 7 minutes and the resulting pellets were treated with 50µL of 1 mg/mL DNaseI (STEMCELL) to avoid clumping. Cells were then depleted of differentiated hematopoietic cells using the EasySep human hematopoietic progenitor enrichment kit (#19056 STEMCELL Technologies) with the following modifications: All samples were processed in 500µL of recommended medium and the progenitor enrichment cocktail was used at 5µL/mL cells, and the sample was only taken through one round of separation[10,11]. Cells were then stained with antibodies to isolate the various progenitor compartments: anti-CD34 Per-CpCy5.5 or PE-Cy7 (clone 581 Biolegend), anti-CD38 FITC (clone HIT2, BD Biosciences), anti-CD90 APC (clone 5E10, BD Biosciences) and anti-CD45RA Horizon V450 (HI100, BD Biosciences). $CD34^+CD38^-$ cells were sorted into microcentifuge tubes containing 500ul of PBS/10%FCS. HSCs were isolated as $Lin^-CD34^+CD38^-CD45RA^-CD90^+$ using a BD Influx sorter equipped with 355nm, 405nm, 488nm, 561nm, and 640nm lasers and

sorted directly into individual wells of a 96-well U-bottomed plate. HSCs were sorted as single cells into StemSpan medium (STEMCELL) supplemented with the cc100 cytokine cocktail (STEMCELL), which was used to maintain the stem and progenitor cell fraction in culture before harvesting and placing into a secondary CFC assay using Methocult 04435 (STEMCELL). For proliferation studies, HSC-derived clones were counted on day 10 (<50 cells were counted directly and larger clones were estimated based on diameter and binned into small (50-500) and medium (500+) categories). To improve genotyping accuracy, 7-10 day single HSC-derived cultures were placed into a CFC assay and the resultant colonies were genotyped for TET2 and JAK2 mutation status.

Primary patient sample gene expression assays

RNA was extracted from sorted $CD34^{+}CD38^{-}$ cells (using the PicoPure RNA isolation kit (Applied biosystems, 15295033) as per the manufacturer's instructions. First strand cDNA synthesis was performed using SuperScript III First-Strand Synthesis System (Invitrogen), using Random Hexamers, as per the manufacturer's guidelines. qPCR was carried out using TaqMan probes (Listed in Supplementary table 2) and TaqMan universal mastermix. qPCR assays were run and analysed using the Applied Biosystems ViiA 7 system.

Statistical Analyses

For calculating stem cell frequency and cluster enrichment scores and their corresponding Chi-squared values (two-tailed, Yates correction), we used the web-based calculator at https://www.graphpad.com/quickcalcs/contingency1/. For all other p values reported, an unpaired Student’s t-test (Microsoft Excel) was used unless otherwise specified.

Supplemental Figure Legends

Supplemental Figure 1

(A) Clone sizes resulting from 14-day single cell cultures of HSCs isolated from WT, JAK2 HET and JAK2 HOM littermate mice. For colonies of less than 50 cells, the total cell number was recorded exactly. Colonies in excess of 50 cells were grouped into three broad categories of small (50-500 cells), medium (500-10,000 cells), and large (>10,000 cells). Estimated clone sizes for medium and large clones were confirmed by individual colony harvest and flow cytometry using fluorescent cell counting beads as previously described[4].

Supplemental Figure 2

Panel A is the loadings plot for the PCA shown in Figure 1. Panel B shows hierarchical clustering of single cell gene expression profiling of HSCs from WT and JAK2 HOM littermate mice. Two main clusters are separated, the first of which is significantly enriched for JAK HOM mutant cells. Self-renewal regulators absent or lowly expressed in this cluster are displayed in the inset and match the genes that identify the distinct region observed in the Principal Component Analysis in Figure 1.

Supplemental Figure 3

A limiting dilution transplantation experiment was performed to determine the lowest dose of JAK HOM TET HOM whole BM required to give a phenotype. 100,000, 50,000 or 10,000 whole BM cells from WT (left panels) or JAK HOM TET HOM (right panels) mice were transplanted into W41 recipients alongside 100,000 CD45.1/2 competitors. Chimerism (B) and hemoglobin (HGB, C) are shown for three months post-transplantation. Chimerism is not different between WT and mutant cells and a red cell phenotype (indicated by a higher hemoglobin) is only observed in mice transplanted with 100,000 or 50,000 double mutant cells.

Supplemental Figure 4

(A) Primary recipients of TET HOM cells (green line) show no difference in platelet count from WT recipients (blue line). Recipients of JAK HOM (red line) and JAK HOM TET HOM (orange line) cells had reduced platelet count at early time points. (B+C) Assessment of blood parameters at 16 weeks after secondary transplantation revealed only JAK HOM TET HOM (orange) cells are able to sustain high hematocrit (HCT) (B) and hemoglobin (HGB) (C) in secondary transplantation recipients.

Supplemental Figure 5

(A) Five main clusters emerge when single HSCs from mice with each of the four genotypes (WT, JAK HOM, TET HOM, and double mutant) are assessed. Individual HSCs and their expression for each self-renewal regulator with HSCs from each genotype are coloured accordingly: Blue (WT), Red (JAK HOM), Green (TET HOM) and Orange (double mutant). (B) Each cluster was assessed for whether or not it contained an increased or decreased number of HSCs from each genotype compared to that expected by random chance by chi-squared analyses and these results are displayed in the table (yellow indicates $p < 0.05$ of a higher than expected observation and purple indicates $p < 0.05$ a lower than expected observation).

Supplemental Figure 6

Gene expression from single HSCs isolated from individual mice are displayed on the PCA plot from Figure 5 (n= 10 mice (5 WT, 3 JAK HOM (RR), 1 TET KO (TKO) and 1 double mutant (RRTKO)).

Supplemental Figure 7

(A) t-Distributed Stochastic Neighbor Embedding (t-SNE) analysis for the same dataset that appears in the PCA in Figure 5. Similar clustering is observed with WT cells scattered across the entire landscape, an area at the top of the plot enriched for JAK2 HOM (RR) cells (and lacking TET KO and double mutant (RRTKO) cells), an area to the left enriched for TET KO cells and double mutant cells clustering either with the TET KO cells or to the right side of the plot. (B) Violin plots for each of the four genes taken forward for functional assays with individual genotypes indicated.

Supplemental Figure 8

Gene expression of *Bmi1* (A), *Runx1* (B), *Pbx1* (C) and *Meis1* (D) from bulk $CD34^{+}CD38^{-}$ patient samples from patients with either no mutations, a JAK V617F mutation or both JAK and TET mutations. Probe IDs are located in Supplemental Table 2.

Supplemental Table 1

List of genes and corresponding Taqman Assay ID for genes used in multiplexed qPCR assays. Assay efficiencies were determined by a dilution series using cDNA prepared from WT BM lineage marker negative cells (5-fold dilutions) and

efficiencies were calculated as described in Pfaffl et al.[12], with a value of 2 representing perfect efficiency.

Supplemental Table 2

List of genes and corresponding Taqman Assay ID for genes used in human $CD34^{+}CD38^{-}$ qPCR.

Supplemental Appendix

Excel workbook including all single cell datasets. Zero values represent the average of two internal housekeeping controls run on every chip.

References

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4. Kent DG, Li J, Tanna H, et al. Self-renewal of single mouse hematopoietic stem cells is reduced by JAK2V617F without compromising progenitor cell expansion. PLoS biology 2013;11:e1001576.
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11. Ortmann CA, Kent DG, Nangalia J, et al. Effect of mutation order on myeloproliferative neoplasms. The New England journal of medicine 2015;372:601-12.
12. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic acids research 2001;29:e45.

A

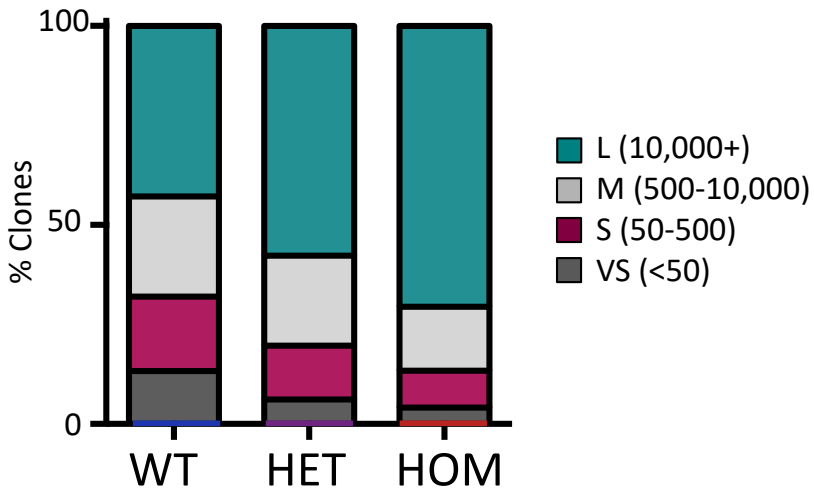

Figure S1

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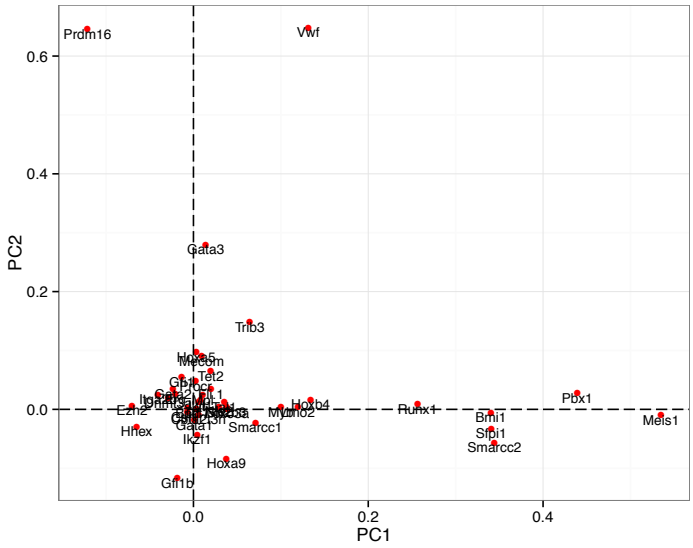
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Gata3
Tnb3
Tet2
Pbx1
Runx1
Bmi1
Sfpi1
Smarcc2
Meis1
Hhex
Ikzf1
Smarcc1
Hoxa9
Gfi1b
PC2
PC1
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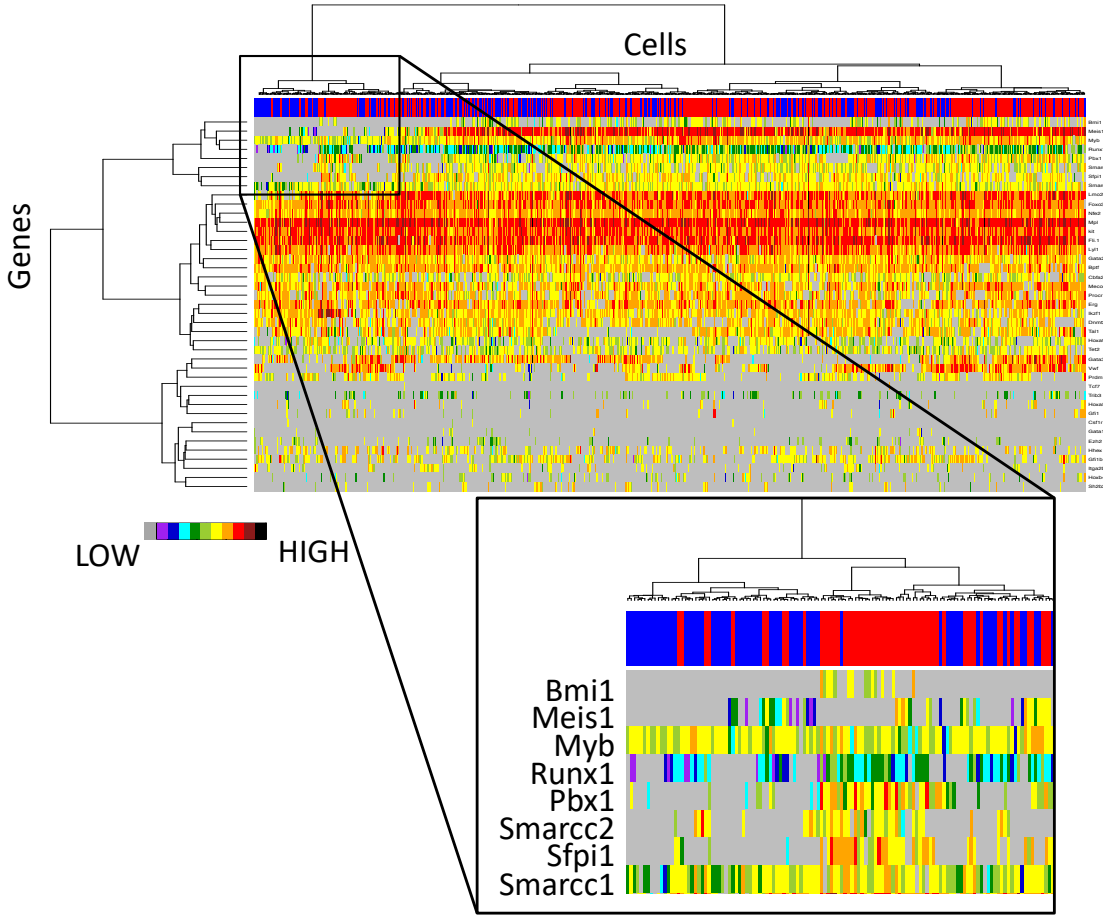
0.4
0.2
0.0
0.0
0.2
0.4

B


Cells
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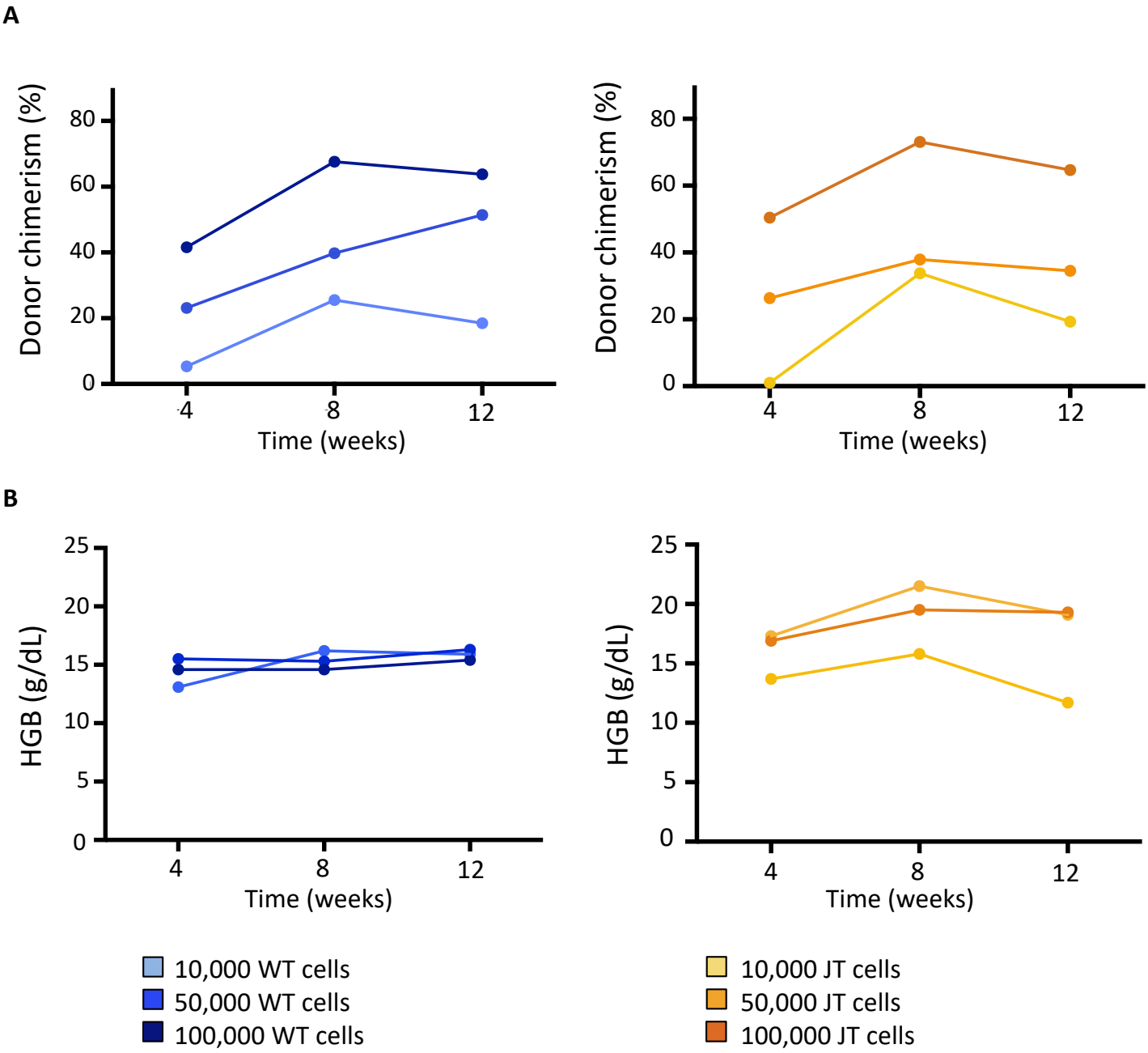
Meis1
Myb
Runx1
Pbx1
Smarcc2
Sfpi1
Smarcc1

Figure S2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Donor chimerism (%)
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20
40
60
80
4
8
12
Time (weeks)
Donor chimerism (%)
0
20
40
60
80
4
8
12
Time (weeks)
B
HGB (g/dL)
0
5
10
15
20
25
4
8
12
Time (weeks)
HGB (g/dL)
0
5
10
15
20
25
4
8
12
Time (weeks)
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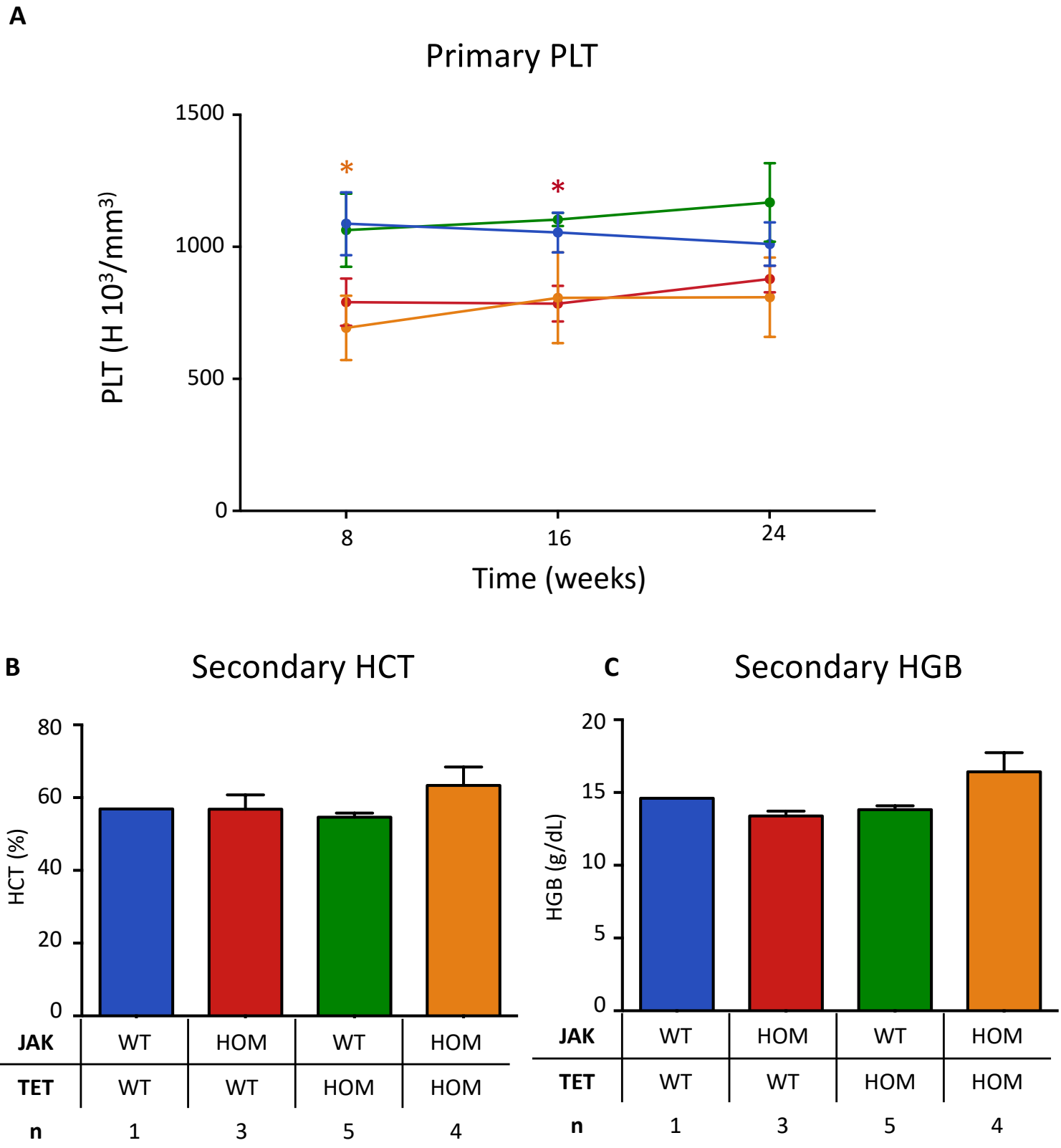
50,000 WT cells
100,000 WT cells
10,000 JT cells
50,000 JT cells
100,000 JT cells

Figure S3


A
Primary PLT
PLT (H 10³/mm³)
1500
1000
500
0
8
16
24
Time (weeks)
*
*
B
Secondary HCT
HCT (%)
80
60
40
20
0
JAK WT HOM WT HOM
TET WT WT HOM HOM
n 1 3 5 4
C
Secondary HGB
HGB (g/dL)
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15
10
5
0
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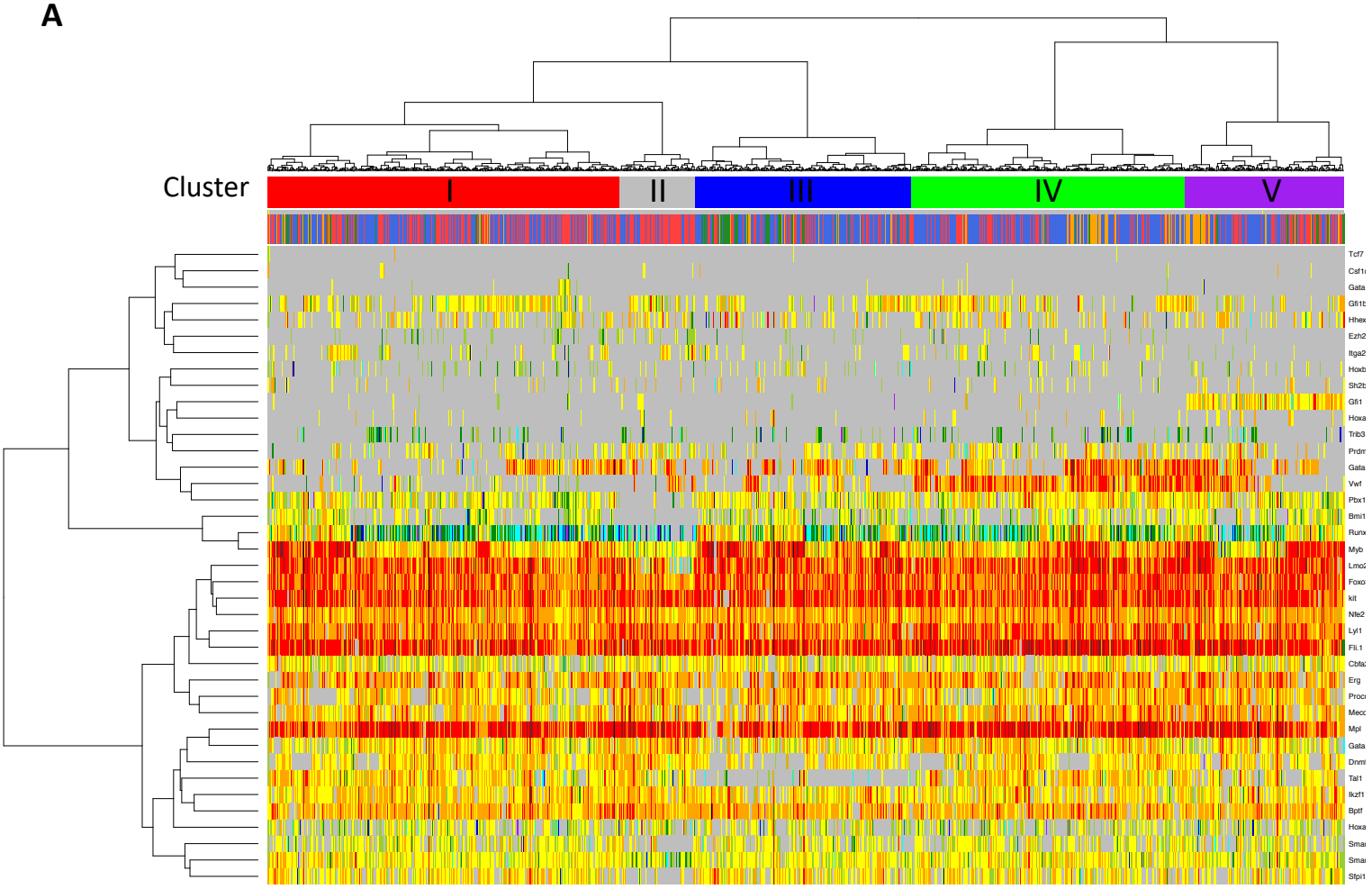
TET WT WT HOM HOM
n 1 3 5 4

Figure S4

A

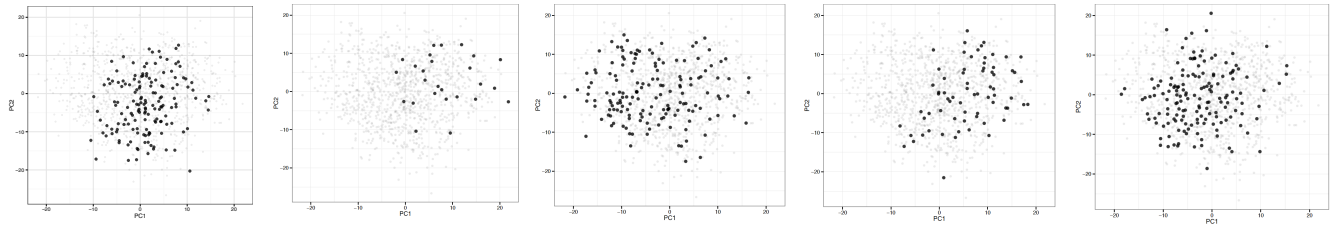


B

	Cluster I	Cluster II	Cluster III	Cluster IV	Cluster V
WT	0.4804	0.1252	0.7124	0.04	0.2938
JAK HOM	0.0282	0.0028	0.0333	0.0057	0.988
TET HOM	0.4677	0.0687	0.0005	0.0015	0.6604
DOUBLE	0.0512	0.0209	0.4562	0.0482	0.0795

Figure S5

A



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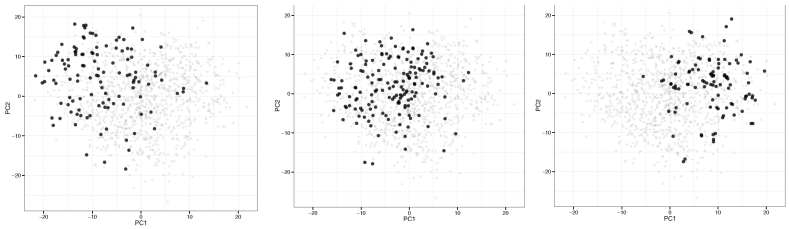

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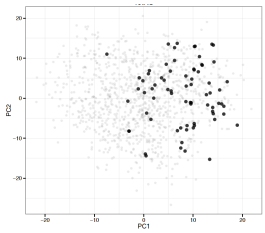

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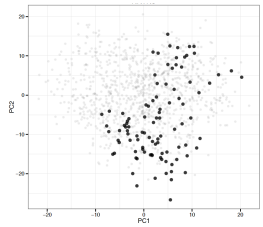
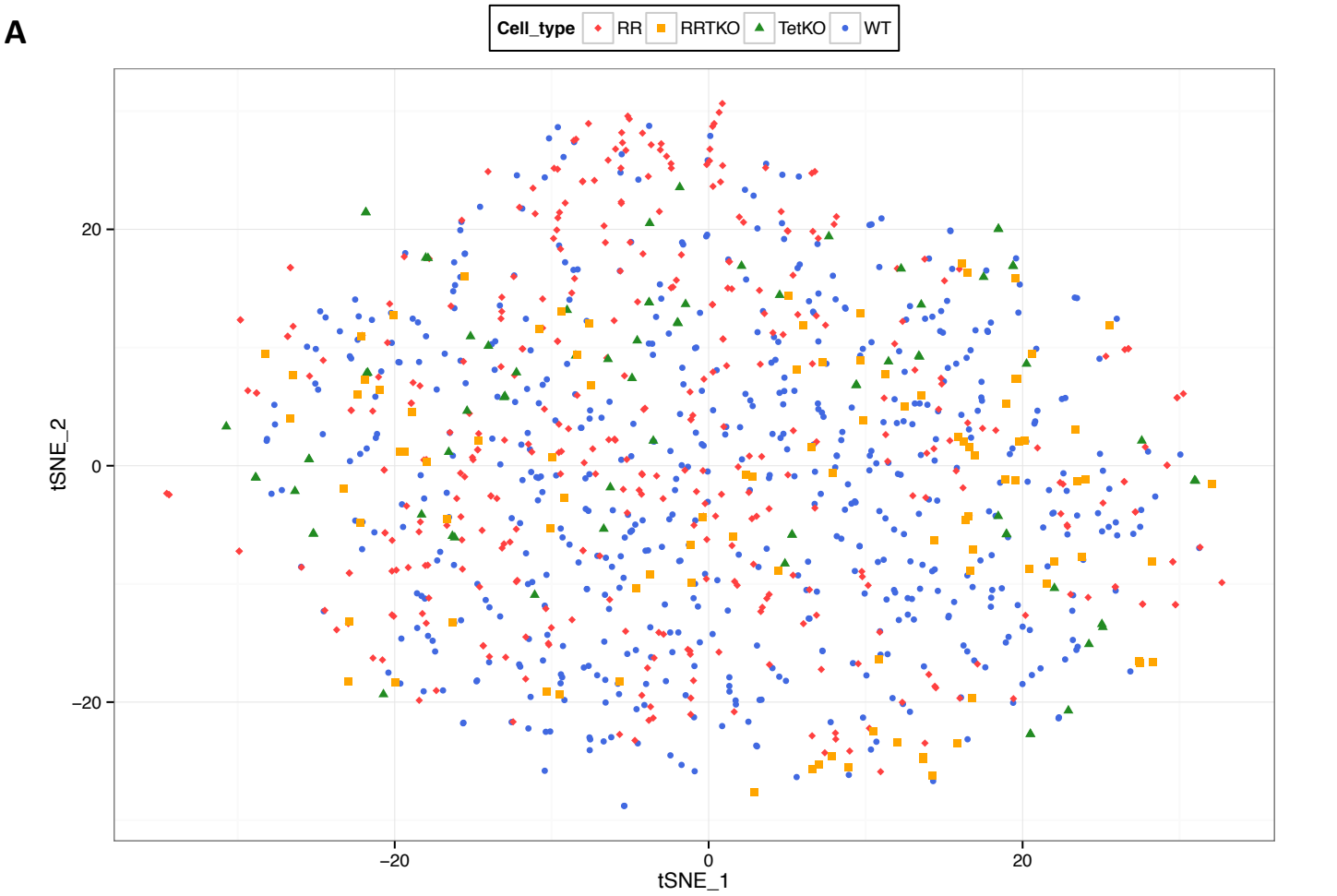


Figure S6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RRTKO
TetKO
WT
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tSNE_1
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−20
−20
0
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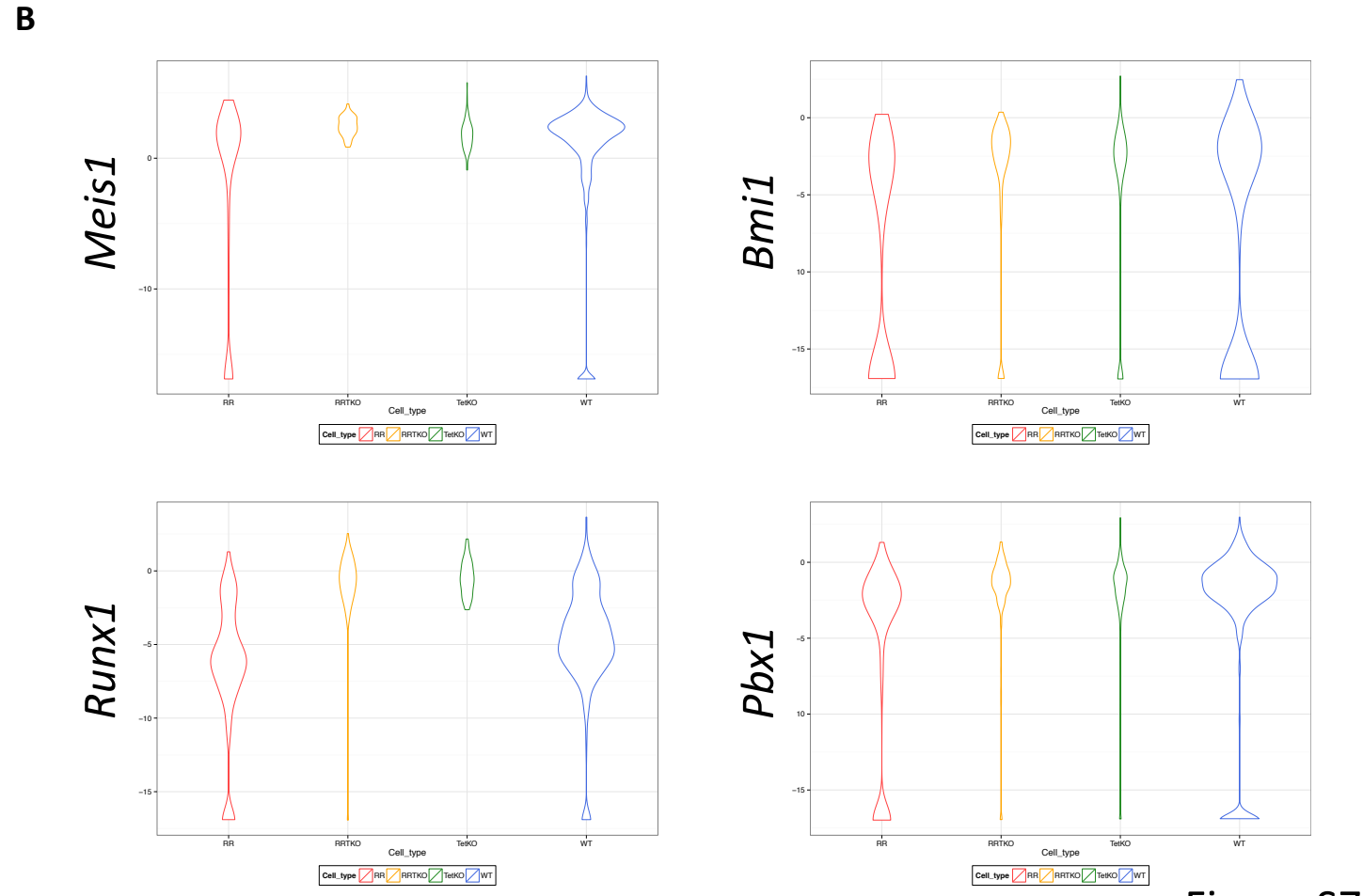
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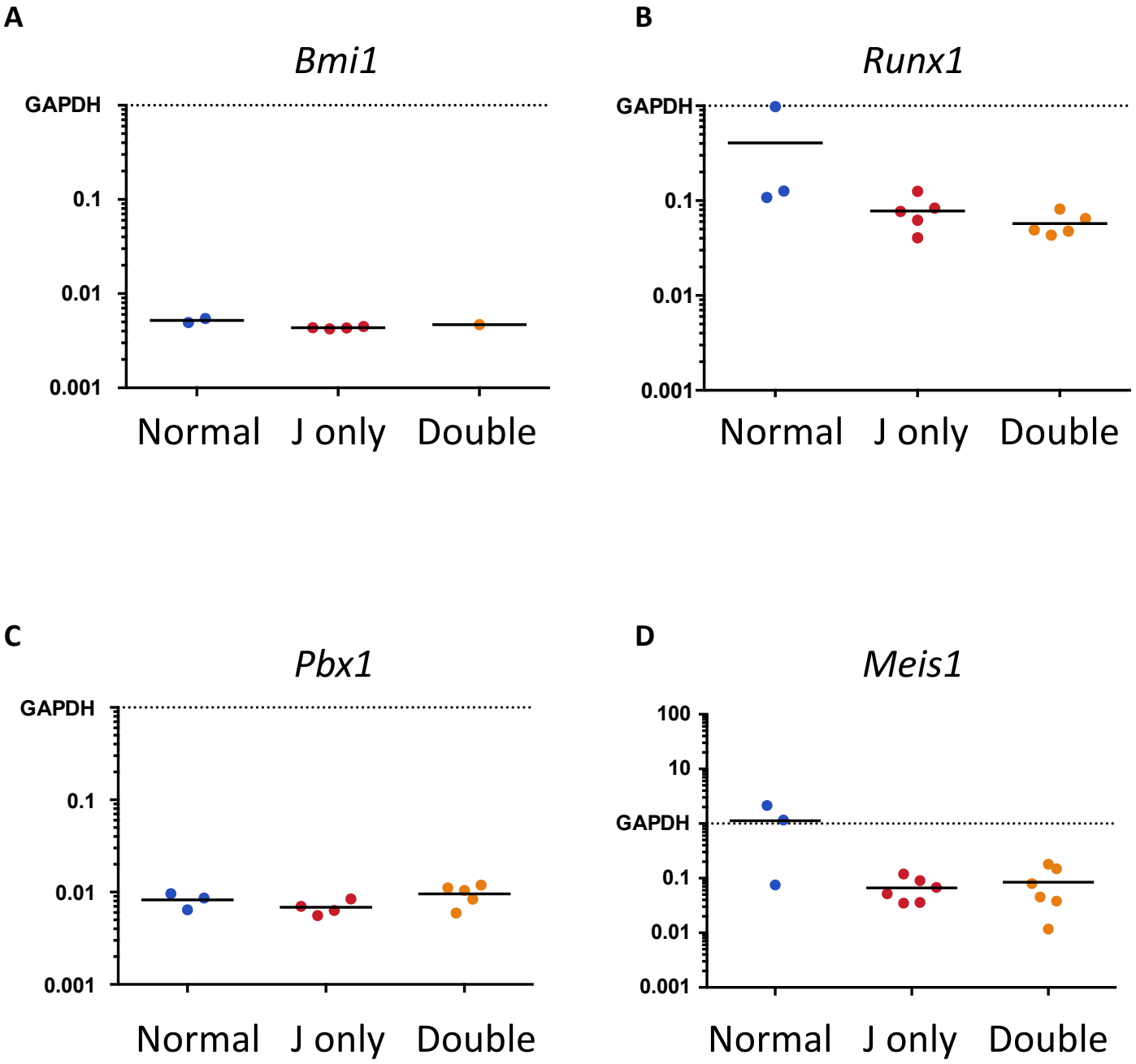
Meis1
Bmi1
Runx1
Pbx1
Cell_type
RR
RRTKO
TetKO
WT

Figure S7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Bmi1
GAPDH
0.1
0.01
0.001
Normal
J only
Double
B
Runx1
GAPDH
0.1
0.01
0.001
Normal
J only
Double
C
Pbx1
GAPDH
0.1
0.01
0.001
Normal
J only
Double
D
Meis1
100
10
GAPDH
0.1
0.01
0.001
Normal
J only
Double

Figure S8

Table S1

Gene	TaqMan order ID	Efficiency
Bmi1	Mm00776122_gH	1.87
Bptf	Mm01251151_m1	1.94
Cbfa2t3h	Mm00486780_m1	1.97
Cbx7	Mm00520006_m1	1.66
Cdkn2a	Mm00494449_m1	1.80
Csf1r	Mm01266652_m1	1.69
Dnmt3a	Mm00432881_m1	1.97
Egfl7	Mm00618004_m1	1.52
Eif2b1	Mm01199614_m1	1.95
Erg	Mm01214246_m1	1.96
Ezh2	Mm00468464_m1	1.52
Fli-1	Mm00484409_m1	1.94
Foxo3a	Mm01185722_m1	1.79
Gata1	Mm00484678_m1	1.94
Gata2	Mm00492300_m1	1.95
Gata3	Mm00484683_m1	2.03
Gfi1	Mm00515855_m1	2.02
Gfi1b	Mm00492318_m1	1.91
Hhex	Mm00433954_m1	1.98
Hoxa5	Mm00439362_m1	1.98
Hoxa9	Mm00439364_m1	2.02
Hoxb4	Mm00657964_m1	1.89
Ikzf1	Mm01187882_m1	1.94
Itga2b	Mm00439768_m1	1.68
kit	Mm00445212_m1	1.79
Lmo2	Mm01281680_m1	1.89
Lyl1	Mm01247198_m1	1.99
Mecom	Mm01289155_m1	2.02
Meis1	Mm00487659_m1	1.95
Mitf	Mm01182480_m1	2.04
Mpl	Mm00440310_m1	1.96
Myb	Mm00501741_m1	1.95
Nfe2	Mm00801891_m1	1.97
Pbx1	Mm04207617_m1	1.90
Polr2a	Mm00839493_m1	1.90
Prdm16	Mm00712556_m1	2.02
Procr	Mm00440993_mH	2.06
Runx1	Mm01213405_m1	1.96
Sfpi1	Mm00488142_m1	1.92
Sh2b3	Mm00493156_m1	1.99
Smarcc1	Mm00486224_m1	2.00
Smarcc2	Mm01159912_m1	1.78
Tal1	Mm01187033_m1	1.98
Tcf7	Mm00493445_m1	2.16
Tet2	Mm00524395_m1	1.93
Trib3	Mm00454879_m1	1.73
UBC	Mm01201237_m1	1.94
Vwf	Mm00550376_m1	2.02

Table S2

Gene	TaqMan order ID
Meis1	Hs00180020_m1
Bmi1	Hs00180411_m1
Runx1	Hs02558380_s1
Pbx1	Hs00231228_m1
GAPDH	Hs02786624_g1