ZRSR1 co-operates with ZRSR2 in regulating splicing of U12-type introns in murine hematopoietic cells

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Supplementary Information

ZRSR1 cooperates with ZRSR2 in regulating splicing of U12-type introns in murine hematopoietic cells

Running title: ZRSR2 & ZRSR1 cooperate in splicing of U12 introns

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Supplementary Methods

Peripheral blood analysis

Peripheral blood was analyzed using Abbott Cell-Dyn 3700 Hematology Analyzer (Abbott Laboratories).

Murine embryonic fibroblasts (MEFs)

Heterozygous *Zrsr2* KO mice were used for timed matings, and embryos from 14.5 days post coitus pregnant females were dissected out. Head and liver were removed, and remaining carcasses were digested for 30 min using Trypsin-EDTA solution with gentle stirring at 37°C. Undigested tissue were removed using 40 µm cell strainer and cell suspension was plated in 10 cm dishes. Adherent cells were expanded in DMEM medium containing 10% FBS, and RNA was extracted using Qiagen RNeasy Mini Kit. Embryos were genotyped for *Zrsr2* KO allele, and their sex was determined using PCR with primers GATGATTTGAGTGGAAATGTGAGGTA and CTTATGTTTATAGGCATGCACCATGTA, as described before.[1] Amplification was performed using GoTaq Green Master Mix (Promega) and PCR products were analysed on 1.5% agarose gel; bands corresponding to 685 bp and 480 bp were obtained for female embryos while 280 bp amplicon was obtained for male embryos.

Competitive and non-competitive repopulation assays

Bone marrow cells were harvested in 5% FBS-PBS and $Lin^{-}Kit^{+}Sca1^{+}$ (LSK) cells were FACS-sorted from the *Zrsr2* KO and WT mice ($CD45.2^{+}$). For competitive transplant assays, 2,000 LSK cells from either *Zrsr2* KO or WT mice were mixed with 500,000

total bone marrow cells from the competitor B6.SJL-Ptprca Pepcb/BoyJ (B6.SJL) strain (CD45.1^{+}), and injected intravenously in lethally irradiated (5.5 Gy x 2) recipient (B6.SJL) mice. Blood was collected every four weeks after transplantation and leukocytes were analyzed for donor chimerism using both lymphoid (CD3 and CD19) and myeloid (CD11b, Gr1 and F4/80) markers.

For non-competitive repopulation assays, two million bone marrow cells from either WT or *Zrsr2* KO mice were injected intravenously in lethally irradiated B6.SJL mice. Peripheral blood counts were analysed at indicated time points post transplantation.

ZRSR1 expression analysis

Expression levels of *ZRSR1* transcripts in human spleen and blood cells were obtained from Genotype-Tissue Expression (GTEx) portal (https://gtexportal.org/home/). Transcript levels of murine *Zrsr1* were gathered either from the Bgee database (spleen),[2] or our RNA-sequencing data (myeloid precursors).

Generation of *Zrsr1* knockdown Lin^{-}Kit^{+} bone marrow cells

Short hairpin RNA (shRNA) sequences targeting mouse *Zrsr1* gene were cloned in AgeI/EcoRI sites of pLKO.1-RFP lentiviral vector. To generate lentiviral particles, 293T cells were transfected using Lipofectamine 2000, and supernatant containing virus particles were collected 48h and 72h after transfection. shRNAs were screened in mouse fibroblast cell line, NIH3T3, and two shRNA sequences (sh1 and sh10), which resulted in significant downregulation of *Zrsr1* transcript levels were selected. The target sequence for mouse *Zrsr1* sh1 and sh10 are 5'-

GCAGAGCGATTACTGGAGATA-3' and 5'-AGTGCAGAAGGGATGACTATG-3', respectively.

Sorted $Lin^{-}Kit^{+}$ bone marrow cells were transduced twice, 24h apart, using RetroNectin reagent (Takara). Cells were cultured in IMDM supplemented with 15% FBS and 10 ng/ml recombinant mouse IL-3, 10 ng/ml recombinant mouse IL-6 and 20 ng/ml recombinant mouse stem cell factor (SCF). RFP^{+} cells were sorted and either used for clonogenic assays or lyzed for RNA/protein extraction. For methylcellulose colony assay, cells were plated in MethoCult GF M3434 medium (STEMCELL Technologies) containing recombinant mouse IL-3, mouse SCF, human IL-6 and human erythropoietin; and colonies were enumerated after 8 days.

Reverse transcription and quantitative PCR analysis for mis-splicing

RNA was treated with DNase I (Thermo Fisher Scientific) to remove genomic DNA contamination followed by reverse transcription using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). To verify intron retention, primers were designed to estimate the levels of both spliced and unspliced transcripts, and retention of U12-type introns was measured as relative quantity of unspliced to spliced transcripts. Quantitative RT-PCR was employed to measure the expression levels of spliced and unspliced transcripts using KAPA SYBR FAST universal kit according to manufacturer's protocol.

Generation of *Zrsr1*/*Zrsr2* double knockout 32D cells

32D cells were cultured in IMDM supplemented with 10% FBS and 2 ng/ml recombinant mouse IL-3. Cells lacking *Zrsr1* and/or *Zrsr2* were generated by sequential deletion of both genes using CRISPR/Cas9. Cells were first transduced with two guide RNAs targeting murine *Zrsr2* gene (LentiCRISPR v2; Addgene #52961), one targeting exon 2 (target sequence GGCGACGGCAGGCGCTCGCT) and the other targeting exon 11 (target sequence AGCATTAACTGATGCTCTCG). Two million cells in 6-well plates were transduced using spinoculation (1000g for one hour) in the presence of 5 µg/ml protamine sulphate. Transduction was repeated after 24h; and cells were selected with 2 µg/ml puromycin for one week. PCR on the cell pool using primers TTCTTCCTTTGCCTCCGAGC and AGAGGTGCACAGGCAGAAAACAGG confirmed deletion of the *Zrsr2* coding sequence. Guide RNA targeting lacZ gene of *Escherichia coli* (TGCGAATACGCCCACGCGAT) was used as control. Single cell clones of *Zrsr2*-deleted (ΔZrsr2) and control cells were expanded and transduced with two guideRNAs targeting murine *Zrsr1* gene (Lenti sgRNA Hygro; Addgene #104991), as described above. The target sequence for murine *Zrsr1* sgRNA1 and sgRNA3 are CCTGGACAGCAACTTCAGGG and AGAAGGGTGGGACTTGACGT, respectively. Cells were selected with 1,100 µg/ml hygromycin; and deletion was assessed using primers CAAACACTGGCCTTCGACTG and TGTCGTCCTGCGTACCATCTT. Single cell clones with deletion of either *Zrsr2* or *Zrsr1* or both were expanded, and RNA and protein were extracted for splicing analyses and western blot, respectively.

Generation of *ZRSR1* knockout K562 cells

K562 cells were transduced with two guideRNAs targeting human *ZRSR1* gene (target sequences ATAAGTAGAATCACTGACAG and CATAGAAATCTAGGAACTGT)

cloned into separate vectors expressing either mCherry or GFP (LentiCRISPRv2-mCherry, Addgene #99154 and LentiCRISPRv2GFP, Addgene #82416). 1-1.5 million cells in 6-well plates were transduced using spinoculation (1000g for one hour) in the presence of 5 µg/ml protamine sulphate. Transduction was repeated after 24h; and cells were FACS-sorted for GFP and mCherry expression. Once deletion of ZRSR1 locus in transduced cell pool was confirmed using PCR, single cell clones were generated for *ZRSR1* guideRNAs- and control guideRNA-transduced cells. Both single cell clone and cell pool of ZRSR1-deleted and control cells were expanded and transduced with shRNAs targeting human *ZRSR2* gene.

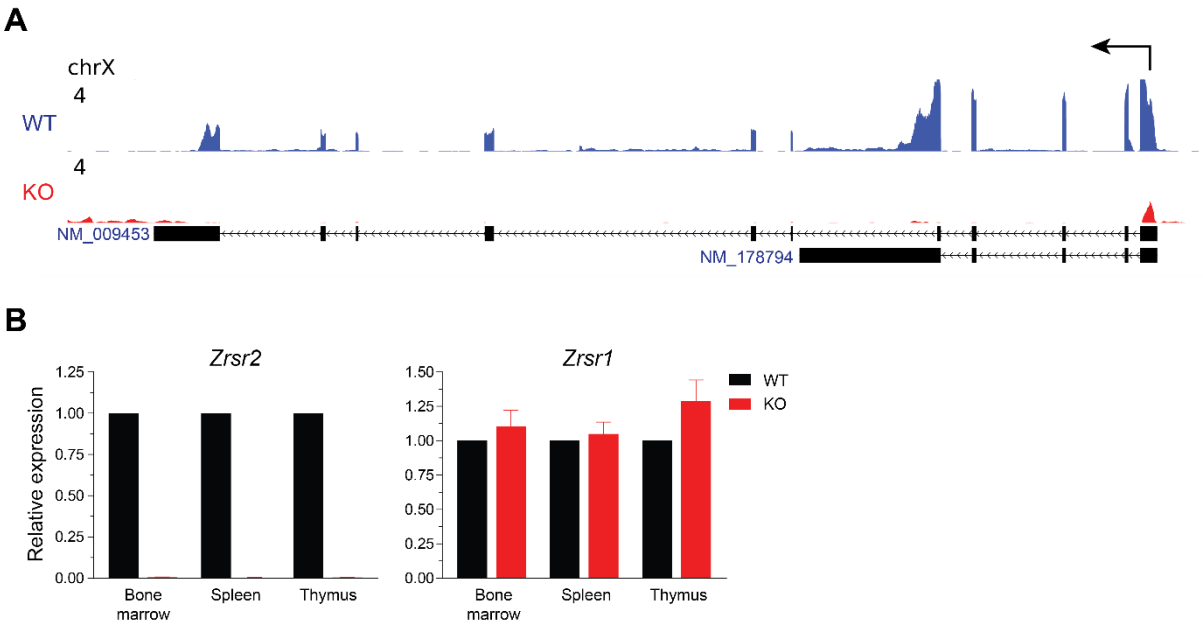
Western blot analysis for MAPK9 and MAPK14

Total protein lysates were prepared using M-PER Mammalian Protein Extraction Reagent (Thermo Scientific) containing protease inhibitor cocktail (Roche) and phosphatase inhibitor (Roche). Protein concentration was measured using Pierce BCA Protein Assay Kit (Thermo Scientific). Proteins resolved on SDS-PAGE gel were transferred to PVDF membrane (Millipore). Antibodies for MAPK9 (JNK2) (4672S) and MAPK14 (p38α) (9218T) were purchased from Cell Signaling Technology. Anti-GAPDH and anti-ACTB antibodies were used as loading controls.

References

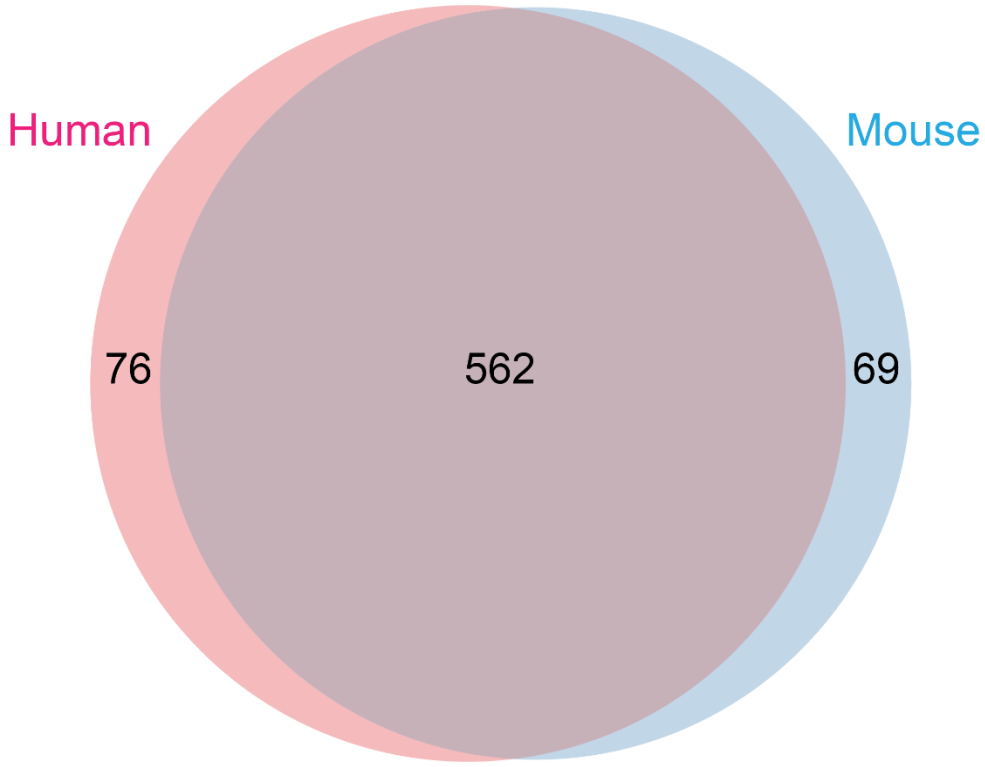
1. McFarlane L, Truong V, Palmer JS, Wilhelm D. Novel PCR assay for determining the genetic sex of mice. Sex Dev. 2013;7(4):207-211.
2. Komljenovic A, Roux J, Wollbrett J, Robinson-Rechavi M, Bastian FB. BgeeDB, an R package for retrieval of curated expression datasets and for gene list expression localization enrichment tests. F1000Res. 2016;5(2748.

Supplementary Figure S1



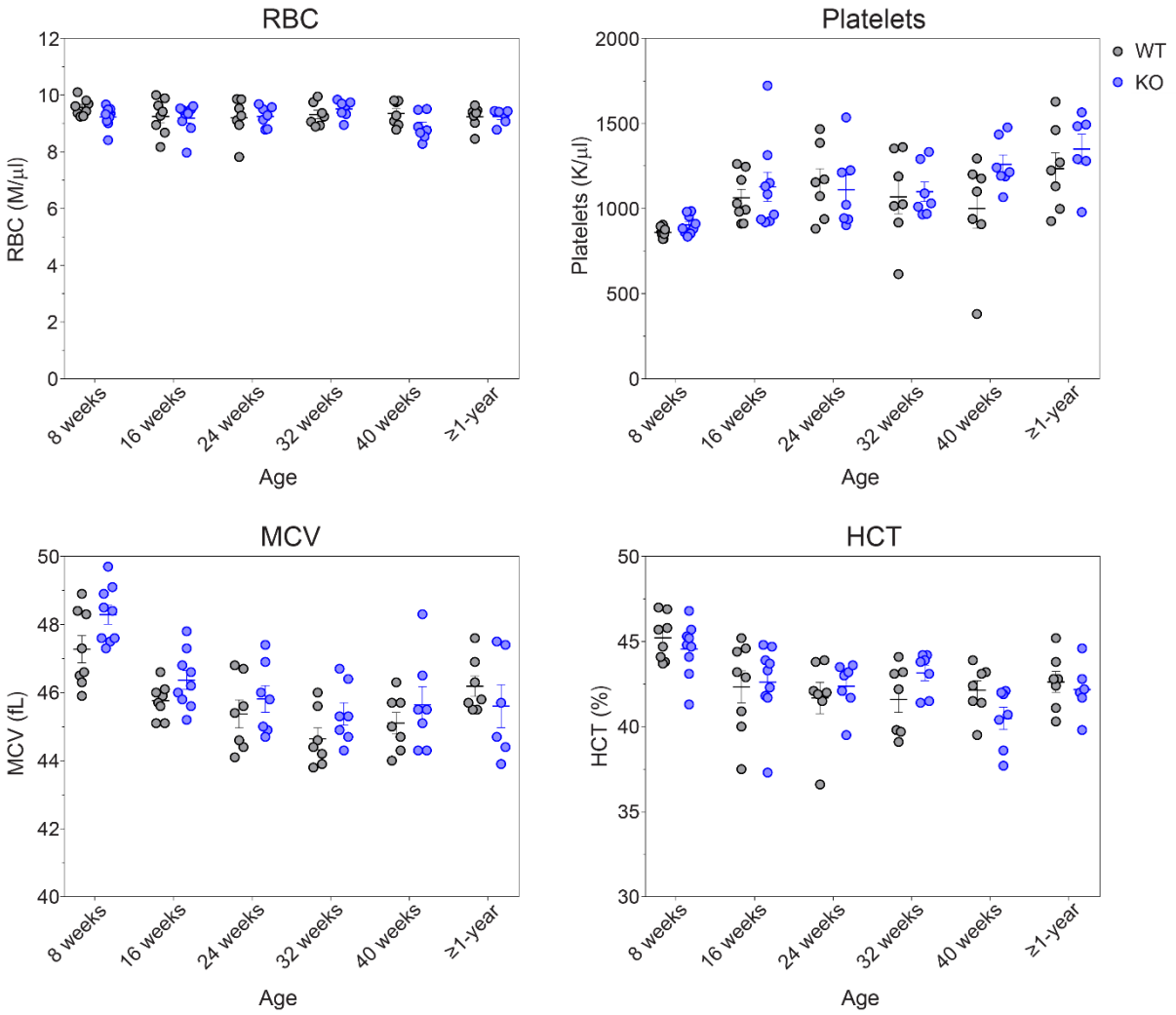
Supplementary Figure S1. (**A**) RNA-seq reads at *Zrsr2* locus in WT and *Zrsr2* KO ES cells. (**B**) Transcript levels of *Zrsr2* and *Zrsr1* in bone marrow, spleen and thymus of WT and *Zrsr2* KO mice. Transcript levels were normalized to those of β-actin. Bars represent mean ± SEM.

Supplementary Figure S2



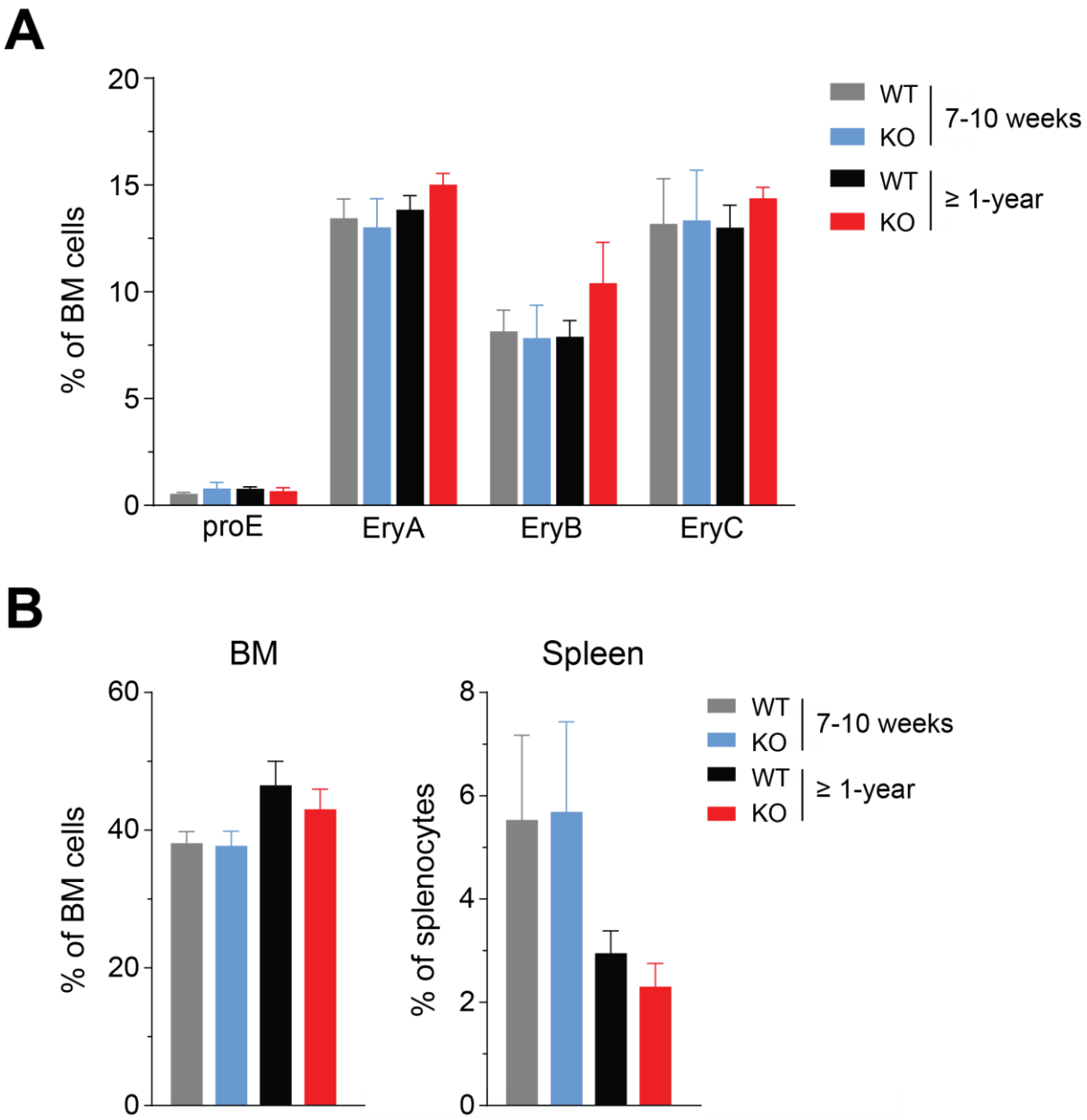
Supplementary Figure S2. Overlap of genes containing U12-type introns between human and murine genomes.

Supplementary Figure S3



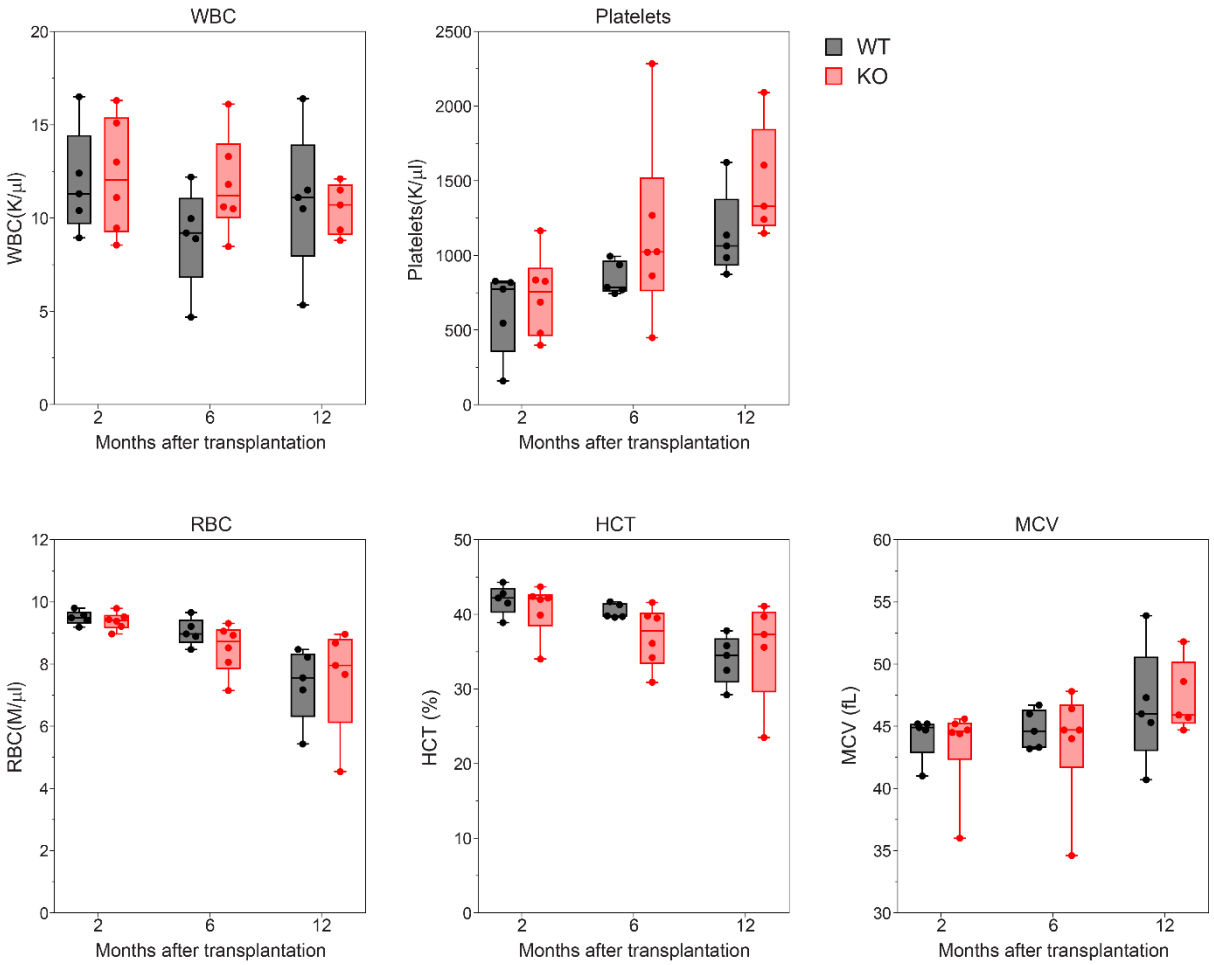
Supplementary Figure S3. Periodic analysis of RBC and platelet counts, mean corpuscular volume (MCV) and haematocrit (HCT) in peripheral blood of WT and *Zrsr2*-deficient mice. Error bars represent SEM.

Supplementary Figure S4



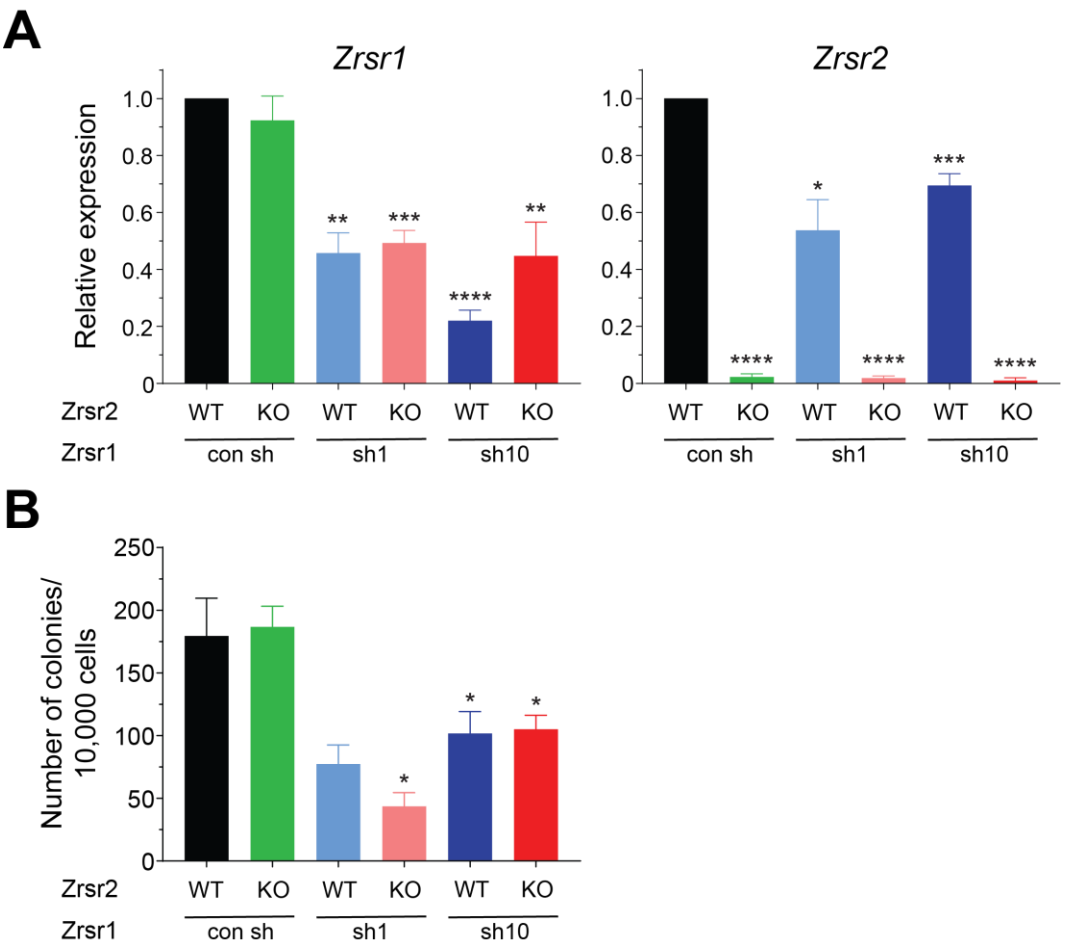
Supplementary Figure S4. (**A**) Frequency of erythroid precursors (proE: $CD71^{+}TER119^{lo}$; EryA: $CD71^{+}TER119^{+}FSC^{hi}$; EryB: $CD71^{+}TER119^{+}FSC^{lo}$ and EryC: $CD71^{-}TER119^{+}FSC^{lo}$) in BM of young (7-10 weeks old) and old (≥1-year old) WT and *Zrsr2* KO mice. (**B**) Proportion of granulocytes ($CD11b^{+}Gr1^{high}$) in the bone marrow and spleen of WT and *Zrsr2* KO mice. Data are from at least four mice of each genotype and age group, and represented as mean ± SEM.

Supplementary Figure S5



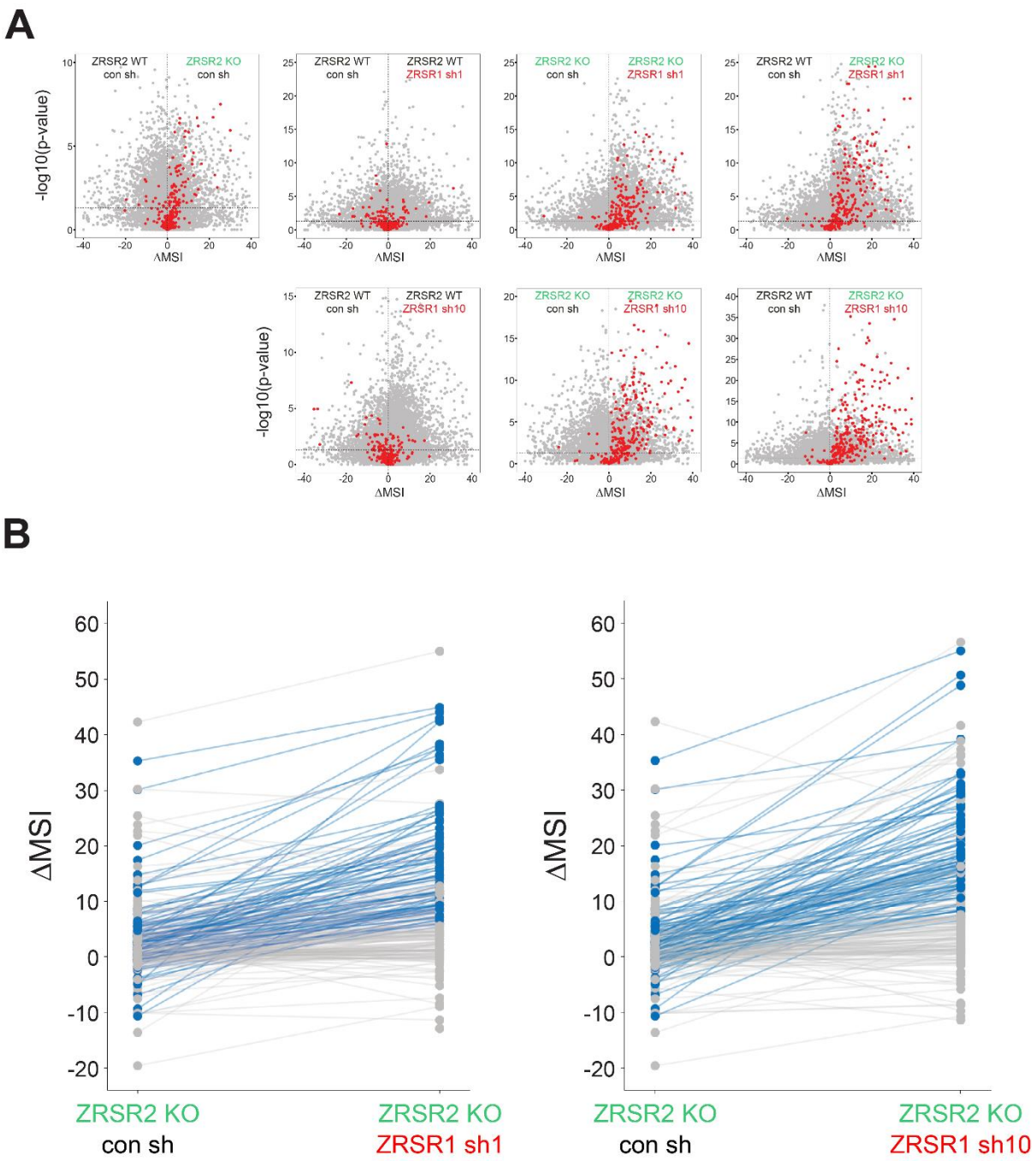
Supplementary Figure S5. Blood cell counts, HCT and MCV measured in the peripheral blood of recipient mice transplanted with either WT or *Zrsr2* KO BM cells in non-competitive repopulation assay. Blood was drawn at indicated time points post transplantation.

Supplementary Figure S6



Supplementary Figure S6. (**A**) Transcript levels of *Zrsr1* and *Zrsr2* in Lin$^-$Kit$^+$ BM cells (from either WT or *Zrsr2* KO mice) transduced with lentivirus expressing shRNAs (either sh1 or sh10) targeting *Zrsr1*. Transduced cells (RFP$^+$) were sorted and RNA was extracted. Transcript levels were normalized to those of β-actin. (**B**) Clonogenic ability of sorted RFP$^+$ Lin$^-$Kit$^+$ BM cells expressing *Zrsr1* shRNA. Data are represented as mean ± SEM. Statistical significance was calculated compared to the control cells (*Zrsr2* WT; con sh). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

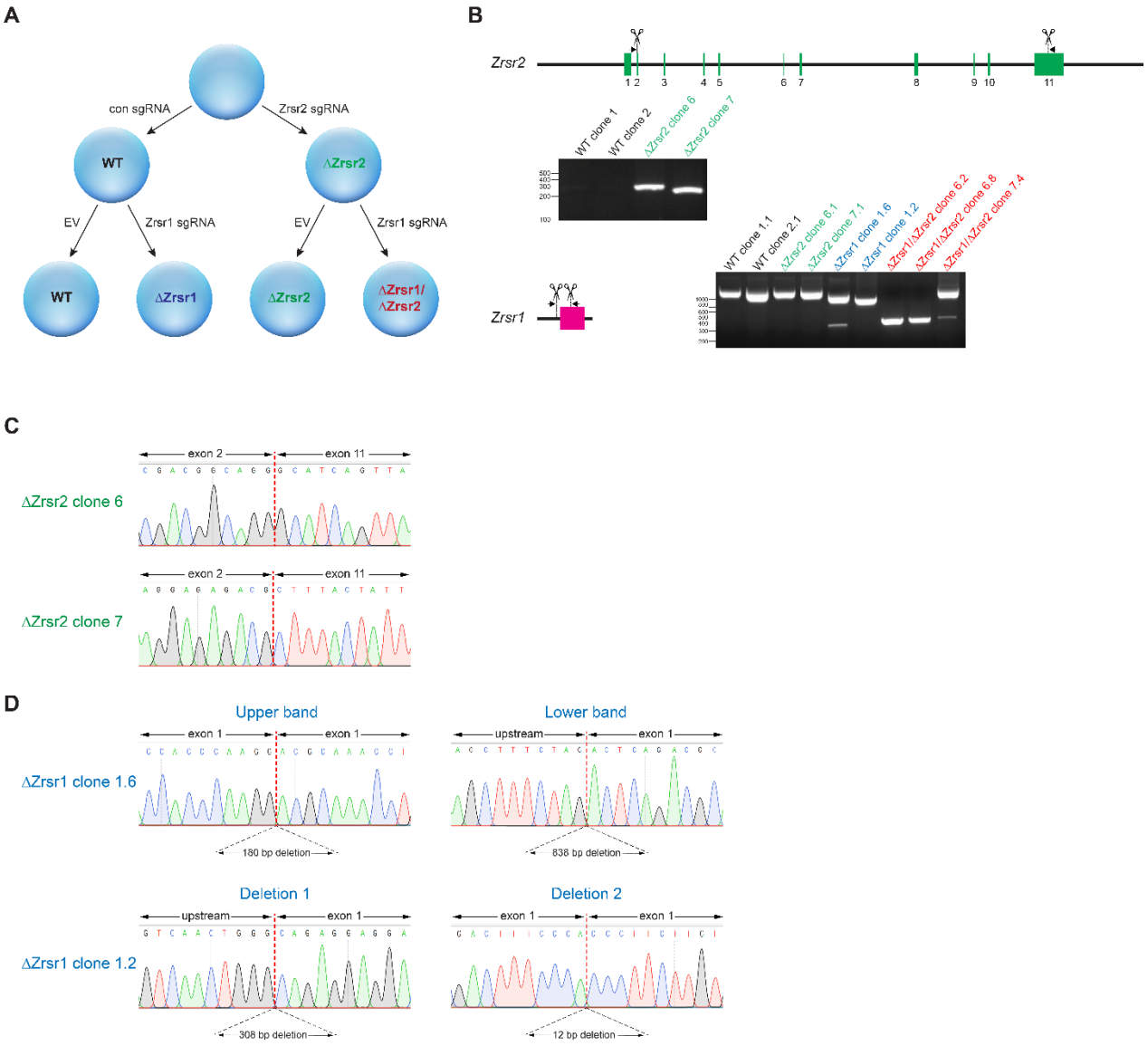
Supplementary Figure S7

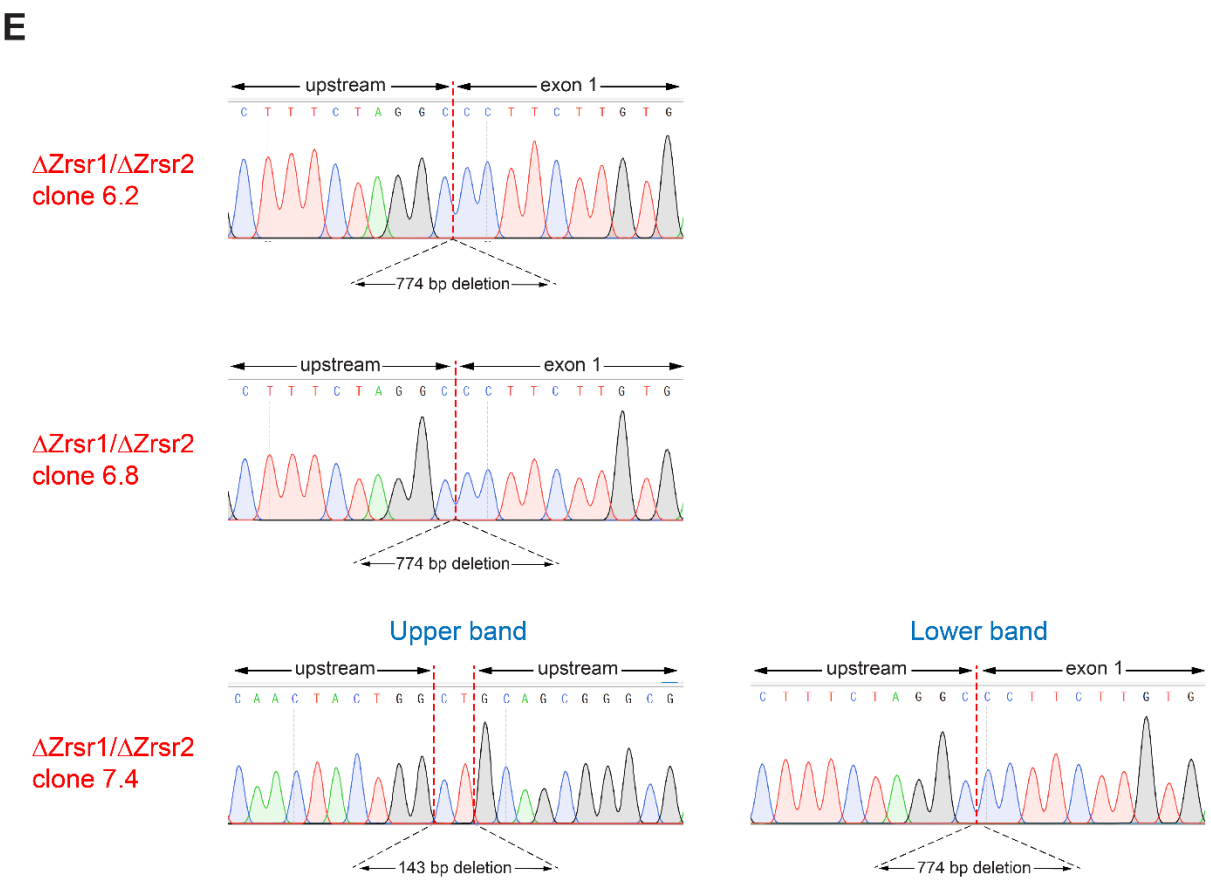


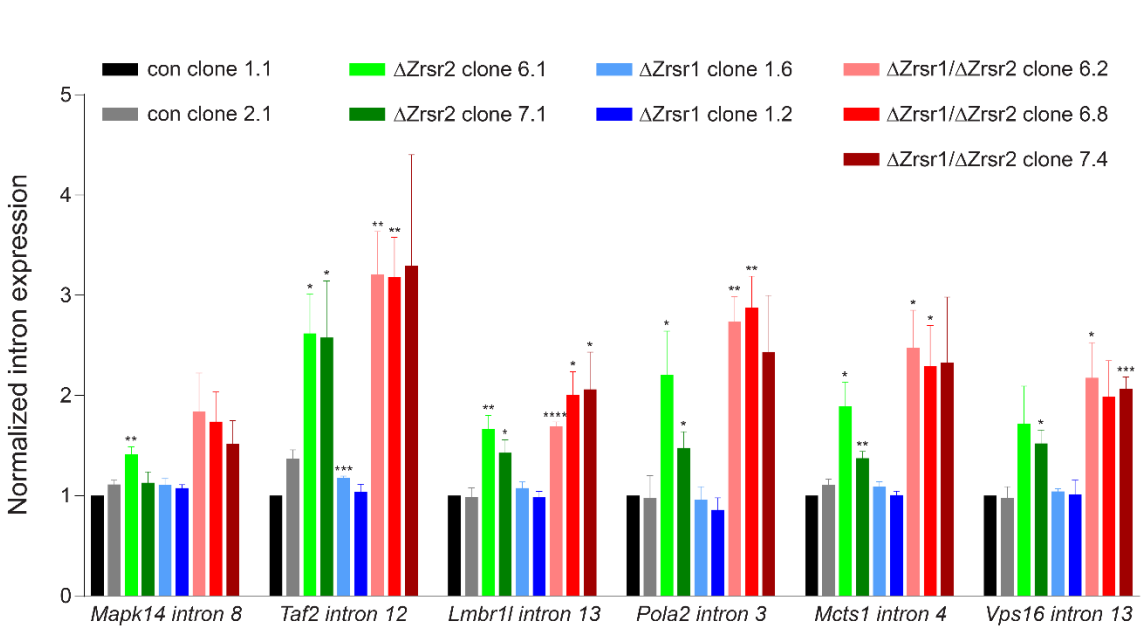
Supplementary Figure S7. (**A**) Dot plots depict intron retention in either *Zrsr2*-deficient, *Zrsr1*-deficient or *Zrsr2*/*Zrsr1* double deficient Lin$^-$Kit$^+$ BM cells. FACS-sorted Lin$^-$Kit$^+$ BM cells from either WT or *Zrsr2* KO mice were transduced with lentivirus expressing shRNAs (shRNA1 or 10) targeting *Zrsr1*. Stably transduced (RFP$^+$) cells were sorted and used for RNA-seq. Red circles represent U12-type introns. (**B**) Change in ΔMSI values for intron retention in *Zrsr2*-deficient and *Zrsr2*/*Zrsr1* double deficient Lin$^-$Kit$^+$ BM cells. Representative plots from one of the two experiments are shown (sh1-targeted cells on left and sh10-targeted cells on right). Introns depicted in blue are those which are retained in both sh1 and sh10-targeted

cells. These introns also have a ΔMSI value greater than 5 in *Zrsr2*/*Zrsr1* double deficient cells, and display an increase in ΔMSI≥5 in *Zrsr2*/*Zrsr1* double deficient cells compared to *Zrsr2* KO cells.

Supplementary Figure S8



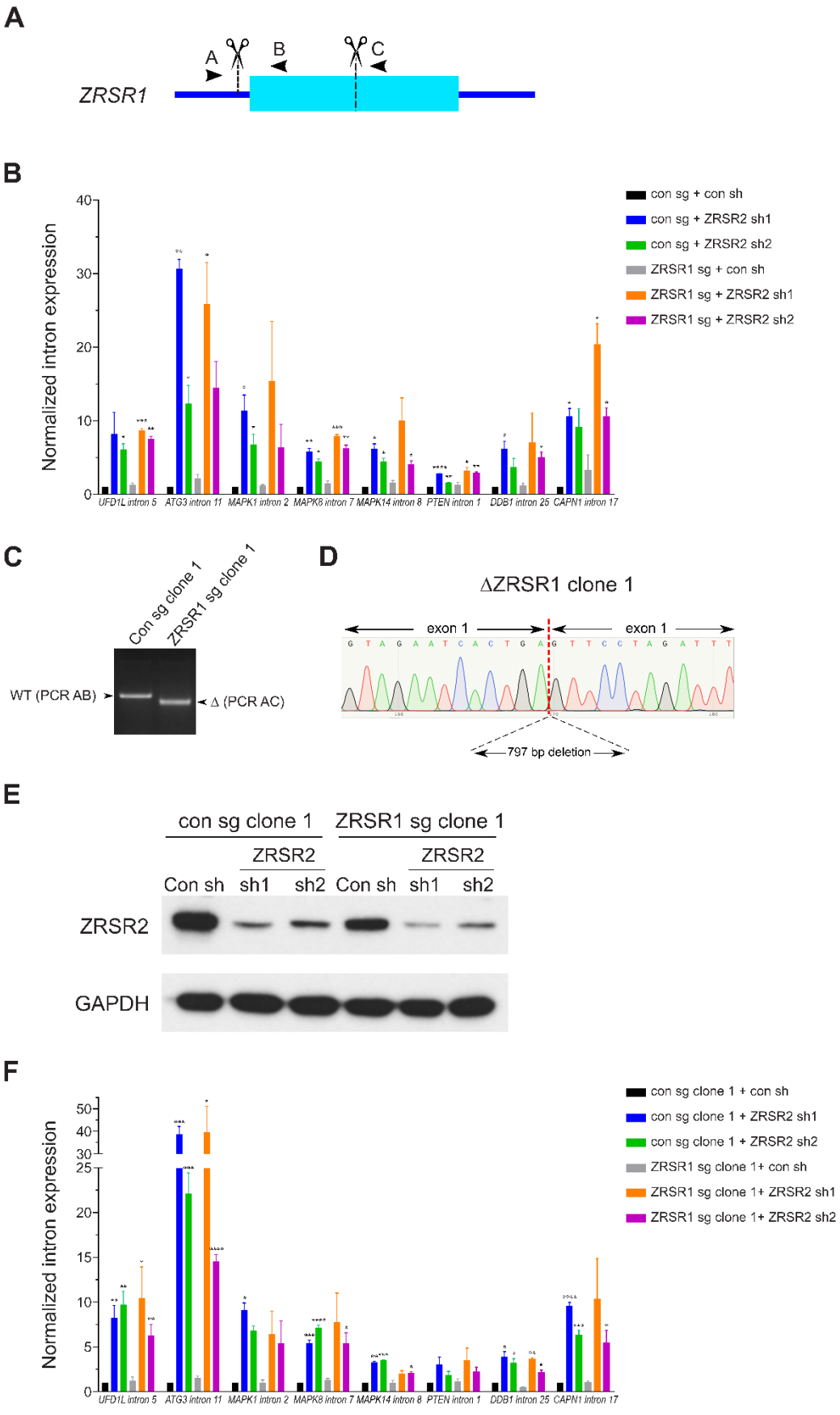




Supplementary Figure S8. (**A**) Schematic representation of strategy used for sequential deletion of *Zrsr2* and *Zrsr1* using CRISPR/Cas9 approach in 32D murine myeloid cells. (**B**) PCR analyses to verify deletion in *Zrsr2* (upper) and *Zrsr1* (lower) genomic loci in 32D cells. Locations of guide RNAs targeting each gene and PCR primers are depicted. (**C**) Sanger sequencing of PCR amplicon verifies deletion in *Zrsr2* gene in two single cell clones. (**D-E**) Sanger sequencing analyses of PCR

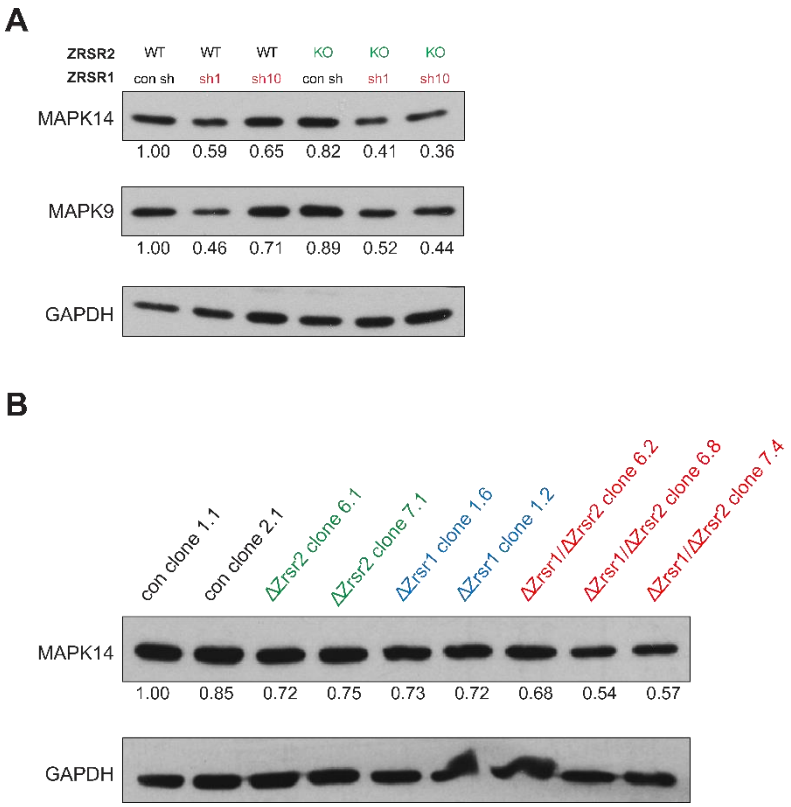
products (shown in b) corresponding to *Zrsr1* genomic deletions in *Zrsr1* single KO (D) and *Zrsr1*/*Zrsr2* double KO (E) single cell clones. (**F**) Quantitative RT-PCR to estimate levels of retained U12-type introns in 32D single cell clones lacking either ZRSR1 or ZRSR2 or both. Data are represented as mean ± SEM. Statistical significance was calculated compared to the control cells (con clone 1.1). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Supplementary Figure S9



Supplementary Figure S9. (**A**) Schematic shows locations of guideRNAs targeting human *ZRSR1* gene and PCR primers used to verify deletion. (**B**) Quantitative RT-PCR to estimate levels of retained U12-type introns in pool of K562 cells expressing guideRNAs targeting *ZRSR1* and transduced with shRNAs targeting ZRSR2. Control cells (con sg + con sh) are used for statistical comparison. (**C**) PCR to verify deletion of *ZRSR1* locus in K562 single cell clone. (**D**) Sanger sequencing of *ZRSR1*-deleted single cell clone revealed excision of 797 bp of genomic sequence. (**E**) Western blot verifies shRNA-mediated knockdown of ZRSR2 in control and ΔZRSR1 K562 single cell clone. (**F**) Quantitative PCR analysis as shown in (B) to estimate retention of U12-type introns in *ZRSR1*-deficient single cell clone transduced with shRNAs targeting *ZRSR2*. Data are represented as mean ± SEM. Statistical significance was calculated compared to the control cells (con sg clone 1 + con sh). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Supplementary Figure S10



Supplementary Figure S10. (**A**) Protein expression of MAPK9 and MAPK14 in WT and *Zrsr2* KO $Lin^{-}Kit^{+}$ BM cells transduced with shRNA (either sh1 or sh10) targeting *Zrsr1*. (**B**) Western blot for MAPK14 in 32D cells lacking either *Zrsr1* or *Zrsr2* or both. Band intensities were measured using ImageJ software and ratio of intensities (relative to GAPDH band intensities) are indicated below the images.

Supplementary Tables

Supplementary Table S1

Genotyping PCR primers

Primer A - mZrsr2-wt-F	ACAAAACCACCCAGAGTCCCAAATC
Primer B - mZrsr2-wt-R	AGAGGTGCACAGGCAGAAAACAGG
Primer C - NeoR-F	GCAGCCTCTGTTCCACATACACTTCA
Primer D - LacZ-F	ACTTGCTTTAAAAAACCTCCCACA

Mouse Zrsr1/Zrsr2 expression analysis

mZrsr1 F	CAAGAAACGTCGGCAGAAAATG
mZrsr1 R	CTATCTCCAGTAATCGCTCTGCAAG
mZrsr2 F	AGACTTCTATTATGATGTCCTGCCTG
mZrsr2 R	GGCTGCTTGACAGTCTTCTTCC
mActb F	GGCACCACACCTTCTACAATGAG
mActb R	GGGTGTTGAAGGTCTCAAACATG

Intron retention analysis (mouse)

Mcts1-ex4-F	AGGAGCCATCAAATTTGTACTCAG
Mcts1-in4-R	TCAGCAGTTAAACCCTGGGA
Mcts1-ex5-R	TGACACCCACACATAAAGCATG
Ccdc28b-ex5-F	GGAAGATCTTAGCAATTCCAT
Ccdc28b-in5-R	TGTGTGAATAGAGATAGGAACG
Ccdc28b-ex6-R	TGAAGAGGAGAGTAGCAGAAGAG
Pola2-ex3-F	AAAAAACTCTCCAAAGCCTG
Pola2-in3-R	GGGAAATTACATAGCACATGTATG
Pola2-ex4-R	GGGTGTGGTATAAGAGCTTAGG
Inpp5b-ex21-F	AGGTCTGAGGCCAGAGTTTG
Inpp5b-in21-R	TGAGCTTTGGGATAGTCTTCTCTC
Inpp5b-ex22-R	GCTCTGGCAGGCTCTCTAGG
Vac14-ex16-F	ACAACCCAGTCACCACCGTG
Vac14-in16-R	CTGGGGGATGGATGACAATG
Vac14-ex17-R	GCACGAGTTTGTCCACCTCTG
Mapk14-ex8-F	TGAGCTGTTGACCGGAAGAAC
Mapk14-in8-R	AGTCTTCCCCACCAATGAGTTAGG
Mapk14-ex9-R	TCTTCAGAAGCTCAGCCCCTG
Ccnt2-ex1-F	GATGAAGAGCTGTCGCATCGC
Ccnt2-in1-R	GACACTAGGCCCGGTCACCC
Ccnt2-ex2-R	CCTGTGCATATAAACAATCGCAGTG
Sfi1-ex10-F	GTCCTGCGGAGGGCCTTTAC

Sfi1-in10-R	AGCTCCTCACACTAGGGACACTCTG
Sfi1-ex11-R	AGGTAGTGCTGGCGGTGTTCAG
Taf2-ex12-F	GAAGTCCATTTCCAATGTCTCTGG
Taf2-in12-R	TCTTCCCTGTCTTCCACACAGC
Taf2-ex13-R	ACTTCCGTAAAACTTTACCACTCCAC
Lmbr1l-ex13-F	TCTCCAAACTGGGATCCTTTG
Lmbr1l-in13-R	CAGTGCTCTCAGGGGTCTATTAAG
Lmbr1l-ex14-R	CCTCAGGCTTCCAAAGAGTG
Mapk9-ex7-F	TCATGGCAGAAATGGTCCTC
Mapk9-ex8-R	TCTTCATGAACTCTGCGGAT
Mapk9-in7-F	GTTTCTGCGGATCCTCTAGGA
Mapk9-in7-R	ACAGTCCTCTCATGCAAAGG
Vps16-ex13-F	ACAGCTTCGTGCACATGTGTC
Vps16-in13-R	TAAACACCCTCATGCCATACATAGG
Vps16-ex14-R	AGCACCTGGATGGTGAGCTG

Intron retention analysis (human)

MAPK9-In7-F	GACAAAGTCGTAAAATCCACTCCAG
MAPK9-In7-R	GTCTCAAAGTCATCACAGGTAGTCAAG
MAPK9-ex7-F	GTGAAAGGTTGTGTGATATTCCAAG
MAPK9-ex8-R	ATAATTCCTCACAGTTGGCTGAAG
MAPK14-In8-F	CTGTATTCCAGTGTCCATGGGTG
MAPK14-In8-R	AGCAAAATACTGAGAGTCTTCCCTAG
MAPK14-ex8-F	ATAATGGCCGAGCTGTTGACTG
MAPK14-ex9-R	GGTTCCAACGAGTCTTAAAATGAGC
UFD1L -In-5F	TATTGAATCTGTGCCTGCTTTGG
UFD1L -In-5R	TGTGCTAGCTGAACCCCATTATTC
UFD1L -Ex-5F	CTGACTTCCTGGACATCACCAAC
UFD1L -Ex-6R	TCATTATAGTTGATGGCAATCACATC
ATG3 -In-11F	ATCCTTCCCTGTATGTAAGATTAGTGG
ATG3 -In-11R	GGACATGTTCTAATCAACTAAGCAAGG
ATG3 -Ex-11F	TCATTGAGACTGTTGCAGAAGGAG
ATG3 -Ex-12R	GTCATATTCTATTGTTGGAATGACAGC
MAPK1 -In-2F	AATTCCACTGCTTGGTAACCTTG
MAPK1 -In-2R	CCAAGATTTTCCATGACTAGACTTAGG
MAPK1 -Ex-2F	GCTTCAGACATGAGAACATCATTG
MAPK1 -Ex-3R	GTGTCTTCAAGAGCTTGTAAAGATCTG
MAPK8 -Ex-7F	ATTATGGGAGAAATGGTTTGC
MAPK8 -In-7R	AGAGAACATAAAGAAGTTCATCGC
MAPK8 -Ex-8R	CAACGTAAGTCCTTACTGTTGGT
PTEN -In-1F	TAGAACGTGGGAGTAGACGGATG
PTEN -In-1R	AGAGTTCCGTCTAGCCAAACACAC
PTEN -Ex-1F	CATTTCCATCCTGCAGAAGAAG
PTEN -Ex-2R	GGAAATCCCATAGCAATAATGTTTG
DDB1 Ex-25F	GCTGGACATGCAGAATCGACTCAA

DDB1 In-25R	AGACATGTAGTAGCTTCCGGGT
DDB1 In-26R	ACTTGTCTGGCCCAGGGTTAAA
CAPN1 -In-17F	TTGTCTCCTGAGTGGGGTTTTG
CAPN1 -In-17R	AACAGGAAGACGTCCAGGGAG
CAPN1 -Ex-17F	GCTTCAGCCTAGAGTCGTGCC
CAPN1 -Ex-18R	AGGTAATTCCGGATGCGGTTC

Primers for checking deletion of *Zrsr2* and *Zrsr1* in 32D cells

mZrsr2-F	TTCTTCCTTTGCCTCCGAGC
mZrsr2-R	AGAGGTGCACAGGCAGAAAACAGG
mZrsr1-F	CAAACACTGGCCTTCGACTG
mZrsr1-R	TGTCGTCCTGCGTACCATCTT

Primers for checking deletion of *ZRSR1* in K562 cells

hZRSR1-F	ACCGCACCTGGCCATAAGTA
hZRSR1-R	GCATTCTTCTTCCGACTGGT
hZRSR1-R	CCTCCTCCTGTGAGAGTCCT

Supplementary Table S2: Introns aberrantly retained in *Zrsr2* KO CMP, GMP, MEP and MEFs

Supplementary Table S3: Introns aberrantly retained in K562 and TF1 cells expressing shRNAs targeting ZRSR2

Supplementary Table S4: Introns aberrantly retained in murine $Lin^{-}Kit^{+}$ bone marrow cells lacking either ZRSR1 or ZRSR2 or both