

Title

Selenoprotein-Mediated Redox Regulation Shapes the Cell Fate of HSCs and Mature Lineages

Running Title

Selenoproteins Shape the Hematopoietic Cell Fate

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KEY POINTS

1. Disruption of antioxidant selenoprotein synthesis exhibited significant defects in B lymphocyte development and HSC function.
2. Cell context and lineage dictate sensitivity to selenoprotein synthesis defects, with lipid peroxidation and ferroptosis serving as drivers.

Abstract

The maintenance of cellular redox balance is crucial for cell survival and homeostasis and is disrupted with aging. Selenoproteins, comprising essential antioxidant enzymes, raise intriguing questions about their involvement in hematopoietic aging and potential reversibility. Motivated by our observation of mRNA downregulation of key antioxidant selenoproteins in aged human hematopoietic stem cells (HSCs) and previous findings of increased lipid peroxidation in aged hematopoiesis, we employed tRNA^{Sec} gene (*Trsp*) knockout (KO) mouse model to simulate disrupted selenoprotein synthesis. This revealed insights into the protective roles of selenoproteins in preserving HSC stemness and B-lineage maturation, despite negligible effects on myeloid cells. Notably, *Trsp* KO exhibited B lymphocytopenia and reduced HSCs' self-renewal capacity, recapitulating certain aspects of aged phenotypes, along with the upregulation of aging-related genes in both HSCs and pre-B cells. While *Trsp* KO activated an antioxidant response transcription factor NRF2, we delineated a lineage-dependent phenotype driven by lipid peroxidation, which was exacerbated with aging yet ameliorated by ferroptosis inhibitors such as vitamin E. Interestingly, the myeloid genes were ectopically expressed in pre-B cells of *Trsp* KO mice, and KO pro-B/pre-B cells displayed differentiation potential toward functional CD11b⁺ fraction in the transplant model, suggesting that disrupted selenoprotein synthesis induces the potential of B-to-myeloid switch. Given the similarities between the KO model and aged wild-type mice, including ferroptosis vulnerability, impaired HSC self-renewal and B-lineage maturation, and characteristic lineage switch, our findings underscore the critical role of selenoprotein-mediated redox regulation in maintaining balanced hematopoiesis and suggest the preventive potential of selenoproteins against aging-related alterations.

Introduction

Age-related alterations in hematopoiesis manifest as a notable shift towards myeloid cell production, commonly referred to as myeloid skewing, alongside diminished levels of lymphoid cells, anemia, and a reduced regenerative capacity of hematopoietic stem cells (HSCs).¹ Several factors contribute to this decline,^{1,2} including increased CDC42 activity in aged HSCs, which is linked to loss of cellular polarity,³ and reduced mitochondrial function coupled with epigenetic reprogramming.¹ A growing body of evidence underscores age-related alterations in HSCs, particularly concerning their levels of reactive oxygen species (ROS). Functional analyses in mice have revealed an increase in the population of HSCs with high ROS levels (ROS^{high}) alongside a decrease in those with low ROS levels (ROS^{low}) compared to their younger counterparts, where ROS^{high} HSCs exhibit a regenerative disadvantage compared to ROS^{low} HSCs.⁴ Indeed, transplantation of ROS^{high} HSCs into recipients resulted in lymphopenia and myeloid skewing, recapitulating the aging phenotypes observed in hematopoiesis.^{1,4} While these findings manifest the critical involvement of ROS in the aging process of HSCs, the precise mechanisms and extent of ROS regulation in hematopoietic aging remain to be fully elucidated.

ROS are chemically reactive molecules, mainly generated as byproducts of the electron transport chain responsible for mitochondrial respiration.^{5,6} These ROS encompass various types, including superoxide anion (O₂^{•-}), hydrogen peroxide (H₂O₂), and hydroxyl radicals (OH[•]), all of which are rapidly metabolized and can modify and disrupt the function of DNA, proteins, and lipids. Notably, ROS can induce the accumulation of secondary products such as lipid peroxides, consequently triggering ferroptosis, an iron-dependent form of programmed cell death.⁷ Excessive ROS levels can deteriorate oxidative stress, a condition implicated in cellular senescence and age-related diseases, including atherosclerosis, diabetes, and cancer.^{5,6,8,9} Therefore, maintaining a delicate balance between oxidants and antioxidants within cells is imperative for avoiding aging phenotype.

Selenoproteins represent a vital group of antioxidant enzymes, encompassing 25 selenocysteine (Sec)-containing proteins in humans and 24 in mice.^{10,11} Selenium, an essential trace element, in the Sec of selenoproteins is crucial for antioxidant activity.^{10,11} Selenocysteine is encoded by the UGA triplet at mRNA level, which also serves as a stop codon.^{10,11} During translation, Sec is integrated into proteins by a selenocysteine tRNA (tRNA^{Sec}), encoded by the *n-Tutca2* gene, known as *Trsp*, only when the 3' untranslated region (UTR) has a specific stem-loop structure.^{10,11} Examples of selenoproteins include thioredoxin reductase (TXNRD) and glutathione peroxidase (GPX), which reduce the active form of thioredoxin and H₂O₂/lipid peroxides, respectively. Within the GPX family, GPX4 emerges as a key regulator of ferroptosis by mitigating lipid peroxides.¹²⁻¹⁴

Lipid peroxide accumulation in aging human HSCs is well documented,¹⁵ but the role of

selenoprotein-regulated ferroptosis in aging phenotype and its reversibility remains unclear. Motivated by recent reports^{15,16} and our observations of impaired selenoprotein synthesis pathways in aged HSCs, we hypothesized that selenoproteins play a crucial role in maintaining hematopoiesis as part of an antioxidant system that counters age-related alterations. By utilizing *Trsp* knockout (KO) mice as a model to simulate disrupted selenoprotein synthesis, we demonstrated that selenoproteins protect HSCs' stemness and facilitate B lymphocyte maturation. This disruption triggered aging-related gene upregulation and a lineage-dependent shift driven by lipid peroxidation, which worsened with age but was mitigated by ferroptosis inhibitors. Notably, both *Trsp* KO and aged wild-type pro-B/pre-B cells also showed a unique potential for myeloid lineage switching. These findings highlight the crucial role of selenoproteins in regulating redox balance and suggest their potential involvement in sustaining hematopoiesis during aging.

Methods

Animals

All animals were housed at the Foundation for Biomedical Research and Innovation (FBRI, Japan) using a 12-hour light/12-hour dark cycle, at ambient temperature of $21.5^{\circ}\text{C} \pm 1^{\circ}\text{C}$ and 30-70% humidity. All animal procedures were followed by the Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committees at FBRI (21-11, 24-08). The *Mx1-Cre* and *R26-CreERT2* strains were obtained from The Jackson Laboratory. *Trsp* floxed mouse strain (RBRC02681) was provided by RIKEN BRC through the National BioResource Project of the MEXT/AMED, Japan. C57BL/6 mice were obtained from Japan SLC.

Data Availability Statement

RNA sequencing data has been uploaded to GEO under Accession ID GSE267189 and GSE267190.

Additional methods

All additional methods are provided in supplemental text and figures.

Results

Lineage- and age-dependent effects of *Trsp* KO hematopoiesis.

Accumulation of ROS stands out as hallmarks of aged hematopoiesis.¹ We observed downregulation of mRNA for key antioxidant selenoproteins in aged human HSCs compared to their younger counterparts (supplemental Figure 1A-B),¹⁷ consistent with previous findings reporting decreased GPX activity and increased lipid peroxidation in peripheral blood mononuclear cells from elderly individuals.^{15,16} We further demonstrated that aging-related DNA damage, induced by treating human cord blood CD34⁺ cells with etoposide,^{3,18-24} resulted in reduced GPX1/2 and GPX4 protein expression and increased lipid peroxides, measured using C11-BODIPY 581/591 (supplemental Figure 1C-D). These results motivated us to examine previously unrecognized roles of selenoproteins in maintaining youthful hematopoiesis. To test this idea, we employed a mouse model enabling time-dependent deletion of the tRNA^{Sec} gene using Cre-loxP system in *Trsp*^{fl/fl}: *Mx1*-Cre mice (Figure 1A-B).^{25,26} Six weeks post-polyinosinic-polycytidylic acid (pl-pC) treatment, *Trsp*^{fl/fl}: *Mx1*-Cre mice exhibited the expected DNA recombination (supplemental Figure 2A) and nearly undetectable tRNA^{Sec} levels across both mature and immature bone marrow (BM) fractions (supplemental Figure 2B). As anticipated, *Trsp* KO mice displayed decreased levels of selenoprotein, such as SEPHS2, GPX1/2, and GPX4, both in the BM and spleen (Figure 1C). This finding was corroborated by mass spectrometry analysis of *Trsp* KO mouse BM cells (supplemental Figure 2C). Staining for 4-hydroxynonenal (HNEJ-1) revealed an accumulation of advanced lipid peroxidation end products across lineages (supplemental Figure 2D-E). Phenotypic analysis unveiled that *Trsp* KO mice developed macrocytic anemia (supplemental Figure 2F-G) with erythrocyte degradation and the presence of erythrocytes with nuclear remnants in the peripheral blood (PB) (supplemental Figure 2G), as well as B lymphocytopenia and splenomegaly filled with erythroblasts and expansion of red pulp (supplemental Figure 2H-I; Figure 1D), consistent with prior reports.²⁶ While the phenotypic changes of heterozygous KO were subtle (supplemental Figure 2J-K), the erythroid and B lineage findings observed in homozygous KO mice were replicated by *Trsp*^{fl/fl}: *R26*-CreERT2 mice upon tamoxifen treatment (supplemental Figure 3A-E), reinforcing the direct impact of *Trsp* KO on the hematopoietic system.

Given that lymphocytopenia and anemia are prominent features in age-related hematopoiesis, we sought to elucidate how aging interacts with *Trsp* KO to manifest these phenotypes. Intriguingly, we observed an accelerated decline in B cell number, subsequently inducing relative myeloid skewing in aged *Trsp* KO mice (Figure 1E; supplemental Figure 4A). We hypothesized that this phenotype could be attributed to the accumulation of ROS in *Trsp* KO B cells. To investigate this, we stained BM B cells with C11-BODIPY 581/591 and CellROX to assess lipid peroxidation and cytoplasmic ROS, respectively. While there was no discernible

difference in ROS accumulation between B and myeloid cells of young control and KO mice (supplemental Figure 4B), aged KO mice exhibited increased accumulation of lipid peroxide and cytoplasmic ROS specifically in B lymphocytes (Figure 1F). On the other hand, myeloid cells showed only cytoplasmic ROS accumulation (Figure 1F).

To ascertain whether the observed phenotypes in *Trsp* KO mice occur in a cell-autonomous manner, we conducted BM transplantation experiments using CD45.2⁺ control *Trsp*^{fl/fl} and *Trsp*^{fl/fl}; *Mx1*-Cre donor cells into lethally irradiated CD45.1⁺ recipient mice (Figure 1G). This approach allowed us to assess the hematopoietic reconstitution capacity of transplanted HSCs. Consistent with the findings in primary mice, three months post-pl-pC treatment, the *Trsp* KO group exhibited B cell reduction and anemia compared to the control group (Figure 1H-I; supplemental Figure 4C-D). Notably, despite the profound B cell phenotypes, *Trsp* KO did not lead to BM failure or shorten the survival (supplemental Figure 4E). These results underscored the lineage-specific effects of *Trsp* KO on hematopoiesis.

***Trsp* KO modulates HSCs' state and elicits NRF2 activation across HSPCs.**

Given the perturbation of mature hematopoietic cells observed in *Trsp* KO, we aimed to investigate whether these effects originate from hematopoietic stem and progenitor cells (HSPCs). We first assessed various HSPC compartments in *Trsp* KO mice, including long-term HSCs (LT-HSCs), short-term HSCs (ST-HSCs), multipotent progenitors (MPP2, MPP3, and MPP4), and common lymphoid progenitors (CLPs). Interestingly, *Trsp* KO led to an increase in ST-HSCs without significant changes in LT-HSCs, MPPs, or progenitors (Figure 2A). Furthermore, cell cycle analysis revealed that *Trsp* KO resulted in a less quiescent state of LT-HSCs (Figure 2B; supplemental Figure 5A), suggesting that the enhanced cell cycle activity in LT-HSCs leads to increase of ST-HSCs (Figure 2A).

To investigate the molecular mechanisms underlying *Trsp* KO effects on HSPCs, we next performed RNA-seq analysis of FACS-purified Lin⁻c-Kit⁺ BM cells from *Trsp*^{fl/fl} and *Trsp*^{fl/fl}; *Mx1*-Cre mice at ten weeks post-pl-pC injection in biological quadruplicate for each model. Transcriptomes of Lin⁻c-Kit⁺ cells exhibited substantial differences, as evident from principal component analysis (PCA) (supplemental Figure 5B; Figure 2C). Differentially expressed genes (DEGs) included a series of downregulated selenoproteins such as *Gpx1*, *Gpx4*, *Selh*, *Selt*, *Sepw1*, or *Sepp1*. This observation can be attributed to the phenomenon of nonsense-mediated mRNA decay (NMD), where UGA-coding Sec serves as a premature stop codon upon the absence of *Trsp*.²⁷

In contrast, DEGs upregulated in *Trsp* KO encompassed pathways related to nuclear factor erythroid 2-related factor 2 (NRF2) (Figure 2C), which are involved in ROS scavenging and detoxification.²⁸ Notable examples included *Sc17a11*, *Nqo1*, *Gclm*, and *Gsta3*. Gene ontology and gene set enrichment analysis (GSEA) of *Trsp* KO versus control RNA-seq results revealed positive enrichment not only for aged HSC characteristics^{29,30} but also for NRF2

pathway (Figure 2D), a finding consistent with observations in selenoprotein-deficient macrophages, liver, and BM cells.^{25,26} To further decipher how *Trsp* loss affects early lineage commitment of HSCs to MPPs, we conducted single-cell RNA-seq (scRNA-seq) targeting 10,000 BM Lin⁻c-Kit⁺ cells each from control and *Trsp* KO primary mice at nine weeks post-deletion. To ensure data quality, we excluded cells with elevated mitochondrial gene expression, as this is indicative of cell death.^{31,32} Uniform manifold approximation and projection (UMAP) analysis integrating both models revealed the expected stem and progenitor clusters, including LT-HSC, MPP, and erythroid, myeloid, and lymphoid progenitors (Figure 2E).³³⁻³⁷ Selenoproteins exhibited uniform downregulation across all HSPC fractions, indicating consistent involvement of the NMD pathway in modulating selenoprotein mRNA levels regardless of lineage variation (Figure 2F). Conversely, NRF2 target genes displayed upregulation across all *Trsp* KO HSPC fractions (supplemental Figure 5C). These findings suggest that in the absence of selenoproteins, NRF2 compensates for impaired ROS scavenging in HSPCs, and the behavior of NRF2 alone cannot entirely account for the lineage-specific phenotype observed in the *Trsp* KO model.

Selenoproteins are required for the maintenance of HSC function.

While detailed examination of primary *Trsp* KO mice revealed altered HSC states and lineage-specific effects, analyzing primary models alone cannot fully evaluate the intrinsic effects of *Trsp*-depleted HSCs compared to normal HSCs. To address this, we conducted an *in vivo* self-renewal assessment of control *Trsp*^{fl/fl} and *Trsp*^{fl/fl}; *Mx1*-Cre mice model in a competitive transplantation experiment. Equal numbers of CD45.2⁺ BM cells from each model were mixed with CD45.1⁺ wild-type BM cells and injected into lethally irradiated CD45.1⁺ recipient mice. Following DNA recombination induced by pl-pC injection, we monitored donor- and competitor-derived chimerism in PB monthly and euthanized all recipients five months later to assess the chimerism in BM (Figure 3A). While *Trsp* KO resulted in a significant disadvantage in PB chimerism, we observed relatively comparable chimerism in BM HSPCs six months post-transplantation (Figure 3B; supplemental Figure 5D). Notably, the competitive disadvantage in the PB and BM was primarily attributed to the near-complete loss of *Trsp* KO cells in B220⁺ and CD3⁺ fractions (Figure 3C; supplemental Figure 5D). To evaluate the long-term reconstitution ability, we also performed a serial transplant assay using the whole BM cells from the first recipients. Remarkably, we observed a profound loss of donor-derived chimerism in all lineages of mature and HSPC fractions (Figure 3D), indicating that *Trsp* KO HSCs exhibited impaired long-term self-renewal and reconstitution ability compared to control HSCs. These results underscore the critical roles of selenoproteins in HSC maintenance *in vivo*. Given that transplant stress can raise ROS levels,³⁸ we examined whether transplantation-induced ROS contributes to the impaired function of *Trsp* KO HSCs in a non-competitive transplant experiment (supplemental Figure 5E). While the control group

accumulated ROS post-transplant, the KO group did not, indicating the impairments are not due to transplant-induced ROS (supplemental Figure 5F).

***Trsp* deficient HSPCs are subject to ferroptosis.**

To examine the self-renewal and proliferation capacity of *Trsp* KO BM cells *in vitro*, we conducted a colony formation assay using whole BM cells from control and *Trsp* KO mice. Despite previous data indicating no decrease in the frequency of HSPCs in *Trsp* KO mice (Figure 2A), we observed that *Trsp* KO completely blocked colony formation capacity in cytokine-supplemented methylcellulose media, including myeloid, erythroid, and pre-B conditions (Figure 4A). To investigate the mechanism underlying cell death in *Trsp* KO BM cells, we targeted various regulatory factors of programmed cell death pathways in MACS-sorted Lin⁻ BM HSPCs (Figure 4B). Baseline viability of HSPCs from *Trsp* KO mice significantly decreased, accompanied by lipid peroxide accumulation during *ex vivo* culture (Figure 4C-E). Using the ferroptosis inhibitor ferrostatin-1 (Fer-1) or α -tocopherol (α -Toc), a prominent component of vitamin E (Vit E), whose concentrations effectively rescued cell death caused by RSL3 (GPX4 inhibitor) in control Lin⁻ cells, we observed significant rescue in KO Lin⁻ cells with Fer-1 or α -Toc (Figure 4E; supplemental Figure 6A-B). Fer-1 also markedly inhibited lipid peroxide accumulation of *Trsp* KO HSPCs (Figure 4C). However, apoptosis (zVAD) or necroptosis (Nec-1s) inhibitors did not prevent cell death in KO HSPCs (Figure 4E; supplemental Figure 6A-B). Intriguingly, aged HSPCs exhibited modestly higher sensitivity to RSL3 compared to young HSPCs (supplemental Figure 6C). These findings suggest ferroptosis is the key cell death pathway in KO HSPCs, further supported by the rescue of colony formation by Fer-1 and α -Toc (Figure 4F). These align with recent reports indicating that GPX4, one of the selenoproteins, serves as a main regulator of ferroptosis,¹² and that *Gpx4* deficiency induced ferroptosis in HSPCs *in vitro*.³⁹

Dysregulation of redox control by *Trsp* KO disrupts B cell differentiation and transcriptional program.

Despite the observed B-cell reduction in *Trsp* KO mice, CLP frequencies remained comparable to controls (Figure 2A), and DEGs specific to MPP4 were not identified. Consequently, we aimed to pinpoint the stage of blocked B-cell maturation. B progenitors differentiate sequentially from prepro-B, pro-B, pre-B, and immature B cells.⁴⁰ Notably, in *Trsp* KO mice, pre-B and immature B cells are significantly reduced, whereas prepro-B stages were comparable to control mice in BM and spleen (Figure 5A; supplemental Figure 7A). *Trsp* KO pre-B cells exhibited impaired IgH V(D)J and IgI Vk-Jk rearrangement, indicating the functional impairment at the pre-B cells stage (Figure 5B, supplemental Figure 7B). However, CD19⁺ cells from *Trsp* KO spleens showed normal proliferation and IgM secretion upon LPS/IL-4 stimulation,⁴¹ suggesting functional integrity under stimulation (supplemental Figure 7C-E).

Importantly, aged *Trsp* KO mice exhibited further pre-B cell reduction and lipid peroxide accumulation (Figure 5C-D; supplemental Figure 7F-G), highlighting the detrimental impact of lipid peroxidation on B-cell differentiation. Similarly, aged wild-type mice accumulated lipid peroxides, particularly in the spleen (supplemental Figure 7H). These findings suggest overlapping lipid peroxidation-induced B-cell phenotypes in *Trsp* KO and aged mice.

To elucidate how *Trsp* KO dysregulates B cell transcriptional program, we next performed RNA-seq analysis of FACS-purified BM prepro-B cells and pre-B cells from control and *Trsp* KO mice, with biological quadruplicate for each model. We observed downregulation of pathways involved in lymphocyte differentiation and B-cell receptor (BCR) signaling in the *Trsp* KO group, particularly pronounced in pre-B cells (Figure 5E; supplemental Figure 8A). For example, Igk germline transcription and Igk rearrangement are regulated by the concerted action of multiple transcription factors at the E κ enhancers, including *Tcf3*, *Pax5*, and *Irf4*, whose downregulation was validated in qRT-PCR (supplemental Figure 8B).^{40,42} In contrast, neutrophil-related genes were ectopically upregulated in *Trsp* KO group, especially in pre-B cells (Figure 5E; supplemental Figure 8A), which are supported by GSEA showing significant upregulation of myeloid transcription factor (TF) CEBPA in pre-B cells (Figure 5F). However, scRNA-seq revealed no alterations in myeloid or lymphoid gene expression within the MPP4/CLP clusters (supplemental Figure 8C). GSEA also demonstrated a positive enrichment of aging HSC characteristics and the NRF2 pathway in pre-B cells (Figure 5F; supplemental Figure 8D).

Direct involvement of selenoproteins in B lineage maturation *in vitro* and *in vivo*.

To determine whether the impaired maturation of B lymphocytes is a direct consequence of *Trsp* KO, we performed *in vitro* differentiation assay (Figure 5G-H; supplemental Figure 8E). BM LSK (Lin⁻c-Kit⁺Sca1⁺) cells of *Trsp*^{fl/fl} and *Trsp*^{fl/fl}; R26-CreERT2 mice were cultured on OP9 stromal cells with IL-7 and FLT3-L. On day 5, 4-hydroxytamoxifen (4-OHT) was added for 24 hours to delete the *Trsp* gene (supplemental Figure 8F). The cells were harvested on day 20 and analyzed by flow cytometry (Figure 5H; supplemental Figure 8E). *Trsp* KO LSK cells showed reduced B lineage differentiation but not myeloid differentiation compared to controls (Figure 5H; supplemental Figure 8E), highlighting the critical role of selenoproteins in early B cell development.

To investigate the differential potential of *Trsp* KO B progenitors *in vivo*, we performed transplantation of CD3⁺CD11b⁺Gr-1⁻Ter-119⁻IgM⁻CD19⁺ (pro-B and pre-B) cells from CD45.2⁺ control or *Trsp* KO model, along with CD45.1⁺ wild-type whole BM cells, into lethally irradiated CD45.1⁺ recipients (Figure 5I). Subsequently, we analyzed the cellular composition of CD45.2⁺ cells in the BM of recipients. Our analysis revealed a significant decrease in CD45.2⁺ donor-derived cells in the *Trsp* KO group, indicating reduced viability of B progenitors from *Trsp* KO mice *in vivo* (Figure 5J; supplemental Figure 8G). Notably, *Trsp* KO pro-B/pre-B cells

demonstrated a significant potential to generate CD11b⁺ myeloid cells in the recipient BM and PB (Figure 5J-K), consistent with the upregulation of myeloid-related genes observed in RNA-seq (Figure 5E-F, supplemental Figure 8A). These CD11b⁺ cells derived from *Trsp* KO pro-B/pre-B cells displayed morphology comparable to those from wild-type mice (supplemental Figure 8H). mRNA expression analysis of *Trsp* KO pro-B/pre-B-derived CD11b⁺ cells manifested downregulation of genes involved in lymphocyte differentiation and BCR signaling pathways, alongside reciprocal upregulation of myeloid genes, to a level similar to that of wild-type CD11b⁺ cells (supplemental Figure 8I). A flow cytometry-based neutrophil extracellular traps (NETs) assay further demonstrated that *Trsp* KO pro-B/pre-B-derived CD11b⁺ cells formed NETs, indicated by MPO⁺ Histone H3⁺ staining,⁴³ suggesting functional similarity to wild-type CD11b⁺ cells (supplemental Figure 8J). These findings highlight the requirement of selenoproteins for B cell maturation and highlight how their deficiency can disturb the normal differentiation program, leading to a B to myeloid lineage switch.

Next, we conducted a similar transplant with pro-B/pre-B cells of young or aged wild-type mice to assess the differentiation potential as observed in *Trsp* KO mice (supplemental Figure 9A). Our analysis revealed a significant reduction in CD45.2⁺B220⁺ cells in the aged group (supplemental Figure 9B-C), indicating impaired engraftment or proliferation of aged pro-B/pre-B progenitors. Interestingly, aged pro-B/pre-B cells showed the potential to generate CD11b⁺ myeloid cells in the recipient BM (supplemental Figure 9B), similar to *Trsp* KO B progenitors. The phenotypes of these uniquely generated CD11b⁺ cells, including morphology, gene expression, and NETs formation, recapitulated those of *Trsp* KO-derived cells (supplemental Figure 9D-F). These findings highlight shared characteristics between physiological aging and *Trsp* KO in B progenitors, further underscoring the functional connection between the two conditions.

Ferroptosis modulation shapes B cell phenotype in *Trsp* KO model.

Given the rescue of *Trsp* KO HSPCs survival by α -Toc (Figure 4E; supplemental Figure 6A), we postulated that dietary Vit E plays a critical role in protecting *Trsp*-depleted hematopoiesis from ferroptosis. To test this hypothesis, mice were fed with a fixed formulation diet containing either 75 mg/kg of Vit E (control diet) or an undetectable amount of Vit E (Vit E-deficient diet) (Figure 6A). Remarkably, we observed an exacerbation of phenotypes in the B, erythroid, and myeloid lineages in the BM of *Trsp* KO mice fed with the Vit E-deficient diet for six weeks, while LSK and LK (Lin⁻c-Kit⁺Sca1⁻) cells were largely unaffected (Figure 6B; supplemental Figure 10A-B). B lymphocytes and B progenitors in the BM exhibited additional accumulation of lipid peroxide and cytoplasmic ROS three weeks after initiation of Vit E-deficient diet, suggesting a cooperative induction of ferroptosis in the B lineage due to deficiency in both selenoproteins and Vit E (Figure 6C-D).

Next, we fed the mice with Vit E-rich diet (500 mg/kg) to clarify whether anti-ferroptotic

effects of Vit E unlock the impaired B cell differentiation of *Trsp* KO mice (Figure 6E). Indeed, Vit E-rich diet administered for six weeks partially rescued the frequency of B220⁺ B cells in *Trsp* KO mice (Figure 6F; supplemental Figure 10C). These results indicate that ferroptosis occurring in the B cells of *Trsp* KO mice serves as the underlying cause of differentiation disorders. Notably, the eight-week administration of a Vit E-rich diet reduced the cell-cycling state of *Trsp* KO LT-HSCs shown in Figure 2B (supplemental Figure 10D-E), consistent with the rescue effect of Vit E (α -Toc) on HSPC ferroptotic cell death induced by RSL3 *in vitro* (supplemental Figure 6B). Together, these findings suggest that the phenotypes observed in *Trsp* KO mice are primarily attributable to ferroptosis and that dietary intervention can modulate the ferroptosis-induced phenotype both *in vitro* and *in vivo*.

Discussion

While the functions of selenoproteins in hematopoiesis have been documented in several contexts,^{26,44} the specific roles they play in HSC stemness and B lymphocyte maturation have remained elusive. Our study uncovers a protective role of selenoproteins in maintaining HSC stemness and highlights their critical involvement in B lymphocyte maturation (supplemental Figure 11A). Disruption of selenoprotein synthesis results in intrinsic impairment of B-lineage maturation, both *in vivo* and *in vitro* settings. Furthermore, B lymphocytes exhibit increased susceptibility to ferroptosis, particularly with aging. Although the *Trsp* KO model presents non-physiological conditions that may amplify this phenotype, our data and others^{15,16} suggest a link between physiological aging and selenoprotein-related traits in impaired HSC self-renewal (Figure 3B-D; supplemental Figure 5D) and B-lineage maturation (Figure 1D; Figure 5A), altered gene expression profiles (Figure 2D; Figure 5F; supplemental Figure 8D), and a unique potential for B-to-myeloid lineage switching (Figure 5J-K; supplemental Figure 9B-C). As age-related declines in selenoprotein expression have been noted in various cell types, including human vascular smooth muscle cells and rat hepatocytes,^{45,46} further studies are expected to fairly evaluate the reduction in selenoproteins and subsequent lipid peroxidation in aged human HSCs or HSCs from patients with age-related blood disorders.

Trsp KO mouse, serving as a model of selenoprotein synthesis failure, exhibited notable phenotypes in erythroid and B lymphocytes, while the myeloid lineage remained largely unaffected. The erythroid phenotypes align with previous findings,^{26,44} showing that selenoproteins modulate stress erythroid progenitors and the splenic microenvironment during stress-induced erythropoiesis.⁴⁴ The erythroid phenotype in *Trsp* KO mice bears resemblance to that of the *Gpx4* KO model, highlighting the importance of GPX4 in erythropoiesis.^{47,48} However, unlike *Gpx4* KO mice, which showed no impairment in B cell maturation in the BM,^{39,49} our experiments consistently demonstrated impaired B cell maturation in *Trsp* KO model (Figure 5A; supplemental Figure 7A). Notably, this B cell phenotype was accompanied by lipid peroxide accumulation, exacerbated upon aging (Figure 1E-F; Figure 5C-D) or Vit E deficiency (Figure 6B-D). While GPX4 is known to reduce lipid peroxides, other selenoproteins, such as TXNRD1, likely contribute to protecting B cells by removing hydrogen peroxide via the peroxiredoxin/thioredoxin system, implicated in ferroptosis suppression.⁵⁰ Myeloid cells did not accumulate lipid peroxides with age or Vit E deficiency, suggesting reliance on alternative antioxidant systems or inherent resistance to lipid peroxidation.

One of the most intriguing phenomena observed in *Trsp* KO mice is the lineage switch from pro-B/pre-B cells to myeloid cells, a trait also shared with aged wild-type pro-B/pre-B cells. As shown in supplemental Figure 8A, RNA-seq data from the *Trsp* KO prepro-B stage revealed modest changes in TFs involved in B and myeloid differentiation, with more pronounced alterations at the pre-B stage (Figure 5E). However, scRNA-seq did not reveal

changes in the expression of myeloid or lymphoid lineage-specific genes within the MPP4/CLP clusters (supplemental Figure 8C), suggesting the switch occurs after the prepro-B stage (supplemental Figure 8A). Notably, CEBPA overexpression in B cells has been shown to drive reprogramming into myeloid cells.⁵¹ The significant upregulation of *Cebpa* in *Trsp* KO pro-B/pre-B cells may underlie this switch. Given the reported effects of oxidative stress on chromatin structure,^{52,53} future studies should investigate whether ROS or lipid peroxides influence lineage-determining TF expression and chromatin states.

Although *Trsp* KO HSPCs displayed cell death *in vitro* (Figure 4D-E), the survival of mice with hematopoietic cell-specific *Trsp* KO was not shortened (supplemental Figure 4E), suggesting protective mechanisms within the BM microenvironment. Factors such as the hypoxic and nutrient-rich environment, cellular density, and support from BM niche cells may contribute to this protection. In our experimental model, Vit E supplementation at least partially ameliorated the phenotype of *Trsp* KO HSPCs (Figure 4E; supplemental Figure 6A) and B cells (Figure 6F), highlighting the protective roles of Vit E within the BM, consistent with reports of Vit E supporting *Gpx4* KO HSPC survival *in vitro*.^{39,48,54} Additionally, high cell density has been shown to increase resistance to ferroptosis induced by cysteine deprivation and GPX4 inhibition.⁵⁵ Cell-cell contact through the NF2-Hippo-YAP pathway also confers ferroptosis resistance in epithelial cells.⁵⁶ Considering the unique partnership between HSCs and mesenchymal stem cells (MSCs) in maintaining healthy hematopoiesis,^{57,58} it is plausible that MSCs help shield HSCs from ferroptosis.

The disruption in B lymphocyte differentiation in *Trsp* KO mice accelerated with aging. RNA-seq analysis revealed upregulation of NRF2 target genes in HSPCs (Figure 2C-D), B progenitors (supplemental Figure 8D), and myeloid cells of *Trsp* KO mice. It has been documented that as aging progresses, NRF2 activation becomes less efficient due to a decline in the oxidative stress response.⁵⁹ Additionally, positive regulators of NRF2 have been shown to decrease with aging in specific contexts.⁶⁰⁻⁶⁴ This reduction in compensatory mechanisms by NRF2 may explain the accelerated aging phenotype of *Trsp* KO mice. Furthermore, numerous cellular and structural changes occur in the BM microenvironment with aging,^{1,65} which may contribute to loss of compensatory mechanisms and progression of phenotypes. Future studies aimed at elucidating these aging-related alterations, alongside defects in selenoprotein synthesis, that drive hematopoietic aging phenotypes are crucial for understanding the underlying mechanisms of aging-related diseases.

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Authorship

Contribution: Y.A., H.Y., K.N., A.T.-K., and D.I. designed the study; T.Shigehiro and T.I. supervised PCR analysis of IgH V(D)J and IgL Vk-Jk rearrangements; B cell differentiation experiments and B progenitor transplantation experiments; Y.A., Y.Kong, and S.T. performed Immunohistochemical staining; Y.A., Y.T., and K.I. performed label-free proteome analysis; Y.A., H.Y., Y.H., Y.M, and A.Toyoda performed scRNA-seq; Y.A., W.Z., and M.N. performed computational analyses of RNA-seq data; Y.A., H.Y., T.Suzuki, W.Z., H.I, Y.Koike, M.F., A.Tanaka, Y.Z., W.S., C.H., S.K., K.I., and M.Y. performed animal experiments. Y.A., H.Y., D.I., M.N., and Y.T. wrote the manuscript with approval from all co-authors.

Disclosure of Conflicts of Interest

The authors declare no competing financial interests.

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Figure Legends

Figure 1. Lineage- and age-dependent effects of *Trsp* KO hematopoiesis.

- (A) Schematic representation of the pathways involved in selenocysteine (Sec) incorporation into selenoproteins. This process involves a multiprotein complex that includes tRNA selenocysteine 1 associated protein 1 (TRNAU1AP), eukaryotic elongation factor (EEFSEC), and Selenocysteine tRNA (tRNA^{Sec}) isoform by SECIS binding protein 2 (SECISBP2). SECISBP2 interacts with the SECIS stem-loop element located in the 3' UTR of mammalian selenoprotein mRNAs, enabling the decoding UGA Sec codons at the ribosomal acceptor site to mediate Sec incorporation into nascent polypeptide.
- (B) Utilization of a mouse model allowing time-dependent deletion of the tRNA^{Sec} gene (*Trsp*) through the Cre-loxP system in *Trsp*^{fl/fl}: *Mx1*-Cre mice. *Trsp* gene is flanked by loxP sequences, shown by arrowheads. *Trsp* excision was induced by intraperitoneal injection (i.p.) of polyinosinic-polycytidylic acid (pl-pC) every other day for 3 times. Control mice were pl-pC-treated *Trsp*^{fl/fl} mice, while *Trsp* knockout (KO) mice represent *Trsp*^{fl/fl}: *Mx1*-Cre mice.
- (C) Western blot analysis for SEPHS2, GPX1/2, GPX4 in whole BM cells and spleen cells derived from Control mice and *Trsp* KO mice. Filled triangles indicate target protein bands, while open triangles represent non-specific bands.
- (D) Absolute number and frequency of CD71⁺Ter-119⁺, CD3⁺, B220⁺, CD11b⁺Gr-1⁺, CD11b⁺Gr-1⁻ cells in the BM are shown. Frequencies of CD71⁺Ter-119⁺, CD3⁺, B220⁺, CD11b⁺Gr-1⁺, CD11b⁺Gr-1⁻ cells, peripheral blood (PB), and spleen of each group are shown. *n* = 5 per group.
- (E) Frequency of B220⁺ and CD11b⁺Gr-1⁺ cells in the BM of each group (young: < 20 weeks of age, aged: 81 weeks of age). *n* = 5 per each group.
- (F) Representative histograms and mean fluorescence intensity (MFI) of C11-BODIPY 581/591 and CellROX staining in B220⁺ or CD11b⁺ BM cells of aged control and *Trsp* KO mice. *n* = 5 per group, 81 weeks of age.
- (G) Schematic representation of non-competitive BM transplantation assays. M: million.
- (H) Counts of white blood cells (WBCs) in whole blood from recipient mice at 3 months post-transplantation. Control group: 6 mice, *Trsp* KO group: 8 mice.
- (I) Frequency of CD45.2⁺ in the PB and of B220⁺ and CD11b⁺Gr-1⁺ cells in CD45.2⁺ cells of recipient mice at 3 months post-transplantation. Control group: 6 mice, *Trsp* KO group: 8 mice.

P-values were calculated by a two-sided Student's *t*-test **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001.

Figure 2. *Trsp* KO modulates HSCs' state and elicits NRF2 Activation across HSPCs.

- (A) Frequency of LSK (Lin⁻c-Kit⁺Sca1⁺), LT-HSCs (CD48⁻CD150⁺ LSK) and ST-HSCs (CD48⁻CD150⁻ LSK), megakaryocytic and erythroid-biased MPP2 (CD135⁻CD150⁺CD48⁺ LSK), myeloid-biased MPP3 (CD135⁻CD150⁻CD48⁺ LSK), lymphoid-biased MPP4 (CD135⁺ LSK), LK (Lin⁻c-Kit⁺Sca1⁻), common myeloid progenitors (CMPs, CD34⁺FcyR⁻ LK), granulocyte-monocyte progenitors (GMPs, CD34⁺FcyR⁺ LK), megakaryocyte-erythrocyte progenitors (MEPs, CD34⁻FcyR⁻ LK), CLPs (Lin⁻c-Kit^{int}Sca1^{int}CD127⁺CD135⁺) in the BM of each group. LSK, LT-HSCs, ST-HSCs, LK, CMPs, GMPs, MEPs: *n* = 5 per group. MPP2, MPP3, MPP4, CLP: *n* = 3 per group. *P*-values were calculated by a two-sided Student's *t*-test.
- (B) Representative FACS plots and percentages of bromodeoxyuridine (BrdU)⁺ (S phase), DAPI⁺ (4N) BrdU⁻ (G2/M phase), and DAPI⁺ (2N) BrdU⁻ (G0/G1 phase) LT-HSCs in the

BM. Control group: 4 mice, *Trsp* KO group: 3 mice. *P*-values were calculated by a two-sided Student's *t*-test.

- (C) Volcano plot illustrating differential mRNA expression in *Trsp* KO Lin⁺Kit⁺ cells, with selenoproteins highlighted in blue and NRF2 pathway genes highlighted in red. Control mice were pl-pC-treated *Trsp*^{fl/fl} mice, while *Trsp* knockout (KO) mice represent *Trsp*^{fl/fl}; *Mx1*-Cre mice. *n* = 4 per group.
- (D) Gene set enrichment analysis (GSEA) enrichment plot for upregulated and downregulated genes in RNA-seq of *Trsp* KO vs. Control in Lin⁺Kit⁺ cells. NES: normalized enrichment score, FDR: false discovery rate. *n* = 4 per group.
- (E) Identification of distinct hematopoietic clusters in Control and KO Lin⁺Kit⁺ cells base on uniform manifold approximation and projection (UMAP) analysis from single-cell RNA sequencing (scRNA-seq). The estimated fractions of stem, progenitor, and mature cells are labeled and highlighted. *n* = 1 per group.
- (F) Violin plots depicting mRNA expression of selenoproteins (*Gpx1*, *Selh*, *Sepw1*) in the indicated clusters (HSC, MPP2, MPP3, MPP4). Box plot and kernel density plot of log₂ expression values are shown. *P*-values were reanalyzed using Loupe Browser. In the box-and-whisker plots, the 0th, 25th, 50th, 75th, and 100th percentiles and mean (dashed lines) are shown.

P* < 0.05, *P* < 0.01, ****P* < 0.001, and *****P* < 0.0001.

Figure 3. Selenoproteins are required for the maintenance of HSC function.

- (A) Schematic representation of competitive BM transplantation assays.
- (B) Percentage of donor-derived (CD45.2⁺) cells in total live cells in PB in primary and serial competitive transplantation. Control group: 7 mice, *Trsp* KO group: 8 mice.
- (C) Percentage of donor-derived (CD45.2⁺) cells in each indicated population in PB in primary and serial competitive transplantation. Control group: 7 mice, *Trsp* KO group: 8 mice.
- (D) The percentage of donor derived cells in each indicated population in the BM following 4 months of serial competitive transplantation. Control group: 4 mice, *Trsp* KO group: 7 mice.

P-values were calculated by a two-sided Student's *t*-test **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001.

Figure 4. *Trsp* deficient HSPCs are subject to ferroptosis.

- (A) Representative pictures showing colony formation of whole BM cells in methylcellulose using M3434 for myeloid colonies. Colonies were scored 7 days after plating in methylcellulose in M3434, M3436, and M3630, for myeloid, erythroid, and pre-B colonies, respectively. *n* = 3 independent experiments.
- (B) Schematic representation of programmed cell death pathways. Ferroptosis (left) is triggered by iron-dependent accumulation of lethal ROS and lipid peroxides. Ferroptosis can be inhibited by iron (Fe²⁺) chelators such as deferoxamine (DFO). α-tocopherol (α-Toc) and ferrostatin-1 (Fer-1) inhibit ferroptosis by suppressing lipid peroxidation. Apoptosis and necroptosis (right) are primarily regulated via TNFR1 signaling. Upon TNF binding, TNFR1 undergoes a conformational change, activating two potential cell death execution mechanisms. Caspase-8 triggers apoptosis by activating the classical caspase cascade and inactivating RIP1 and RIP3 through cleavage. As an alternative pathway, phosphorylated RIP1 and RIP3 initiate necroptosis independently of caspase-8. zVAD inhibit apoptosis by inhibiting caspase-8, while Necrostatin-1s (Nec-1s) inhibits necroptosis by inhibiting RIP1.
- (C) Relative MFI of C11-BODIPY 581/591 staining in Lin⁺ cells from Control mice and *Trsp* KO

mice 24 hours after culturing in the presence of Fer-1 (1 μ M). $n = 5$ independent experiments.

- (D) Frequency of DAPI⁺ cells in Lin⁻ cells measured 48 hours following treatment with Fer-1. $n = 5$ independent experiments.
- (E) Heatmap illustrating relative cell number of viable Lin⁻ cells compared to day 0, as determined by the CellTiter-Glo Luminescent Cell Viability Assay, 48 hours after culturing in the presence of Fer-1 (1-5 μ M), α -Toc (1-2 mM), zVAD (25-50 μ M), and Nec-1s (50-100 μ M). $n = 3$ per group.
- (F) Colonies were scored 7 days after plating in methylcellulose (MethoCult M3434) in the presence of Fer-1 (5 μ M) and α -Toc (2 mM). $n = 3$ per group.

P -values were calculated by a two-sided Student's t -test or one-way ANOVA test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

Figure 5. Dysregulation of redox control by *Trsp* KO disrupt B cell differentiation and transcriptional program.

- (A) Frequency of prepro-B (CD3⁻CD11b⁻Gr-1⁻Ter-119⁻IgM⁻CD19⁻CD43⁺), pro-B (CD3⁻CD11b⁻Gr-1⁻Ter-119⁻IgM⁻CD19⁺CD43⁺), pre-B (CD3⁻CD11b⁻Gr-1⁻Ter-119⁻IgM⁻CD19⁺CD43⁻), and immature B (CD3⁻CD11b⁻Gr-1⁻Ter-119⁻IgM⁺CD19⁺CD43⁻) cells in the BM of Control and *Trsp* KO mice. $n = 3$ per group. P -values were calculated by a two-sided Student's t -test.
- (B) Representative image of PCR amplification. pre-B cells were isolated from BM by cell sorting and Igh V(D)J gene rearrangement was determined by PCR amplification with *Gapdh* as a loading control. $n = 2$ per group.
- (C) Frequency of pre-B cells in the BM of each group categorized by age (young: < 20 weeks of age, aged: 81 weeks of age). $n = 3$ mice per young group, $n = 5$ mice per aged group. P -values were calculated by a two-sided Student's t -test.
- (D) MFI of C11-BODIPY 581/591 staining in pre-B, and immature B cells in the BM cells of aged control mice and aged *Trsp* KO mice. $n = 5$ mice per group, 81 weeks of age. P -values were calculated by a two-sided Student's t -test.
- (E) Heatmap showing genes that regulate neutrophil and B cell differentiation evaluated by RNA-seq in *Trsp* KO BM pre-B cells. The listed genes are from gene ontology biological process. Neutrophil: neutrophil degranulation (GO:0043312) and *Cebpa*. B lymphocyte: lymphocyte differentiation (GO:0030098), lymphocyte differentiation (GO:0030098), regulation of B cell receptor signaling pathway (GO:0050855), B cell receptor signaling pathway (GO:0050853), regulation of B cell proliferation (GO:0030888), and *Ebf1*, *Irf4*, *Irf8*. $n = 4$ per group.
- (F) GSEA enrichment plot for upregulate genes in RNA-seq of *Trsp* KO vs. Control in pre-B cells. $n = 4$ per group.
- (G) Schematic representation of the experimental procedure. LSK cells were sorted from the BM of *Trsp*^{fl/fl} or *Trsp*^{fl/fl}; R26-CreERT2 mice, expanded until day 6, and cultured under B cell differentiation conditions with OP-9 stromal cells in the presence of IL-7 and FLT3-L. Cells were treated with 4-OHT for 24 hours on day 5. On day 20, they were analyzed by FACS.
- (H) The frequency of B220⁺, B220⁺CD19⁺, and CD11b⁺ cells in each group is shown. P -values were calculated by one-way ANOVA test.
- (I) Schematic of BM transplantation assays. CD3⁻CD11b⁻Gr-1⁻Ter-119⁻CD19⁻IgM⁻ (pro-B/pre-B) BM cells sorted from the BM of Control (*Trsp*^{fl/fl}) or *Trsp* KO (*Trsp*^{fl/fl}; Mx1-Cre) mice at 8 weeks post-pl-pC injection, with rescue cells (CD45.1⁺ whole BM cells) were injected into lethally irradiated CD45.1 mice.
- (J) Flow cytometric profiles for B220⁺ and CD11b⁺ in respective conditions and frequency of

CD45.2⁺B220⁺ and CD45.2⁺CD11b⁺ cells in the BM of each group were assessed following 1 month of transplantation. $n = 5$ per group. P -values were calculated by a two-sided Student's t -test.

- (K) The appearance of donor pro-B/pre-B-derived CD45.2⁺CD11b⁺ fractions in recipient PB is shown in each group (defined as >0.3% in live PB cells). P -values were calculated using Fisher's exact test.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

Figure 6. Ferroptosis modulation shapes B cell phenotype in *Trsp* KO model.

- (A) Schematic representation of feeding a vitamin E (Vit E) deficient diet.
- (B) Frequency of B220⁺, CD11b⁺Gr-1⁺ and CD11b⁺Gr-1⁻ cells in the BM of each group administered Vit E-deficient diet for 6 weeks. *Trsp* KO/Vit E-deficient diet group: 2 mice, other groups: 3 mice per group.
- (C) MFI of C11-BODIPY 581/591 and CellROX staining in B220⁺ BM cells of each group administered Vit E-deficient diet for 3 weeks. For C11-BODIPY 581/591: Control/control diet group: 4 mice, other groups: 5 mice per group, For CellROX: Control/control diet group: 3 mice, other groups: 4 mice per group. Representative histograms of C11-BODIPY 581/591 and CellROX staining in B220⁺ BM cells of each group are provided.
- (D) MFI of C11-BODIPY 581/591 and CellROX staining in prepro-B, pro-B, pre-B, and immature B cells in BM cells of each group. Control/control diet group: 3 mice, other groups: 4 mice per group.
- (E) Schematic representation of feeding a Vit E-rich diet.
- (F) Frequency of B220⁺ CD11b⁺Gr-1⁺ and CD11b⁺Gr-1⁻ cells in the spleen of each group administered Vit E-rich diet for 6 weeks. $n = 3$ per group.

P -values were calculated by a two-sided Student's t -test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

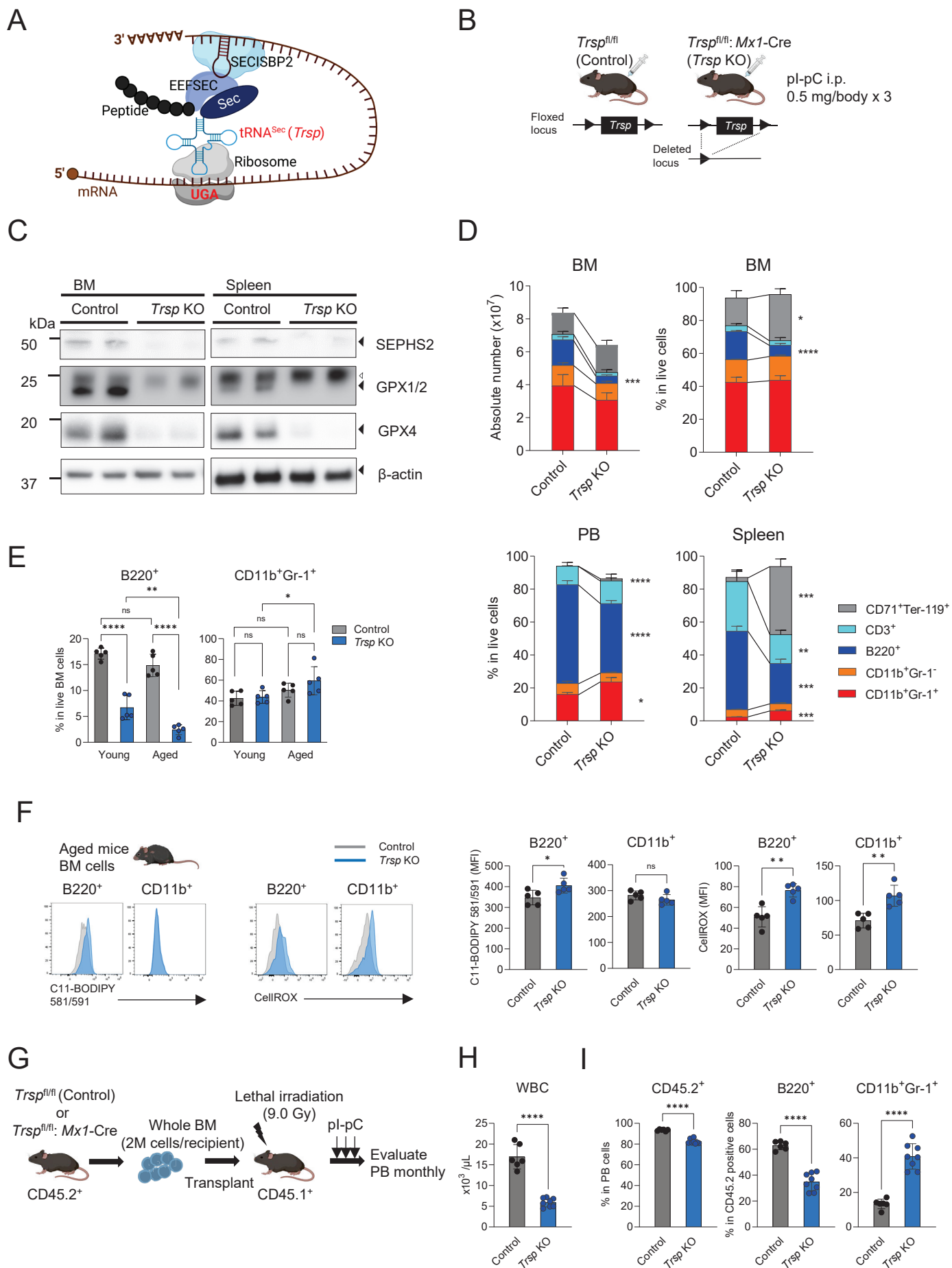


Figure 1

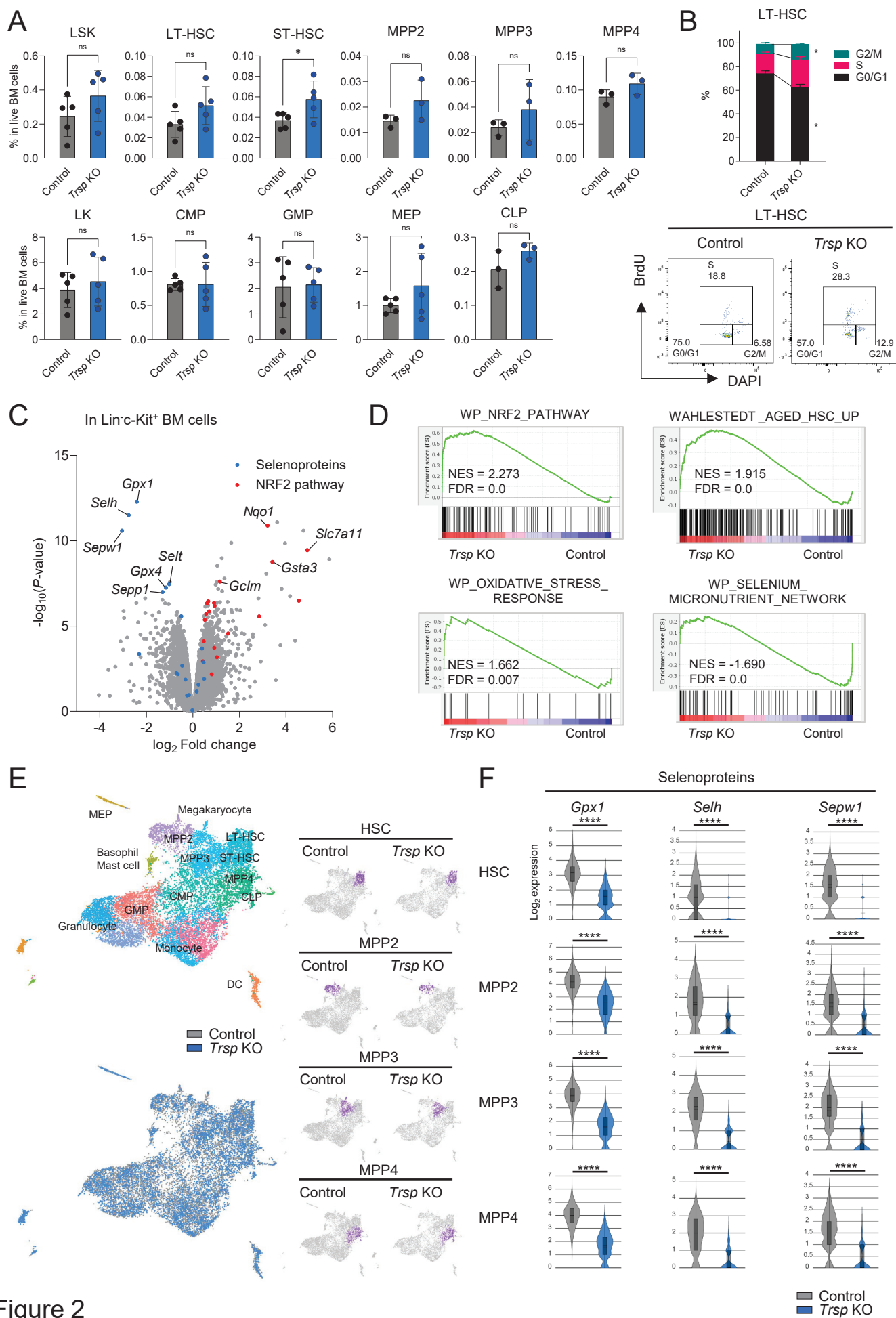


Figure 2

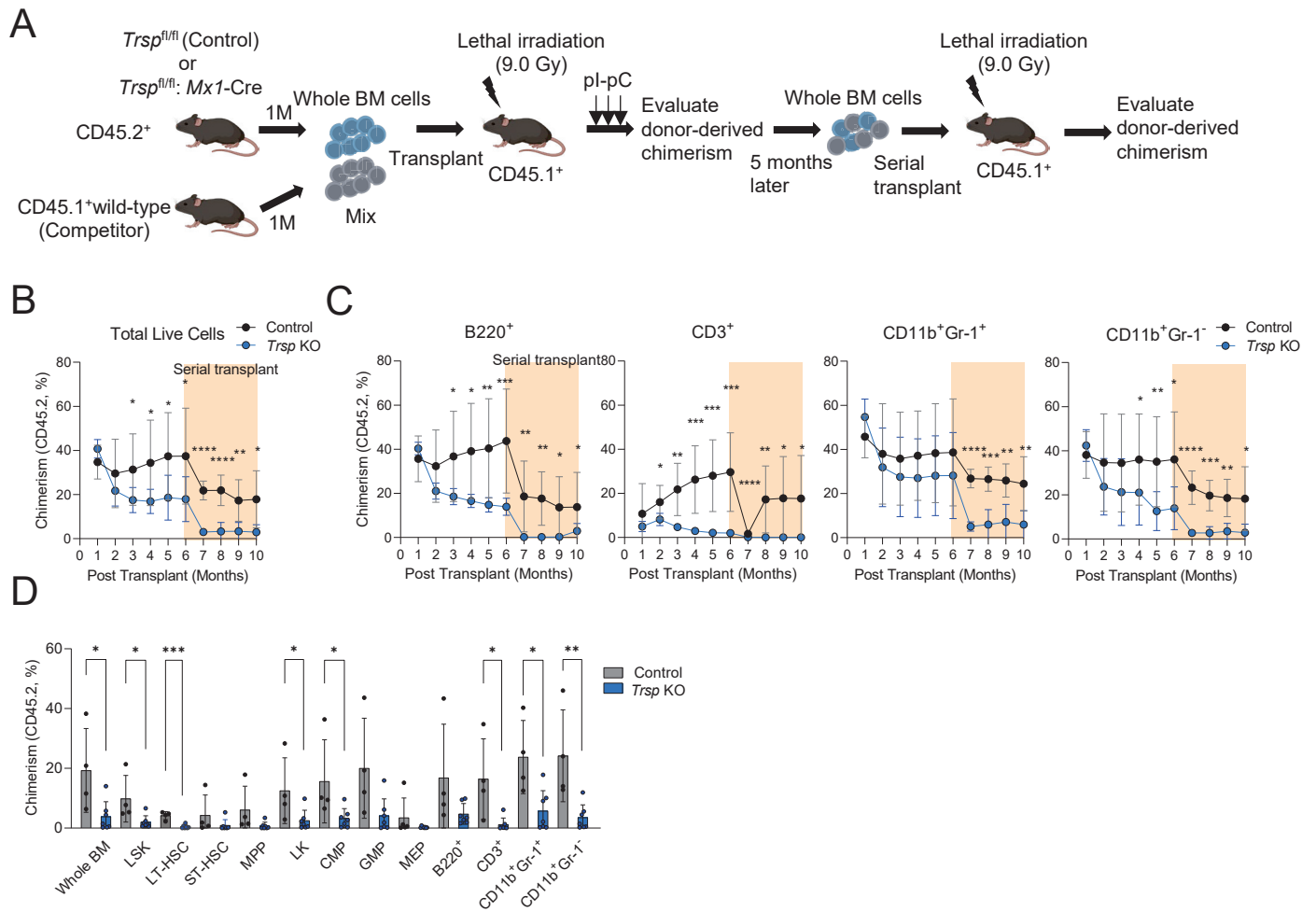


Figure 3

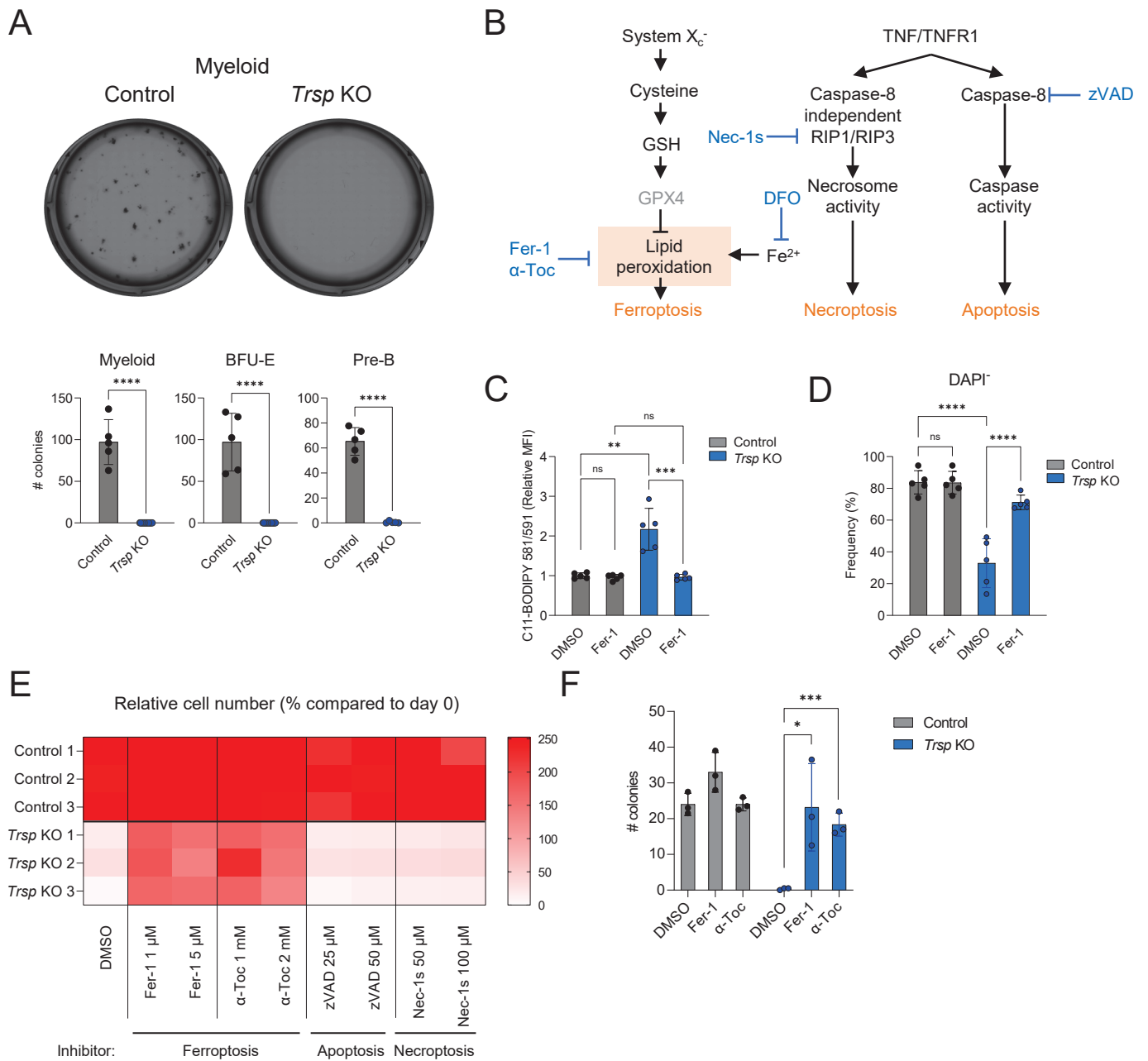


Figure 4

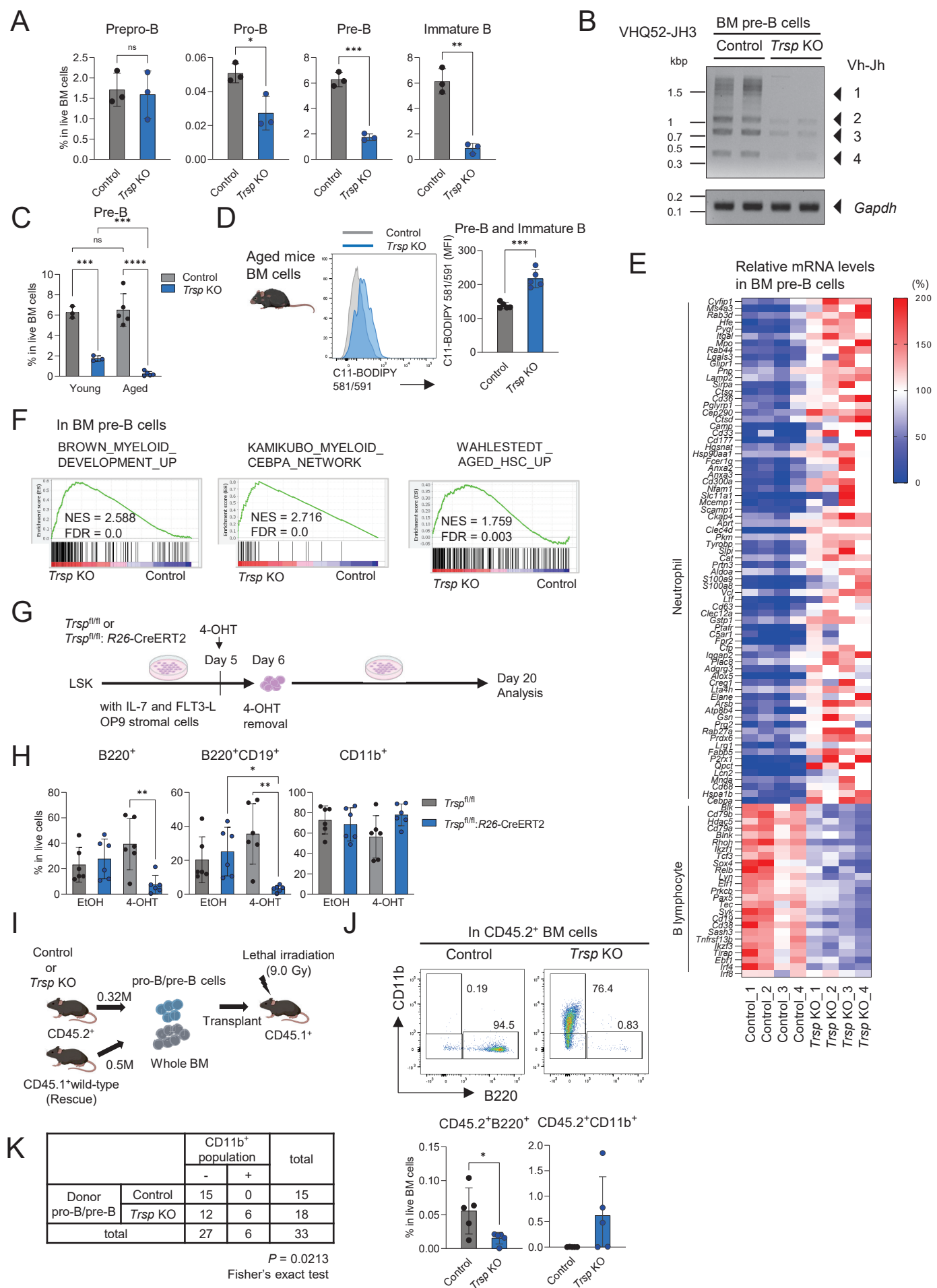


Figure 5

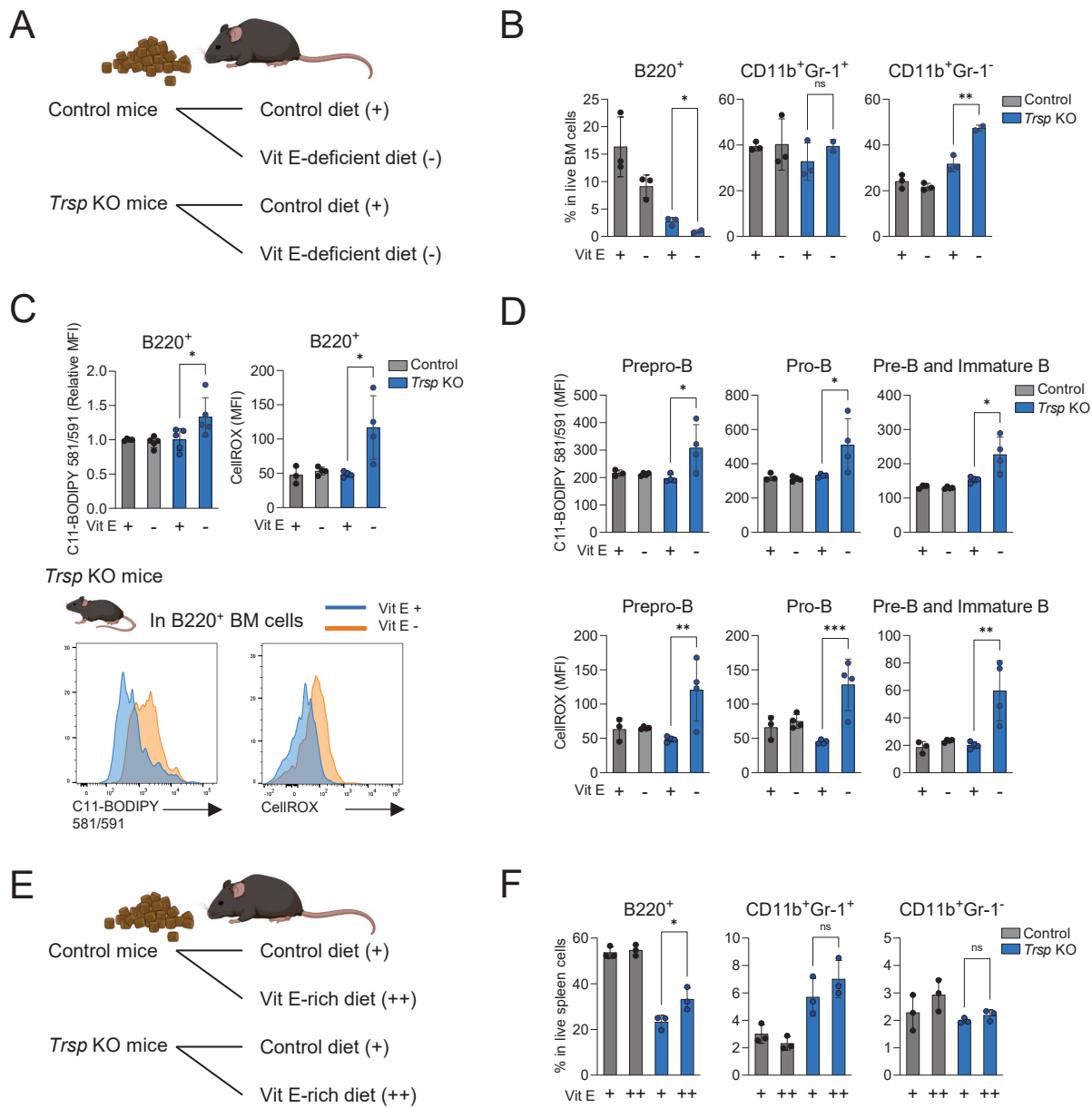


Figure 6

Supplemental Materials

Selenoprotein-Mediated Redox Regulation Shapes the Cell Fate of HSCs and Mature Lineages

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This supplement contains:

Supplemental Methods

Supplemental References

Supplemental Figure Legends

Supplemental Table 1-2

Supplemental Figure 1-11

Supplemental Methods

Mouse treatment

For *Trsp^{fl/fl}*: *Mx1*-Cre mice, Cre expression and *Trsp* excision were induced with intraperitoneal injections of polyinosine-polycytidylic acid (pl-pC, Sigma-Aldrich; p1530, 500 µg/mouse, every other day, 3 times). In *Trsp^{fl/fl}*: *R26*-CreERT2 mice, deletion was achieved with intraperitoneal injections of tamoxifen (Sigma-Aldrich; T5648, 100 mg/kg, daily for 10 days). Vitamin E depletion or repletion experiments were conducted with a fixed formulation diet with 0 mg/kg (vitamin E-deficient diet), 75 mg/kg (control diet), or 500 mg/kg vitamin E (vitamin E-rich diet), based on AIN-93M (Clea-Japan). Primers used for PCR are listed in Supplemental Table 1, and the ingredient lists of the mouse diets are in Supplemental Table 2.

Peripheral blood analysis

Mouse peripheral blood samples were collected from the submandibular vein by animal lancet (MEDpoint). Automated peripheral blood counts were obtained using a pocH-100iV (Sysmex). Differential blood counts were realized on blood smears stained using Wright-Giemsa staining and visualized using an Axio Observer A1 microscope (Carl Zeiss).

Bone marrow (BM) transplantation

For competitive transplantation experiments, freshly dissected femora and tibiae were isolated from *Trsp^{fl/fl}*, *Trsp^{fl/fl}*: *Mx1*-Cre CD45.2⁺ mice. The BM was centrifuged at 6000 x g, and red blood cells (RBCs) were lysed in ammonium chloride-potassium bicarbonate lysis buffer for 1 min. After centrifugation, cells were resuspended in PBS with 2% FBS, passed through a cell strainer, and counted. Finally, 1.0 x 10⁶ total BM cells from *Trsp^{fl/fl}* and *Trsp^{fl/fl}*: *Mx1*-Cre mice were mixed with 1.0 x 10⁶ wild-type CD45.1⁺ support BM and transplanted via tail vein injection into lethally irradiated (4.5 Gy, twice) CD45.1⁺ recipient mice. Chimerism in the peripheral blood was measured using flow cytometry (FACS) at 4 weeks after transplant (week 0, pre-pl-pC). After confirming the engraftment, the recipient mice were treated with pl-pC. Chimerism was followed via FACS from the peripheral blood every 4 weeks. Additionally, for each bleeding, whole blood cell counts were measured on a blood analyzer, and peripheral blood smears were scored. For the serial transplant, whole BM samples collected six months after the initial transplant were transplanted into lethally irradiated (4.5 Gy, twice) wild-type recipients (2 x 10⁶ cells per recipient). For non-competitive transplantation experiments, 2 x 10⁶ total BM cells from *Trsp^{fl/fl}* and *Trsp^{fl/fl}*: *Mx1*-Cre mice were injected into lethally irradiated (4.5 Gy, twice) CD45.1⁺ recipient mice. After confirming the engraftment, the recipient mice were treated with pl-pC. For the non-competitive transplantation experiments to evaluate reactive oxygen species (ROS), 2 x 10⁶ total BM cells from *Trsp^{fl/fl}* and *Trsp^{fl/fl}*: *Mx1*-Cre mice, collected ten weeks post-pl-pC injection, were transplanted into lethally irradiated (4.5 Gy,

twice) CD45.1⁺ recipient mice. For B progenitor transplantation experiments, 3.2×10^5 CD3⁺Gr-1⁺CD11b⁺Ter-119⁺CD19⁺IgM⁺ BM cells sorted from pl-pC-treated *Trsp*^{fl/fl} or *Trsp*^{fl/fl}:*Mx1*-Cre mice, or 5.0×10^5 CD3⁺Gr-1⁺CD11b⁺Ter-119⁺CD19⁺IgM⁺ BM cells sorted from CD45.2⁺ young (5 weeks) or aged (58 weeks) wild-type C57BL/6 mice, were mixed with 5.0×10^5 wild-type CD45.1⁺ support BM and injected into lethally irradiated (4.5 Gy, twice) CD45.1⁺ recipient mice.

***In vitro* colony-forming assays**

Whole BM cells from pl-pC-treated *Trsp*^{fl/fl} or *Trsp*^{fl/fl}:*Mx1*-Cre were seeded at a density of 2×10^4 , 1×10^5 , and 2×10^5 cells per replicate into cytokine-supplemented methylcellulose medium (MethoCult M3434, M3436, and M3630, respectively; STEMCELL Technologies; ST-03434, ST-03436, ST-03630) in a 6-well plate. Colonies propagated in culture were scored on day 7. For the inhibitor experiments, whole BM cells were seeded at a density of 2.5×10^4 cells/mL in MethoCult M3434 and plated in a 12-well plate. Colonies formed in culture were then scored on day 7. The following inhibitors were used: ferrostatin 1 (Cayman chemical; 17729; 5 μ M), and ($\pm$)- α -tocopherol (α -tocopherol, Sigma-Aldrich; T3251; 2 mM).

FACS

All FACS antibodies were purchased from BD Biosciences, eBioscience, Invitrogen, or BioLegend. BM mononuclear cells were stained with a lineage cocktail comprised of antibodies targeting CD3, CD4, CD8, B220, CD19, NK1.1, Gr-1, CD11b, and Ter-119. Cells were also stained with antibodies against c-Kit, Sca1, CD150, and CD48. Cell populations were analyzed using a FACSLyric (BD Biosciences) and sorted with a FACS Aria II (BD Biosciences), FACSMelody (BD Biosciences), and MA900 (Sony). The following antibodies were used for analysis: B220-PECy7 (clone: RA3-6B2; BD Pharmingen; catalog #: 10322; dilution: 1:300); B220-PE (RA3-6B2; BD Pharmingen; 553090; 1:200); CD3-APCCy7 (145-2c11; BioLegend; 100330; 1:300); CD3-FITC (145-2c11; BD Pharmingen; 553062; 1:300); CD3-PECy7 (145-2c11; BioLegend; 100320; 1:300); Gr-1-APC (RB6-8C5; BD Pharmingen; 553129; 1:300); Gr-1-APCCy7 (RB6-8C5; BioLegend; 108424; 1:300); CD11b-PerCPCy5.5 (M1/70; BioLegend; 101228; 1:300); CD11b-FITC (M1/70; BD Pharmingen; 553310; 1:300); CD11b-PECy7 (M1/70; BioLegend; 101216; 1:300); c-Kit-APC (2B8; BD Pharmingen; 553356; 1:200); c-Kit-PE (2B8; BD Pharmingen; 553356; 1:200); c-Kit-PECy7 (2B8; BioLegend; 105814; 1:400); c-Kit-APCCy7 (2B8; BioLegend; 105826; 1:400); Sca1-PECy7 (D7; BioLegend; 108114; 1:400); Sca1-FITC (D7; BioLegend; 108106; 1:200); Streptavidin-PerCPCy5.5 (BioLegend; 405214; 1:400); Streptavidin-BV605 (BioLegend; 405229; 1:400); Streptavidin-BV421 (BioLegend; 405226; 1:400); CD45.1-FITC (A20; BD Pharmingen; 553775; 1:300); CD45.1 biotin (A20; BD Pharmingen; 553774; 1:100); CD45.2-PE (104; BD Pharmingen; 560695; 1:300); CD45.2-FITC (104; BD Pharmingen; 553772; 1:300); CD45.2-APC (104; BD Pharmingen; 561875; 1:300); CD45.2-APCCy7 (104; BD Pharmingen; 560694;

1:300); CD48-APCCy7 (HM48-1; BioLegend; 103432; 1:400); CD150-BV605 (TC15-12F12.2; BioLegend; 115927; 1:100); CD150-PE (TC15-12F12.2; BioLegend; 115904; 1:100); CD16/CD32 (FcγRII/III)-Alexa700 (93; eBioscience; 56-0161-82; 1:200); CD34-eFluor450 (RAM34; eBioscience; 48-0341-82; 1:50); CD135-PE (A2F10.1; BD Pharmingen; 553842; 1:100); CD127 (IL-7Rα)-APCCy7 (A7R34; BioLegend; 135039; 1:100); CD19-PECy7 (6D5; BioLegend; 115520; 1:200); CD19-APCCy7 (6D5; BioLegend; 115530; 1:200); IgM-FITC (RMM-1; BioLegend; 406505; 1:200); CD43-PerCPCy5.5 (S7; BD Pharmingen; 562865; 1:200); CD43-FITC (S7; BD Pharmingen; 561856; 1:200); NK1.1-PerCPCy5.5 (PK136; BioLegend; 108728; 1:300); CD24-PECy7 (M1/69; BD Pharmingen; 560536; 1:50); CD93 (AA4.1)-PerCPCy5.5 (AA4.1; Invitrogen; 45-5892-82; 1:50); Ter-119-APCCy7 (TER-119; BD Pharmingen; 560509; 1:300); Ter-119-PE (TER-119; BD Pharmingen; 553673; 1:300); Ter-119-PerCPCy5.5 (TER-119; BioLegend; 116228; 1:300); Ter-119-FITC (TER-119; BioLegend; 116206; 1:300); CD71-PECy7 (RI7217; BioLegend; 113811; 1:300); CD71-FITC (RI7217; BioLegend; 113806; 1:300); CD45.1-FITC (A20; BD Pharmingen; 553775; 1:400); CD45.1-PE (A20; BD Pharmingen; 553776; 1:400); CD45.2-PE (A20; BD Pharmingen; 553776; 1:400); CD45.2-BV480 (104; BD Pharmingen; 566077; 1:400); CD45.2-APC (104; BD Pharmingen; 558702; 1:400); BODIPY 581/591 C11 (Invitrogen; D3861; 1 μM), CellROX Deep Red (Invitrogen; C10422; 5 μM), DAPI (BD Pharmingen; 564907; 1:10,000), 7-AAD (BD Pharmingen; 51-68981E; 1:100), Propidium Iodide (BD Pharmingen; 51-66211E; 1:10,000).

Western blot analysis

Cells were lysed with RIPA buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS), and this was mixed with Pierce Lane Marker Reducing Sample Buffer (Thermo Fisher Scientific; 39000) and denatured. The mixture was loaded onto 4–12% Bis-Tris NuPAGE Gels (Thermo Fisher Scientific; NW04125) followed by wet transfer. The following antibodies were used for Western blot analysis: SEPHS2 (Novus Biologicals; NBP1-77776; 1:1000), GPX1/2 (Santa Cruz; sc-133160; 1:400), GPX4 (Abcam; ab125066, 1:1,000), Actin (Sigma-Aldrich; A-5441; 1:5,000), rabbit IgG-HRP (Cell Signaling Technology; 7074; 1:10,000), and mouse IgG-HRP (Cell Signaling Technology; 7076; 1:10,000).

Label-free proteome analysis

1 x 10⁷ whole BM cells were lysed with RIPA buffer. Cell lysates containing 50 μg total protein was precipitated with acetone. The precipitate was then denatured with 50% trifluoroethanol. Disulfide bonds were reduced with dithiothreitol, alkylated with iodoacetamide, and digested with trypsin. After desalting and purification of the resulting peptides using MonoSpin C18 columns (GL Sciences Inc.), LC-MS/MS was performed using a nanoLC system (Eksigent 400, AB Sciex) connected online to a mass spectrometer (TripleTOF 6600, AB Sciex). Peptide separation was performed using a nano C18 reverse-phase capillary tip column (75 μm × 125

mm, 3 μ m, Nikkyo Technos CO). A DIA-NN software version 1.8.1 was used to extract quantitative data for proteins from the SWATH runs with a library-free workflow.¹ A predicted library from a Swiss-Prot human database was built in silico with the options 'FASTA digest for library-free search/library generation' and 'Deep learning-based spectra, RTs and IMs prediction'. Quantitative data were output using RT-dependent cross-run normalization and filtering with a 1% FDR threshold.

Histological analysis

Mice were sacrificed and autopsied, and the dissected tissue samples were fixed in 4% paraformaldehyde (FUJIFILM Wako; 163-20145) for 48 h, dehydrated, and embedded in paraffin. Paraffin blocks were sectioned at 4 μ m and stained with hematoxylin and eosin (H&E). Wright-Giemsa and immunohistochemical staining were performed on 3 μ m-thick paraffin-embedded sections after 10% neutral buffered formalin (FFPE). Briefly, after deparaffinization, the specimens were incubated with 0.3% H₂O₂ in methanol for the inhibition of endogenous peroxidase. Then, normal rabbit serum (diluted 1:75; Dako) for inhibition of non-specific binding of the secondary antibody, mAbs against HNE-modified proteins (HNEJ-1),² biotin-labeled rabbit anti-mouse IgG serum (diluted 1:300; Dako) and avidin-biotin complex (diluted 1:100; Vector Laboratories, Burlingame, CA) were sequentially used. 3,3-Diaminobenzidine solution (Dako Liquid DAB, K3466) was used for visualizing the antibody complexes. Images were acquired using an Axio Observer A1 microscope (Carl Zeiss) and a microscope (BZ-X800, Keyence).

RNA-seq

Total RNA was prepared from sorted cells using RNeasy Micro Kit (Qiagen; 74004) per manufacturer's instructions. RNA was then Poly(A)- selected, and stranded illumina libraries were prepared using SMART-Seq HT Plus Kit (Clontech; R400748) or NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs; E7490) and NEBNext UltraTMII Directional RNA Library Prep Kit for Illumina (New England Biolabs; E7760). The libraries were sequenced with Illumina NovaSeq 6000 at a depth of 40M 2 x 150 bp reads per sample. RNA-seq data were analyzed using BioJupies for the volcano plot.³⁻⁵ For differential expression analysis, sequenced reads were mapped to mm10 using nf-core/rnaseq v3.0 pipeline⁶ with star_rsem aligner. RSEM gene counts were normalized and differentially expressed genes were identified with Adjusted *P*-value < 0.1 by DESeq2 v1.26.0,⁷ where independent hypothesis weighting was applied.⁸ The normalized count was used in a PC analysis and heatmap analysis. With differentially expressed gene sets, gene set enrichment analysis (GSEA) pathway analysis was performed by the Molecular Signatures Database (MSigDB),⁹⁻¹² aging-related gene sets,^{13,14} and GSEA software.

scRNA-seq

Single cell RNA-seq libraries of sorted lineage-negative (Lin⁻) c-Kit⁺ BM cells from *Trsp*^{fl/fl}, *Trsp*^{fl/fl}; *Mx1*-Cre mice, at 9 weeks post-pl-pC injection were prepared according to the manufacturer's protocol using the 10x Genomics Chromium Next GEM Single Cell 3' Kit v3.1 and Dual Index Kit. The libraries were pooled together and sequenced with NovaSeq 6000 (illumina) at a depth of ~ 647M reads per sample. Raw sequencing data was demultiplexed and converted to the standard FASTQ files by executing cell ranger mkfastq (6.1.2, 10x Genomics).

Gene counts of each sample were generated from the FASTQs by executing cellranger count (6.1.2, 10x Genomics) with mm10-2020-A as the reference dataset. The filtered feature barcode matrices of the samples were analyzed using Seurat (4.1.1) R package¹⁵ on R (4.1.3).¹⁶ After calculating QC metrics including mitochondrial percentage, low quality cells not meeting the following criteria were removed: nFeature_RNA > 2000 and percent.mt < 12. As a result 13649 cells were retained. SCTransform v2 function with the parameters variable.feature.n = 3000 and vars.to.regress = "percent.mt" was applied for the first normalization and cell cycle scores were calculated by CellCycleScoring function with cc.genes.updated.2019 cell cycle genes, where difference of cell cycle scores (CC.Difference) between S phase and G2M phase were obtained. Then, SCTransform function with the parameters variable.feature.n = 3000 and vars.to.regress = c("percent.mt", "CC.Difference") was applied for the second normalization. Finally, each data was integrated through executing SelectIntegrationFeatures with nfeatures = 3000, PrepSCTIntegration, FindIntegrationAnchors with normalization.method = "SCT" and reduction = "cca" and dims = 1 : 100 and IntegrateData. Principal component analysis (PCA) was performed with the integrated data followed by uniform manifold approximation and projection (UMAP) dimensional reduction, FindNeighbors and FindClusters. Cell clusters and UMAP coordinates were exported from the Seurat object and imported into the Loupe Browser file created by cellranger aggr for visualization. Box plot and kernel density plot of log₂ expression values are shown. *P*-values were reanalyzed using Loupe Browser. In the box-and-whisker plots, the 0th, 25th, 50th, 75th and 100th percentiles and mean (dashed lines) are shown.

Quantitative RT-PCR

For cDNA synthesis, total RNA was reverse transcribed to cDNA with the Verso cDNA Synthesis kit (Thermo Scientific; AB-1453/B). Quantitative RT-PCR (RT-qPCR) was performed with SYBR Green PCR Master Mix (Thermo Scientific; A25742). All RT-qPCR analyses were performed on the CFX Connect Real-Time PCR Detection System (BioRad). Relative gene expression levels were calculated using the comparative CT method, and the values were corrected with expression levels of the internal control, *Gapdh*. The primers used for RT-qPCR are listed in Supplemental Table 1.^{17,18}

Cell culture and in vitro cell viability assays

Human cord blood CD34⁺ cells were prepared from cord blood mononuclear cells (RIKEN BRC) using the CD34 MicroBead Kit (Miltenyi Biotech; 130-046-702) and cultured in StemSpan SFEM (STEMCELL Technologies; ST-09600) supplemented with CD34⁺ Expansion Supplement (STEMCELL Technologies; ST-02691). For premature aging experiments, CD34⁺ cells were cultured with 1 μ M etoposide (Abcam; ab120227) for 24 hours. After 7 days of culture, cells were subjected to Western blot and FACS analysis using C11-BODIPY 581/591.

For cell viability assays, Lin⁻ BM cells from *Trsp^{fl/fl}*, *Trsp^{fl/fl}; Mx1-Cre* mice at 7 weeks post-pl-pC injection were prepared using the Lineage Cell Depletion Kit (Miltenyi Biotech, 130-090-858) and cultured in RPMI 1640(Wako; 189-02025) supplemented with 10% FBS, GlutaMax (Gibco; 35050-061), NEAA (Gibco; 11140-050), 2-ME (Sigma-Aldrich; M3148; 50 μ M), 5 ng/mL recombinant murine IL-3 (Peprotech; 213-13), recombinant murine 5 ng/mL IL-6 (Peprotech; 216-16), and 10 ng/mL recombinant murine SCF (Peprotech; 250-03). Cells were seeded in white flat-well 96-well plates (Falcon; 351172) at a density of 2.5×10^4 cells per well. ATP luminescence readings were taken 48 hours after seeding, using CellTiter-Glo (Promega; G7570) and GloMAX Navigator microplate luminometer (Promega) according to the manufacturer's instructions. For FACS analysis, these cells were seeded in 24-well plates (Falcon; 351147) at a density of 2.5×10^5 cells per well. For the inhibitor experiments, the following inhibitors were used: ferrostatin 1 (Cayman chemical; 17729; 1-5 μ M), ($\pm$)- α -tocopherol (α -tocopherol, Sigma-Aldrich; T3251; 1-2 mM), Z-VAD(OH)-FMK (zVAD, Abcam; ab120382, 25-50 μ M) 7-Cl-O-Nec1 (Nec-1s, Abcam; ab221984; 50-100 μ M). Control cells were cultured with RSL3 (Cayman chemical; 19288; 1 μ M) as a ferroptosis inducer, or TNF α (Thermo Fisher; PMC3014; 10 ng/mL), zVAD (50 μ M), and BV-6 (Selleckchem; S7597; 0.5 μ M) as necroptosis inducers.¹⁹

For cell viability assays, Lin⁻ BM cells from young (10 weeks of age) and aged (104 weeks of age) wild-type C57BL/6 mice were prepared using the Lineage Cell Depletion Kit and cultured in IMDM (Sigma-Aldrich; I3390) supplemented with 10% FBS, 10 ng/mL recombinant murine IL-3, 10 ng/mL recombinant murine IL-6, 10 ng/mL recombinant murine TPO (Peprotech; AF-315-14), and 20 ng/mL recombinant murine SCF. Cells were seeded in 96-well plates at a density of 2.5×10^4 cells per well and treated with RSL3 (0.5 μ M or 1 μ M) for 24 hours. ATP luminescence readings were taken 24 hours after seeding, using CellTiter-Glo.

For the functional assay on B cell maturation, CD19⁺ cells were sorted from the spleens of *Trsp^{fl/fl}*, *Trsp^{fl/fl}; Mx1-Cre* mice at 7 weeks post-pl-pC injection. These cells were labeled with CFSE(Invitrogen; C34554 A; 5 μ M) for 20 minutes at 37°C and cultured in RPMI 1640 supplemented with 10% FBS, sodium pyruvate (Nacalai; 06344-56; 1 mM), sodium

bicarbonate (Nacalai; 31213-15; 2 mg/mL), nonessential amino acid solution (Nacalai; 0644-56; 0.1 mM), 2-ME (50 μ M), LPS (Sigma-Aldrich; L2630; 100 μ g/mL), and 10 ng/mL recombinant murine IL-4 (R&D Systems; 404-ML-010/CF). After 48 and 96 hours, cells were assessed for cell division by flow cytometry, and IgM secretion was measured by ELISA using a mouse IgM ELISA Kit (ENZ; ADI-900-120).²⁰

For B cell differentiation experiments, LSK cells sorted from the BM of *Trsp*^{fl/fl} or *Trsp*^{fl/fl}; *R26-CreERT2* mice were co-cultured with OP9 stromal cell feeders in RPMI 1640 (Sigma; R8758) supplemented with 10% FBS, 30 ng/mL recombinant murine IL-7 (BioLegend; 577808), 30 ng/mL recombinant murine FLT3-L (BioLegend; 550706), 30 ng/mL recombinant murine SCF (BioLegend; 579708), and 30 ng/mL recombinant murine TPO (BioLegend; 593302). On day 5, cells were treated with (Z)-4-Hydroxytamoxifen (4-OHT, Sigma-Aldrich; H7904; 1 μ M) for 24 hours. On day 6, cells were seeded at a density of 2×10^4 cells per well and cultured in MEM α (Gibco; 11900073) supplemented with 20% FBS, 10 ng/mL recombinant murine IL-7, 5 ng/mL recombinant murine FLT3-L, and OP9 stromal cell feeders. After an additional 14 days, cells were analyzed by FACS.

BrdU injection and cell cycle analysis

Trsp^{fl/fl}, *Trsp*^{fl/fl}; *Mx1-Cre* mice, at 7 weeks post-pl-pC injection were intraperitoneally injected with 1 mg BrdU (Abcam; ab142567) to label dividing cells and were sacrificed one day post-injection. BM cells were harvested and Lin⁻ cells were enriched using the Lineage Cell Depletion Kit (Miltenyi Biotech, 130-090-858). For cell cycle analysis of *Trsp* KO mice fed either a control or a vitamin E-rich diet for eight weeks, one million Lin⁻ BM cells, enriched using the Lineage Cell Depletion Kit, were incubated with 10 μ M BrdU (Abcam; ab142567) for 60 minutes to label the cells. Following labeling and enrichment, cells were stained with antibodies against c-Kit, Sca1, CD150, and CD48 and fixed in BD Cytofix/Cytoperm Buffer (BD Biosciences; 51-2090KZ). Then cells were permeabilized with BD Cytoperm Permeabilization Buffer Plus (BD Biosciences; 51-2356KC) and treated with DNase I and stained in BD Perm/Wash Buffer containing diluted anti-BrdU-FITC (3D4; BioLegend; 364104; 1:10). Finally, the cells were resuspended in PBS containing DAPI (BD Pharmingen; 564907; 1:10,000) and analyzed by FACS.

PCR analysis of IgH V(D)J and IgI Vk-Jk rearrangements

DNA was extracted from CD3⁺Gr-1⁻CD11b⁻Ter-119⁻CD19⁺CD43⁻IgM⁻ BM cells of pl-pC-treated *Trsp*^{fl/fl} or *Trsp*^{fl/fl}; *Mx1-Cre* mice by proteinase K (Nacalai; 29442-14) extraction and ethanol precipitation. Thermocycling conditions were as follows: 30 cycles of 10 sec at 98°C, 15 sec at 60°C, and 1 min at 68°C.²⁰⁻²⁴ PCR primers are listed in Supplemental Table 1.

Flow cytometry-based NETs assay

CD45.2⁺CD11b⁺ cells, sorted from recipients transplanted with *Trsp* KO pro-B/pre-B cells or wild-type C57BL/6 mice, were stimulated with 100 nM phorbol-12-myristate-13-acetate (PMA, Sigma-Aldrich; P1585) for 4 hours, fixed in 2% paraformaldehyde, and blocked with 2% bovine serum albumin (BSA; Sigma-Aldrich; A3059) in DPBS (Gibco; 14040-133). Without permeabilization, cells were stained with antibodies against histone H3 (Abcam; ab1791; 1:300), goat anti-rabbit IgG (Heavy chain) superclonal secondary antibody, Alexa Fluor 647 (Invitrogen; A27040; 1:300), and MPO (Abcam; ab90812; 1:300).²⁵

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Supplemental Figure Legends

Supplemental Figure 1. Age-dependent effect of Selenoprotein synthesis in hematopoiesis.

- (A) Reanalysis of mRNA levels for selenoproteins and their synthesis pathway in young and aged human bone marrow (BM) Lin⁻(B220/CD3/CD11b/Gr-1/Ter-119)⁻CD34⁺CD38⁻ cells. $n = 10$ per group. Young samples: ages < 31 years, aged samples: ages 65-75 years.
- (B) Heatmap illustrating mRNA levels of selenoproteins and their synthesis pathway in young and aged human BM Lin⁻CD34⁺CD38⁻ cells. $n = 10$ per group. Young samples: ages < 31 years, aged samples: ages 65-75 years.
- (C) Western blot analysis for GPX1/2 and GPX4 in CD34⁺ cord blood cells cultured with DMSO or etoposide (1 μ M).
- (D) Mean fluorescence intensity (MFI) of C11-BODIPY 581/591 staining in CD34⁺ cord blood cells cultured with DMSO or etoposide (5 μ M).

P -values were calculated by a two-sided Student's t -test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

Supplemental Figure 2. Characterization of *Trsp* knockout mouse model.

- (A) PCR analysis of *Trsp*^{fl/fl} and *Trsp*^{fl/fl}: *Mx1*-Cre mice bone marrow (BM) cells at 6 weeks after the pl-pC treatment. Fragments derived from floxed allele (494 bp; filled triangles) and recombined allele (465 bp; open triangles). $n = 3$ per group.
- (B) Expression levels of tRNA^{Sec} level in whole BM and each BM fraction, including Lin⁻c-Kit⁺, prepro-B, pre-B, and CD11b⁺ cells from *Trsp*^{fl/fl} and *Trsp*^{fl/fl}: *Mx1*-Cre mice after the pl-pC treatment. whole BM: $n = 3$ per group, each BM fraction: $n = 4$ per group.
- (C) Label-free proteome analysis of whole BM cells, with selenoproteins highlighted (blue). A value of 0.1 (corresponding to 25% of the lowest value) was assumed to be below the detection limit for the entire proteome. $n = 2$ per group.
- (D) Wright-Giemsa staining in femur from recipient mice following 2 months of non-competitive transplantation. Scale bar = 100 μ m. $n = 3$ per group.
- (E) Immunohistochemical examination in femur from recipient mice following 2 months of non-competitive transplantation using 4-hydroxynonenal (HNEJ-1) antibody. Scale bar = 100 μ m. $n = 3$ per group.
- (F) Whole blood counts of Hb, MCV, WBCs, and platelets in each group. $n = 5$ per group.
- (G) Peripheral blood (PB) smears of mice at 6 weeks after the final pl-pC injection were stained with Wright-Giemsa. Scale bar = 20 μ m. $n = 3$ per group.
- (H) Macroscopic appearance of spleens at 6 weeks after the final pl-pC injection, accompanied by plots of body weight, spleen, thymus, and liver body weight ratio. $n = 5$ per group.
- (I) Representative hematoxylin and eosin (H&E) stain of spleens at 6 weeks after the final pl-pC injection. Scale bar = 200 μ m. $n = 3$ per group.
- (J) Whole blood counts of Hb, MCV, WBCs, and platelets in *Trsp*^{fl/fl}, *Trsp*^{fl/wt}: *Mx1*-Cre and *Trsp*^{fl/fl}: *Mx1*-Cre mice at 4 weeks after the pl-pC treatment. *Trsp*^{fl/fl}: *Mx1*-Cre group: 5 mice, other groups: 3 mice per group.
- (K) Frequency of B220⁺, CD3⁺, CD11b⁺Gr-1⁺, CD11b⁺Gr-1⁻ cells in the PB cells of *Trsp*^{fl/fl}, *Trsp*^{fl/wt}: *Mx1*-Cre and *Trsp*^{fl/fl}: *Mx1*-Cre mice at 4 weeks after the pl-pC treatment. *Trsp*^{fl/fl}: *Mx1*-Cre group: 5 mice, other groups: 3 mice per group.

P -values were calculated by a two-sided Student's t -test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

Supplemental Figure 3. Macrocytic anemia and B lymphocytopenia were replicated in *Trsp*^{fl/fl}: *R26*-CreERT2 mice with tamoxifen treatment.

- (A) *Trsp*^{fl/fl}: *R26*-CreERT2 mice were utilized as an additional mouse model enabling time-dependent deletion of *Trsp*. *Trsp* excision was induced by intraperitoneal injection (i.p.) of tamoxifen daily for 10 days. Control mice represent tamoxifen-treated *Trsp*^{fl/fl} mice, while *Trsp* knockout (KO) mice represent *Trsp*^{fl/fl}: *R26*-CreERT2 mice.
- (B) PCR analysis of *Trsp*^{fl/fl} and *Trsp*^{fl/fl}: *R26*-CreERT2 mice BM cells at 6 weeks after the tamoxifen treatment. Fragments derived from the floxed allele (494 bp; filled triangles)

- and recombined allele (465 bp; open triangles).
- (C) Frequency of CD71⁺Ter-119⁺, CD3⁺, B220⁺, CD11b⁺Gr-1⁺, CD11b⁺Gr-1⁻ cells in the BM and spleen of each group. $n = 5$ per group.
 - (D) Whole blood counts of Hb, MCV, WBCs, and platelets in each group. $n = 5$ per group.
 - (E) Body weight, Spleen, thymus, and liver body weight ratio in each group. $n = 5$ per group.
- P -values were calculated by a two-sided Student's t -test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

Supplemental Figure 4. Despite the profound B cell phenotypes, *Trsp* KO did not lead to BM failure or shorten survival.

- (A) Frequency of CD3⁺, CD11b⁺Gr-1⁻ cells in the BM of each group. The groups are categorized by age: young (< 20 weeks of age) and aged (81 weeks of age). $n = 5$ per group. P -values were calculated by a two-sided Student's t -test.
 - (B) MFI of C11-BODIPY 581/591 and CellROX staining in B220⁺ or CD11b⁺ BM cells of young control mice and young *Trsp* KO mice. Young < 20 weeks of age, Control group: 2 mice, *Trsp* KO mice 4 mice. P -values were calculated by a two-sided Student's t -test.
 - (C) Plots showing Hb, MCV, and the proportion of CD3⁺, CD11b⁺Gr-1⁻ in PB of recipient mice following 3 months of transplantation. Control group: 6 mice, *Trsp* KO group: 8 mice. P -values were calculated by a two-sided Student's t -test.
 - (D) Frequency of CD3⁺, CD11b⁺Gr-1⁻ in CD45.2⁺ PB cells of recipient mice following 3 months of transplantation. Control group: 6 mice, *Trsp* KO group: 8 mice. P -values were calculated by a two-sided Student's t -test.
 - (E) Kaplan-Meier survival curves of each recipient group. P -values were calculated by a log-rank test.
- * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

Supplemental Figure 5. Analysis of HSPCs in *Trsp* KO mice *in vivo*.

- (A) Percentages of BrdU⁺ (S phase), DAPI⁺ (4N) BrdU⁻ (G2/M phase), and DAPI⁺ (2N) BrdU⁻ (G0/G1 phase) LSK, LK, ST-HSCs and MPPs in the BM. Control group: 4 mice, *Trsp* KO group: 3 mice.
 - (B) Principal component analysis (PCA) plots of RNA-seq of Lin⁻c-Kit⁺ BM cells. $n = 4$ per group.
 - (C) Violin plots depicting mRNA expression of NRF2 target genes (*Gclm*, *Gclc*, *Ftl1*) in the indicated clusters (HSC, MPP2, MPP3, MPP4, CMP, GMP, MEP, CLP). Box plot and kernel density plot of log₂ expression values are shown. P -values were reanalyzed using Loupe Browser. In the box-and-whisker plots, the 0th, 25th, 50th, 75th, and 100th percentiles and mean (dashed lines) are shown.
 - (D) Percent of donor-derived (CD45.2⁺) cells in each indicated population in the BM following 6 months of primary competitive transplantation. Control group: 7 mice, *Trsp* KO group: 8 mice. P -values were calculated by a two-sided Student's t -test.
 - (E) Schematic representation of non-competitive BM transplantation assays from *Trsp*^{fl/fl} and *Trsp*^{fl/fl}; *Mx1*-Cre mice after the pl-pC treatment, followed by ROS evaluation.
 - (F) Mean fluorescence intensity (MFI) of CellROX staining in c-Kit⁺ BM cells of each group. primary: $n = 3$ per group, BMT: $n = 5$ per group. P -values were calculated by a two-sided Student's t -test.
- * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

Supplemental Figure 6. Analysis of HSPCs in *Trsp* KO mice *in vitro*.

- (A) Relative number of viable Lin⁻ cells, compared to day 0, calculated using CellTiter-Glo Luminescent Cell Viability Assay 48 hours after culturing in the presence of α -Toc (1-2 mM), Fer-1 (1-5 μ M), zVAD (25-50 μ M) and Nec-1s (50-100 μ M). $n = 3$ per group.
- (B) Heatmap showing relative number of viable Lin⁻ cells, compared to day 0, calculated using CellTiter-Glo Luminescent Cell Viability Assay 48 hours after culturing in the presence of TNF α (10ng/mL), BV-6 (0.5 μ M), zVAD (50 μ M), α -Toc (2 mM), Fer-1 (5 μ M) and Nec-1s (100 μ M). $n = 3$ $n = 3$ per group.
- (C) Relative number of viable Lin⁻ cells from young or aged wild-type mice, compared to day 0, calculated by CellTiter-Glo Luminescent Cell Viability Assay 24 hours after culturing in

the presence of RSL3 (0.5 μ M or 1 μ M). $n = 3$ per group. P -values were calculated by a two-sided Student's t -test or one-way ANOVA test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

Supplemental Figure 7. Analysis of B progenitors in *Trsp* KO mice.

- (A) Frequency of prepro-B, pro-B, pre-B, and immature B cells in the spleen of Control mice and *Trsp* KO mice. $n = 3$ per group.
- (B) Representative image of PCR amplification. pre-B cells were isolated from BM by cell sorting and Igh V(D)J and Igl Vk-Jk gene rearrangement was determined by PCR amplification with *Gapdh* as loading control. $n = 2$ per group.
- (C) Schematic representation of the experimental procedure. CD19⁺ cells were sorted from the spleens of control and *Trsp* KO mice, labeled with CFSE, and cultured with LPS (100 μ g/mL) and IL-4 (10 ng/mL). After 48 and 96 hours, cells were assessed for cell division by flow cytometry, and IgM secretion was measured by ELISA.
- (D) Representative histograms of CFSE labeling for each group. The frequency of proliferative cells was evaluated by CFSE dilution in live cells. $n = 3$ mice per group.
- (E) IgM levels were measured by ELISA for each group. $n = 3$ mice per group.
- (F) Frequency of prepro-B, pro-B, immature B cells in the BM of each group. Young < 20 weeks of age, aged: 81 weeks of age. $n = 3$ mice per young group, $n = 5$ mice per aged group.
- (G) MFI of C11-BODIPY 581/591 staining in prepro-B, pro-B, pre-B, and immature B cells in the BM cells of each group. Young: $n = 3$ mice per group, 37 weeks of age. Aged: $n = 5$ mice per group, 81 weeks of age.
- (H) MFI of C11-BODIPY 581/591 staining in whole BM, whole spleen, B220⁺, prepro-B, pro-B, pre-B, and immature B cells in the BM and spleen cells of young wild-type mice and aged wild-type mice. $n = 3$ mice per group, young: 7 weeks of age, aged: 80 weeks of age.

P -values were calculated by a two-sided Student's t -test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

Supplemental Figure 8. *Trsp* KO B progenitors exhibited myeloid characteristics.

- (A) Heatmap showing the genes that regulate myeloid and B cell differentiation evaluated by RNA-seq in BM prepro-B cells. The genes list up from gene ontology biological process. Neutrophil: neutrophil degranulation (GO:0043312) and *Cebpa*. B lymphocyte: lymphocyte differentiation (GO:0030098), lymphocyte differentiation (GO: 0030098), regulation of B cell receptor signaling pathway (GO:0050855), B cell receptor signaling pathway (GO:0050853), regulation of B cell proliferation (GO:0030888), and *Ebf1*, *Irf4*, *Irf8*. $n = 4$ per group.
- (B) Relative expressions of IgC κ , IgG λ , *Tcf3*, *Pax5*, and *Irf4* mRNA level of pre-B cells in the BM of each group. $n = 4$ per group. P -values were calculated by a two-sided Student's t -test.
- (C) Violin plots depicting mRNA expression of *Spi1*, *Cebpa*, and *Irf7* in MPP4 and CLP cluster. Box plot and kernel density plot of log₂ expression values are shown. In the box-and-whisker plots, the 0th, 25th, 50th, 75th, and 100th, with the mean represented by dashed lines.
- (D) GSEA enrichment plot for upregulated genes in RNA-seq of *Trsp* KO vs. control in pre-B cells.
- (E) Flow cytometric profiles for CD19 and CD11b in respective conditions.
- (F) PCR analysis of LSK cells from *Trsp*^{fl/fl} and *Trsp*^{fl/fl}; R26-CreERT2 mice, cultured under B cell differentiation conditions and treated with 4-OHT (1 μ M). Fragments derived from floxed allele (494 bp; filled triangles) and recombined allele (465 bp; open triangles).
- (G) Frequency of donor-derived (CD45.2⁺) prepro-B cells, pro-B cells, pre-B cells, and immature B cells in the BM of each group were assessed following 1 month of transplantation. $n = 5$ per group. P -values were calculated by a two-sided Student's t -test.
- (H) Cytospin images of primary CD11b⁺ cells and CD11b⁺ cells derived from *Trsp* KO pro-B/pre-B cells, stained with Wright-Giemsa. Scale bar = 20 μ m. Counts of segmented neutrophils, banded neutrophils, metamyelocytes, and monocytes are provided for

- primary CD11b⁺ cells and *Trsp* KO pro-B/pre-B-derived CD11b⁺ cells.
- (I) Relative expression levels of *IgkGL*, *Tcf3*, *Pax5*, *Ebf1*, *Irf4*, *Cebpa*, and *Mpo* mRNA in each group. *Trsp* KO pro-B/pre-B-derived CD11b⁺ group was technically triplicated.
 - (J) Flow cytometric profiles for MPO and Histon H3 in CD11b⁺ cells from each group stimulated with 100 nM phorbol-12-myristate-13-acetate (PMA). Primary CD11b⁺ group: *n* = 3, *Trsp* KO pro-B/pre-B-derived CD11b⁺ group: *n* = 2.
- **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001.

Supplemental Figure 9. Pro-B/pre-B cells from aged wild-type exhibited the potential for the myeloid lineage switch.

- (A) Schematic of BM transplantation assays. CD3⁺CD11b⁺Gr-1⁺Ter-119⁺CD19⁺IgM⁺ BM cells (pro-B/pre-B cells) sorted from the BM of young or aged wild-type mice along with rescue cells (CD45.1⁺ whole BM cells) were injected into lethally irradiated CD45.1⁺ recipient mice.
 - (B) Flow cytometric profiles for B220⁺ and CD11b⁺ under respective conditions.
 - (C) Frequency of CD45.2⁺B220⁺ and CD45.2⁺CD11b⁺ cells, donor-derived (CD45.2⁺) prepro-B cells, pro-B cells, pre-B cells, and immature B cells in the BM of each group were assessed one month post-transplantation. *n* = 5 per group.
 - (D) Cytospin images of primary CD11b⁺ cells and CD11b⁺ cells derived from aged wild-type pro-B/pre-B cells, stained with Wright-Giemsa. Scale bar = 20 μm. Count of segmented neutrophils, banded neutrophils, metamyelocytes, and monocytes in cytospin of primary CD11b⁺ cells and CD11b⁺ cells derived from aged wild-type pro-B/pre-B cells.
 - (E) Relative expression levels of *IgkGL*, *Tcf3*, *Pax5*, *Ebf1*, *Irf4*, *Cebpa*, and *Mpo* mRNA in each group. *n* = 2 per pro-B/pre-B from aged wild-type mice derived CD11b⁺ group and *n* = 3 per the other group.
 - (F) Representative flow cytometric profiles for MPO and Histon H3 in CD11b⁺ cells and frequency of MPO and Histon H3 expression in CD11b⁺ cells from each group stimulated with 100 nM phorbol-12-myristate-13-acetate (PMA). Primary CD11b⁺ group: *n* = 3, pro-B/pre-B from aged wild-type derived CD11b⁺ group: *n* = 4.
- P*-values were calculated by a two-sided Student's *t*-test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001.

Supplemental Figure 10. Vitamin E depletion or repletion experiments in *Trsp* KO mice.

- (A) Plots showing Hb, MCV, WBCs, and platelets of each group administered Vit E-deficient diet for 6 weeks. *Trsp* KO/Vit E-deficient diet group: 2 mice, other groups: 3 mice per group.
 - (B) Frequency of LSK, LK, CD3⁺, prepro-B, pro-B, pre-B, and immature B cells in the BM of each group administered Vit E-deficient diet for 6 weeks. *Trsp* KO/Vit E-deficient diet group: 2 mice, other groups: 3 mice per group.
 - (C) Frequency of CD3⁺ cells in the spleen of each group administered Vit E-rich diet for 6 weeks. *n* = 3 per group.
 - (D) Schematic representation of the cell cycle analysis of Lin⁺ BM cells from control and *Trsp* KO mice fed either control or Vit E-rich diet.
 - (E) Percentages of bromodeoxyuridine (BrdU)⁺ (S phase) LT-HSCs in the BM. Control group: 3 mice, *Trsp* KO group: 4 mice.
- P*-values were calculated by a two-sided Student's *t*-test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001.

Supplemental Figure 11. Disrupted selenoprotein synthesis in hematopoiesis.

- (A) Graphical summary. Disrupted selenoprotein synthesis in hematopoiesis caused by *Trsp* gene deletion recapitulates certain aspects of the aged phenotype. In *Trsp* KO mice, lipid peroxide accumulation impairs HSC stemness and B-lineage maturation. This oxidative stress drives B cell progenitors to acquire myeloid characteristics and a unique potential for myeloid lineage switch. Pharmacological and dietary interventions can modulate these oxidative effects.

Supplemental Table 1. Primer sequences for mice genotyping, RT-PCR, Igh V(D)J and Igl Vk-Jk rearrangements assay.

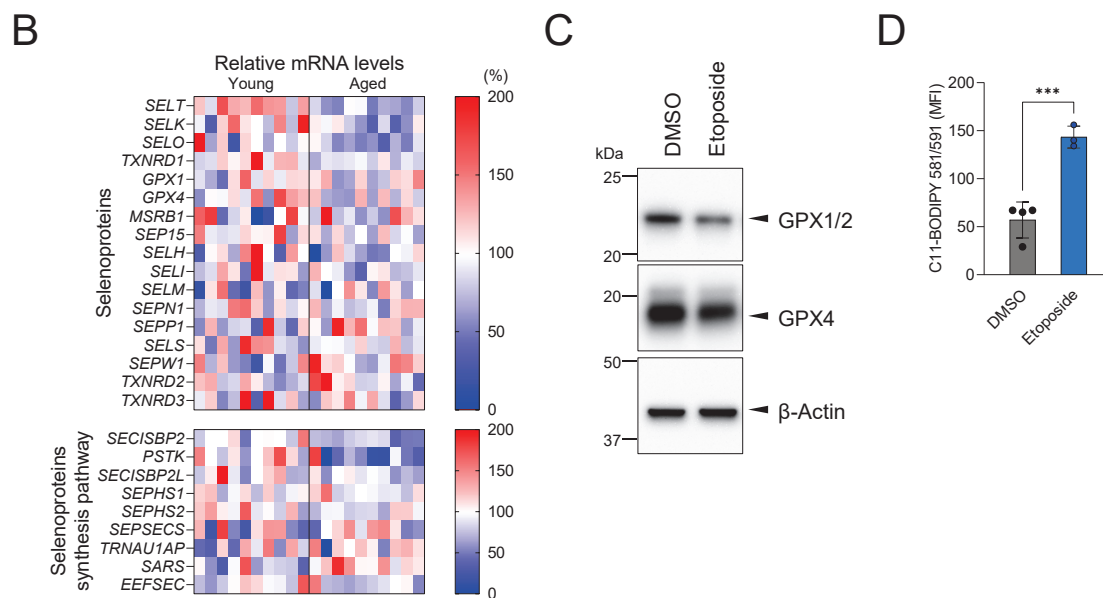
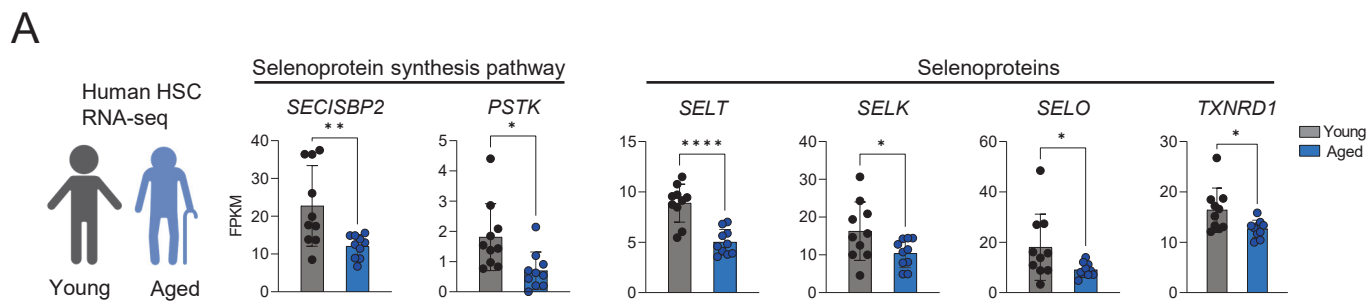
Target		Primer sequence (5' to 3')
Genotyping		
<i>Trsp</i>	SeCF1	GGCTGACTTACAGTTTCAGAGGCA C
	SeCR1	ACCAACTCCCCTTGAGTTTAGACGC
<i>Mx1</i> -Cre	E2A Cre 550F	CAAGTGACAGCAATGCTGTTTCAC
	E2A FOS control F	GGCTGGCCCTGTATTCCTGAT
	E2A Cre 550 R	CAGGTATCTCTGACCAGAGTCATC
	E2A FOS control R	TCTTCTGACCCTTCCCTACTGAGC
<i>R26</i> -CreERT2	R26-F1	CTTCCCTCGTGATCTGCAACT
	R26-R1	AAACTCGGGTGAGCATGTCTT
	Cre-R2	CGACCGGTAATGCAGGCAAAAT
PCR for confirming recombination		
<i>Trsp</i>	SeCF1	GGCTGACTTACAGTTTCAGAGGCA C
	SeCR1	ACCAACTCCCCTTGAGTTTAGACGC
	SeCR2	GACTGAGCCGGAGTGAACAAATGAAC
RT-PCR for confirming tRNA^{Sec} expression		
tRNA ^{Sec}	Forward	GATGATCCTCAGTGGTCTGG
	Reverse	CGAAAGGTGGAATTGAACC
<i>Gapdh</i>	Forward	AGGTCGGTGTGAACGGATTTG
	Reverse	TGTAGACCATGTAGTTGAGGTCA
PCR for Igh V(D)J and Igl Vk-Jk gene rearrangements		
VHQ52-JH3	VH Q52	ACTGARCATCASCAGGACAAYTCC
	JH3	CTCACAAGAGTCCGATAGACCCTGG
DHL-JH3	DHL	MTTTTGTSAAGGGATCTACTACTGTG
	JH3	CTCACAAGAGTCCGATAGACCCTGG
Igl Vk-Jk	Vdk(degenerate primer for Vk)	GGCTGCAGSTTCAGTGGCAGTGGRTCWGGRAC
	Igk intron primer	AACACTGGATAAAGCAGTTTATGCCCTTTC
RT-PCR		
IgCk	Forward	GATGTCTTGTGAGTGGCCCTC
	Reverse	CCAAAGACATCAATGTCAAGTGGA
Igk germ-line	Forward	AGGAGGGTTTTTGTACAGCCA
	Reverse	TGGATGGTGGGAAGATGGAT
<i>Tcf3</i> E2A	Forward	CCAACACTGGTGTCTCTCCCAAAG
	Reverse	TCCTTTGACCCTAGCCGGACATAC
<i>Pax5</i>	Forward	GGGCTCCTCATACTCCATCA
	Reverse	CTGCTGCTGTGTGAACAGGT
<i>Ebf1</i>	Forward	CATGTCCTGGCAGTCTCTGA
	Reverse	CAACTCACTCCAGACCAGCA
<i>Cebpa</i>	Forward	GGTGACAAGAACAGCAACGAG
	Reverse	TAGAGATCCAGCGACCCGAAAC
<i>Mpo</i>	Forward	CGCTTCTCCTTCTTCACTGG
	Reverse	CTGCCATTGTCTTGAATCG
<i>Irf4</i>	Forward	CAATGTCCTGTGACGTTTGG
	Reverse	GGCTTCAGCAGACCTTATGC
<i>Gapdh</i>	Forward	AGGTCGGTGTGAACGGATTTG
	Reverse	TGTAGACCATGTAGTTGAGGTCA

Supplemental Table 2. The ingredient lists of the mouse diets.

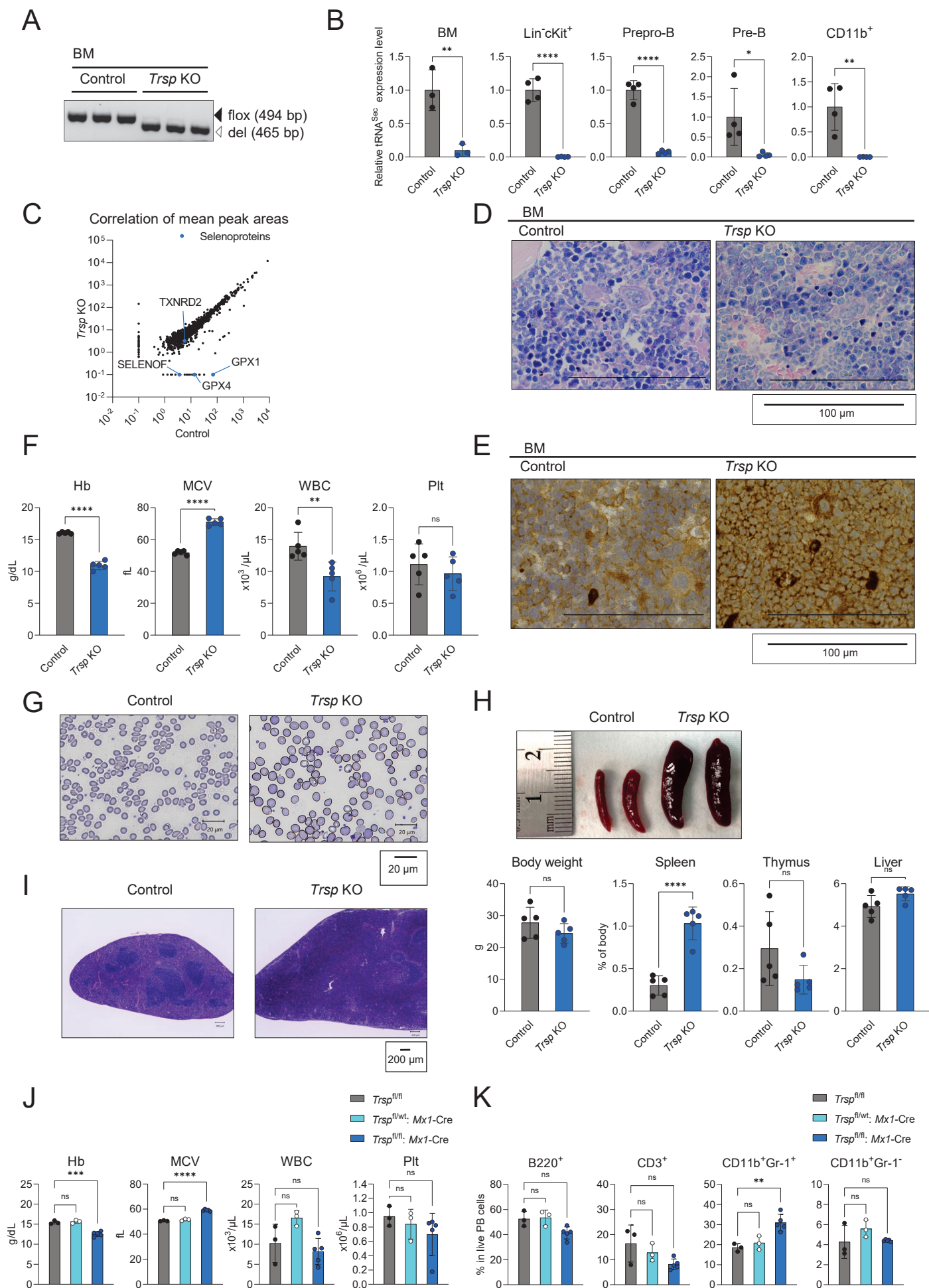
AIN-93M based diet	(%)
cornstarch	46.5
alpha cornstarch	15.5
vitamin-free casein	14
sucrose	10
crystal cellulose	5
purified lard	4
mineral mix	3.5
vitamin mix	1
choline bis(tartrate)	0.25
L-Cystine	0.18
Tertiary butylhydroquinone	0.0008

	Vit E-rich diet	control diet	Vit E-dificient diet
detail of vitamin mix	(%)	(%)	(%)
vitamin E (50%)	10	1.5	0
sucrose	88.84742	97.34742	98.84742
nicotinic acid	0.3	0.3	0.3
vitamin B ₁₂ (0.1%)	0.25	0.25	0.25
D-Pantothenic acid Ca	0.16	0.16	0.16
vitamin A (3.25 x 10 ⁵ IU/g)	0.12308	0.12308	0.12308
vitamin D ₃ (1 x 10 ⁵ IU/g)	0.1	0.1	0.1
vitamin B ₆	0.07	0.07	0.07
vitamin B ₂ (>98%)	0.06	0.06	0.06
vitamin B ₁	0.06	0.06	0.06
folic acid	0.02	0.02	0.02
vitamin K ₁	0.0075	0.0075	0.0075
D-biotin(100%)	0.002	0.002	0.002

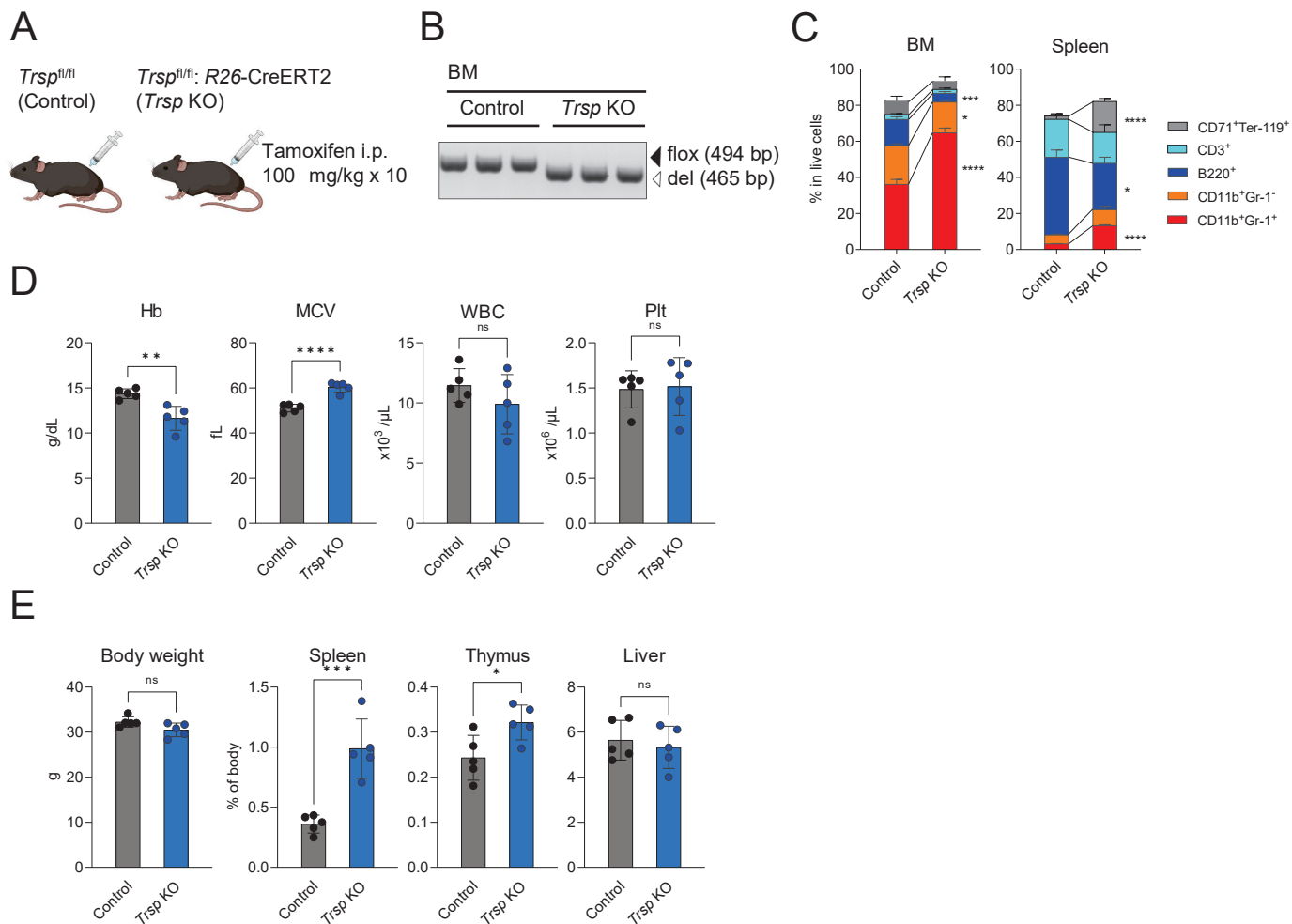
detail of mineral mix	(%)
CaCO ₃	35.7
KH ₂ PO ₄	25
sucrose	20.97832
NaCl	7.4
K ₂ SO ₄	4.66
K ₃ C ₆ H ₅ O ₇ · H ₂ O	2.8
MgO	2.4
FeC ₆ H ₅ O ₇ · XH ₂ O	0.606
ZnCO ₃	0.165
NaSiO ₃ · 9H ₂ O	0.145
MnCO ₃	0.063
CuCO ₃ Cu(OH) ₂ · H ₂ O	0.0324
Crk(SO ₄) ₂ · 12H ₂ O	0.0275
H ₃ BO ₃	0.00815
NaF	0.00635
NiCO ₃ · 2Ni(OH) ₂ · 4H ₂ O	0.00306
LiCl	0.00174
Na ₂ SeO ₄	0.001025
KIO ₃	0.001
(NH ₄) ₆ Mo ₇ O ₂₄ · 4H ₂ O	0.000795
NH ₄ VO ₃	0.00066



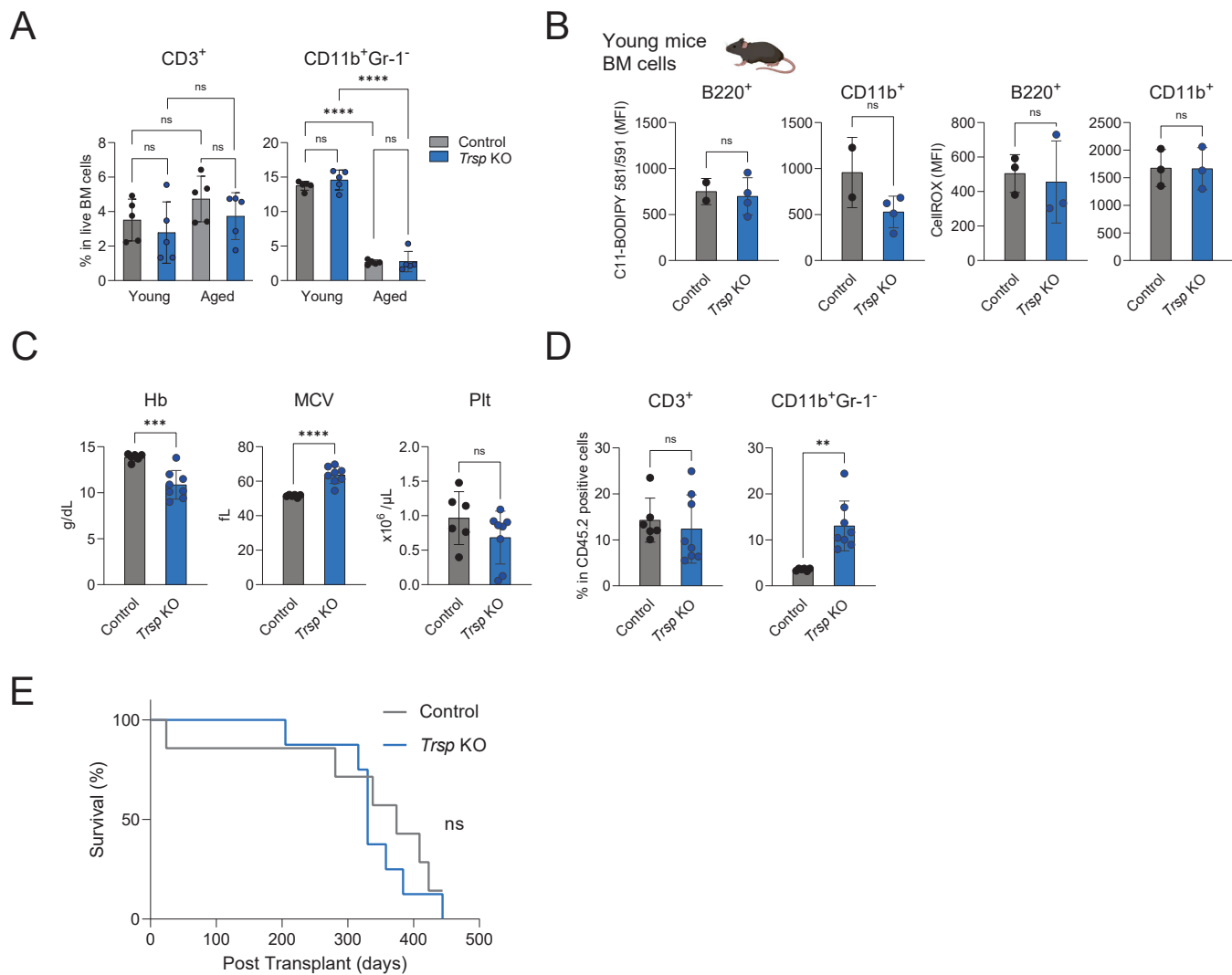
Supplemental Figure 1



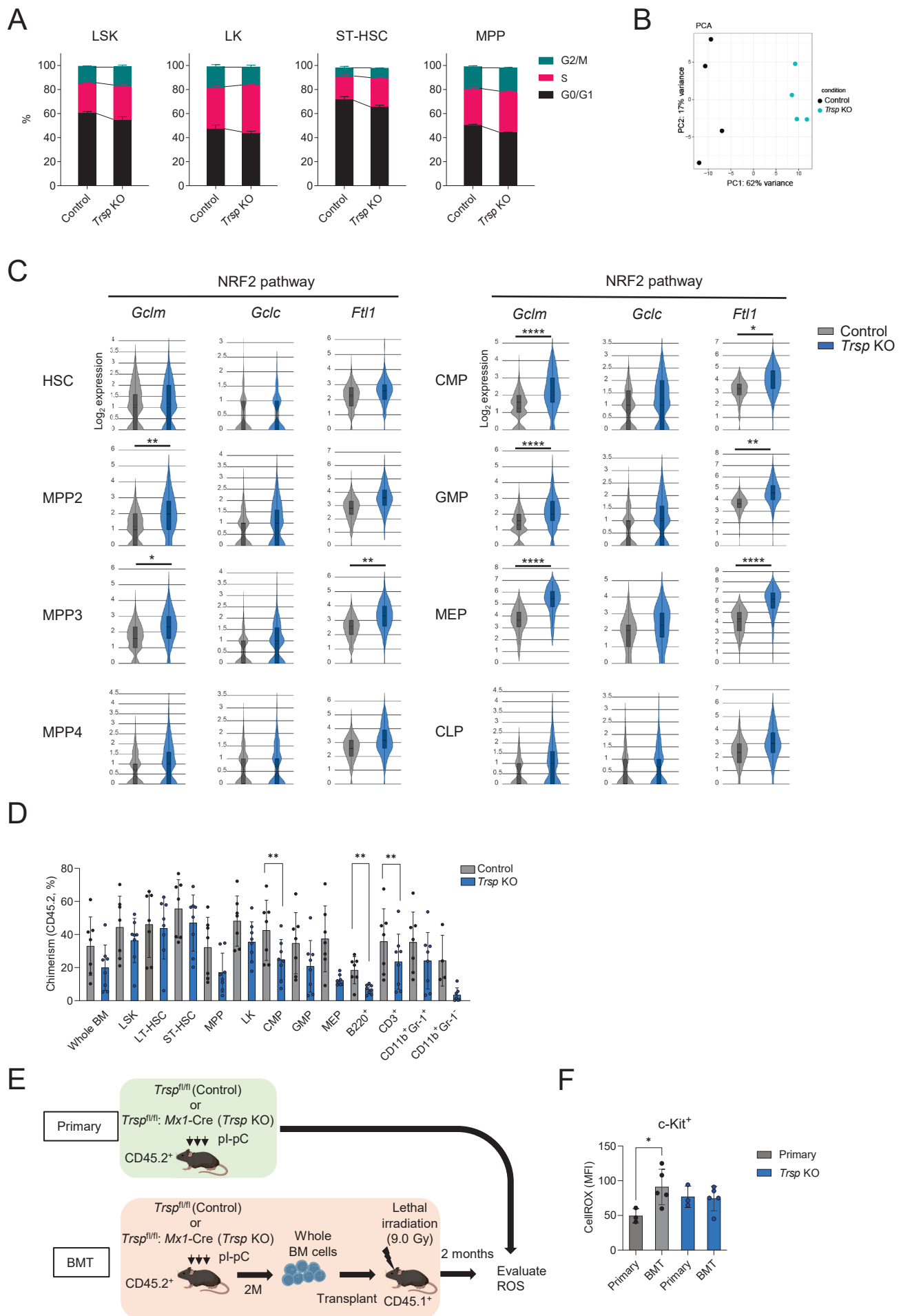
Supplemental Figure 2



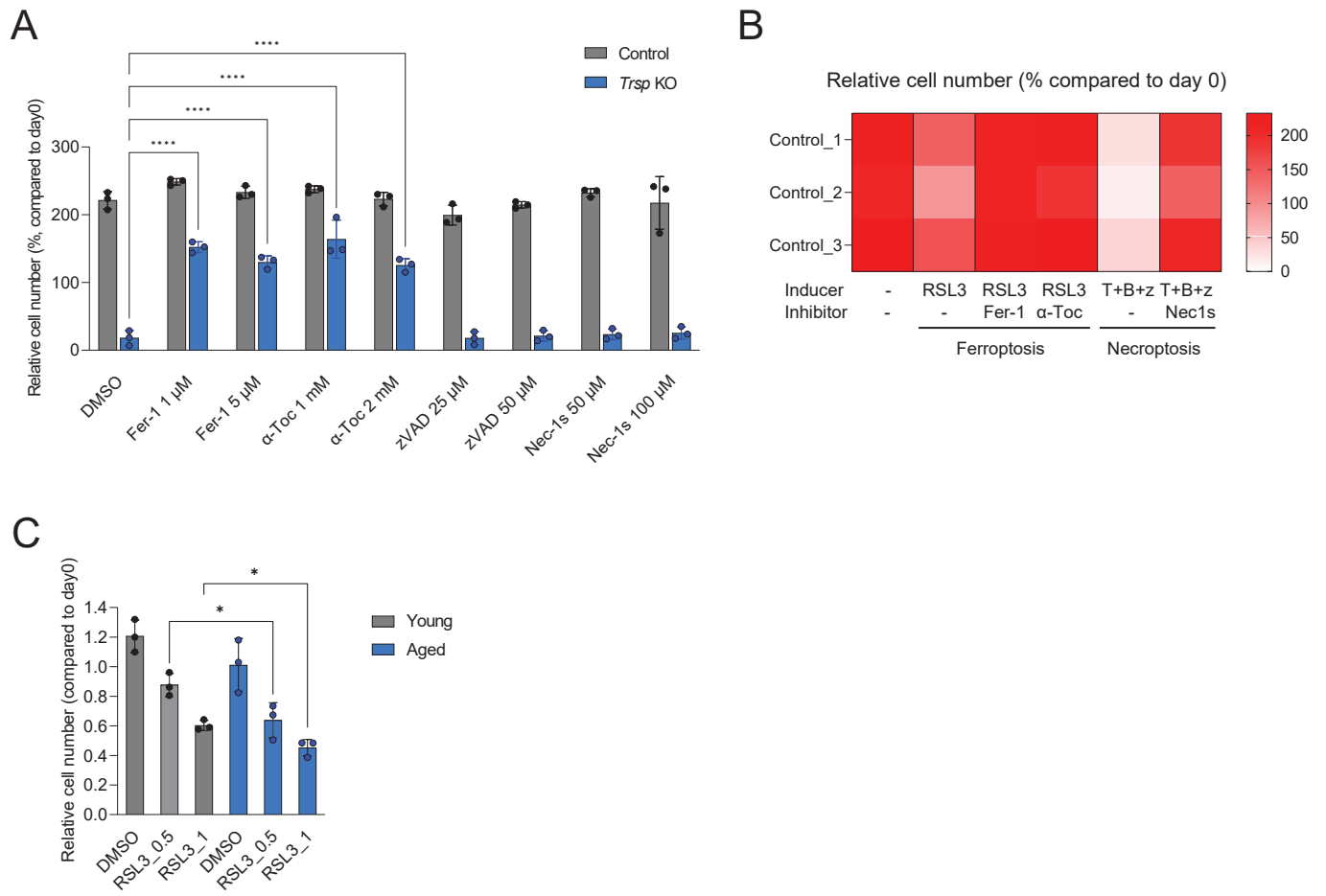
Supplemental Figure 3



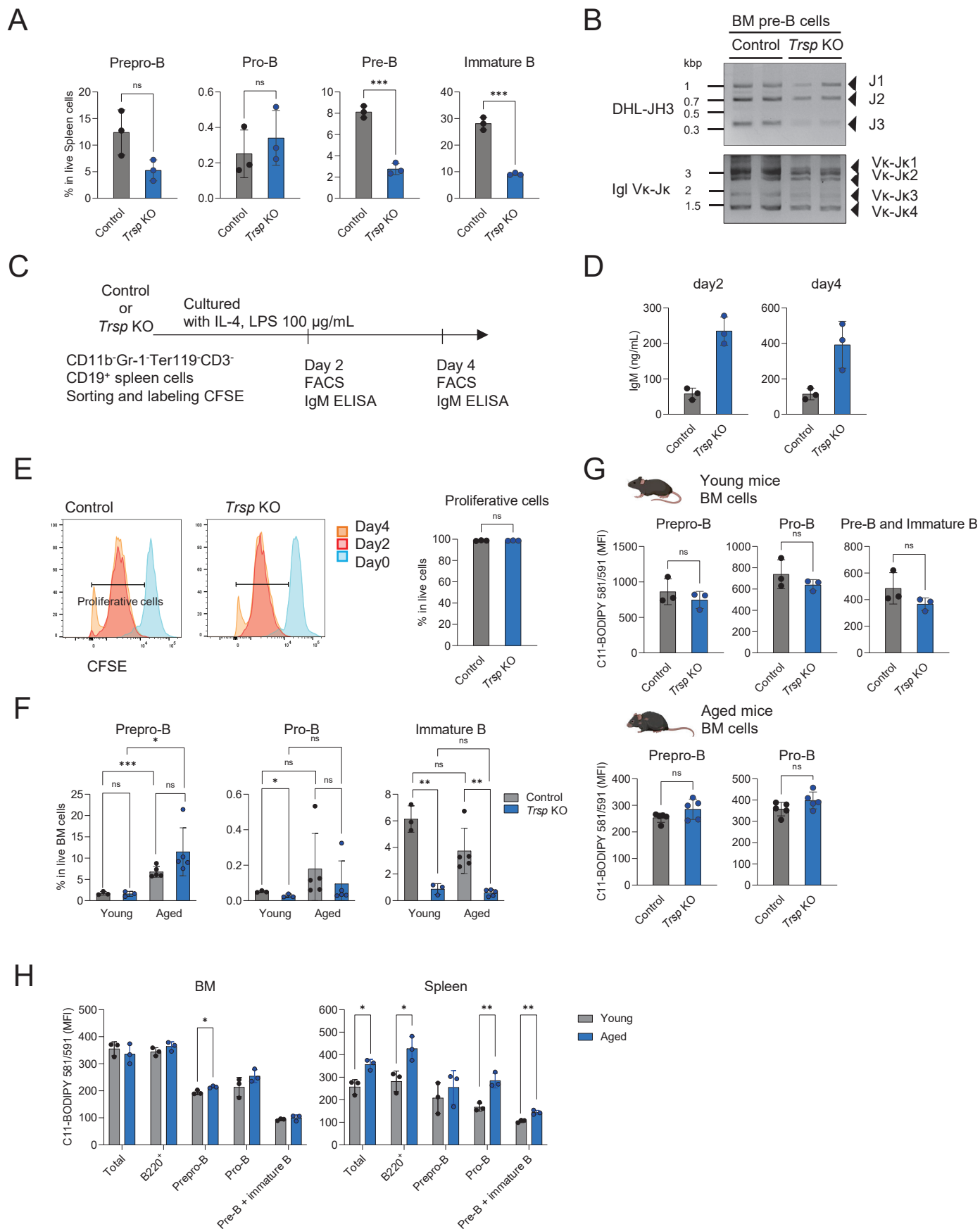
Supplemental Figure 4



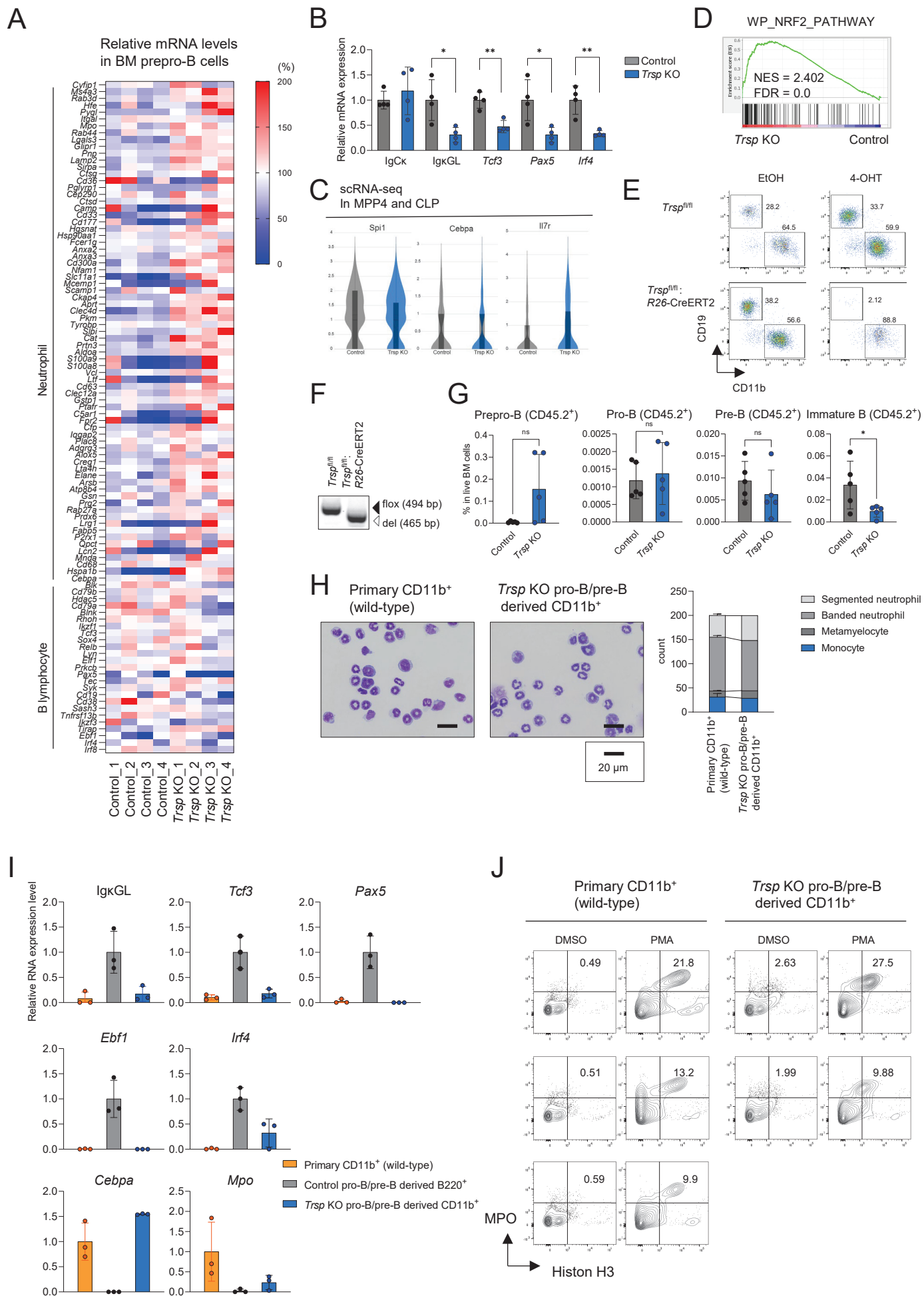
Supplemental Figure 5



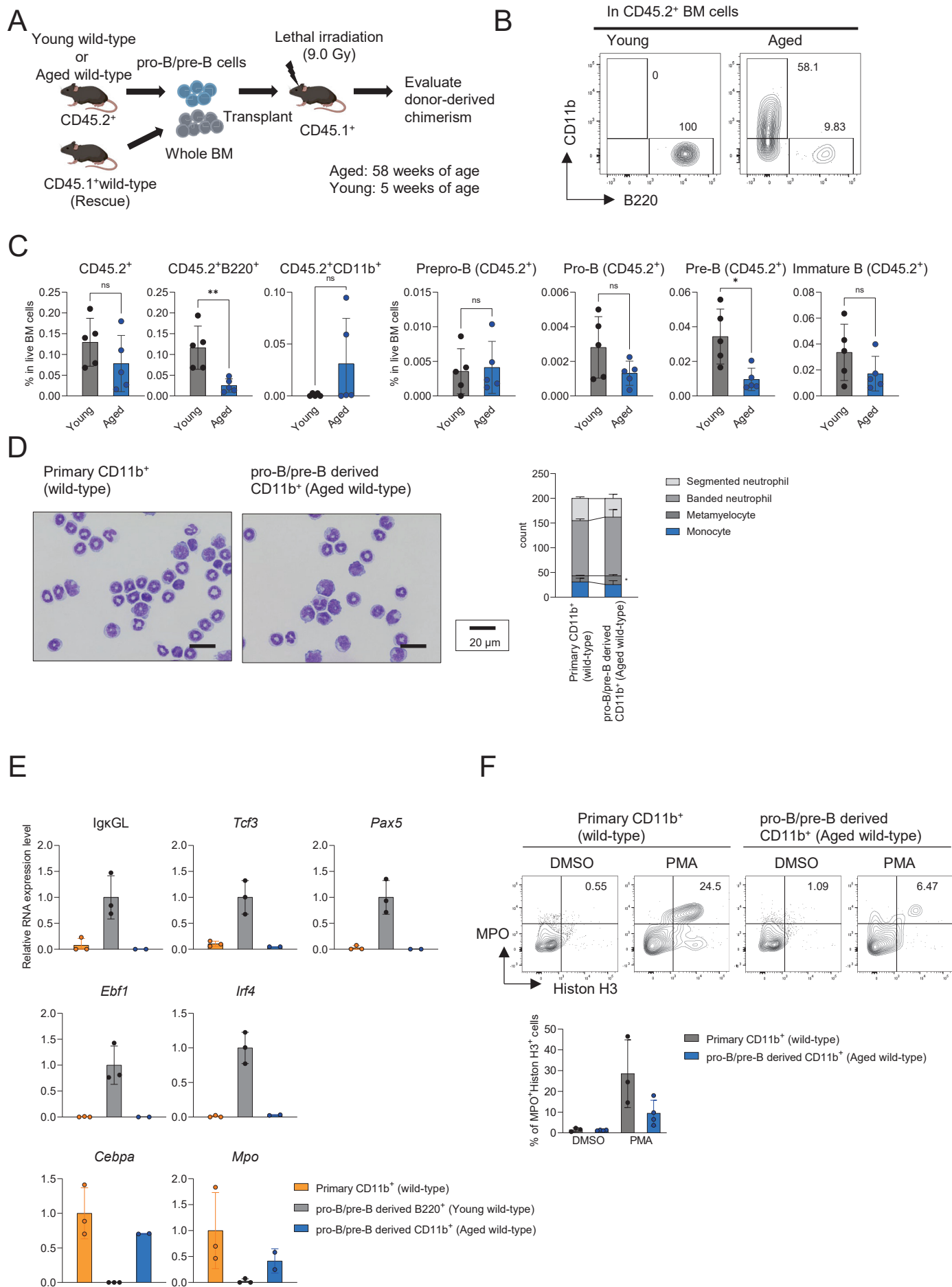
Supplemental Figure 6



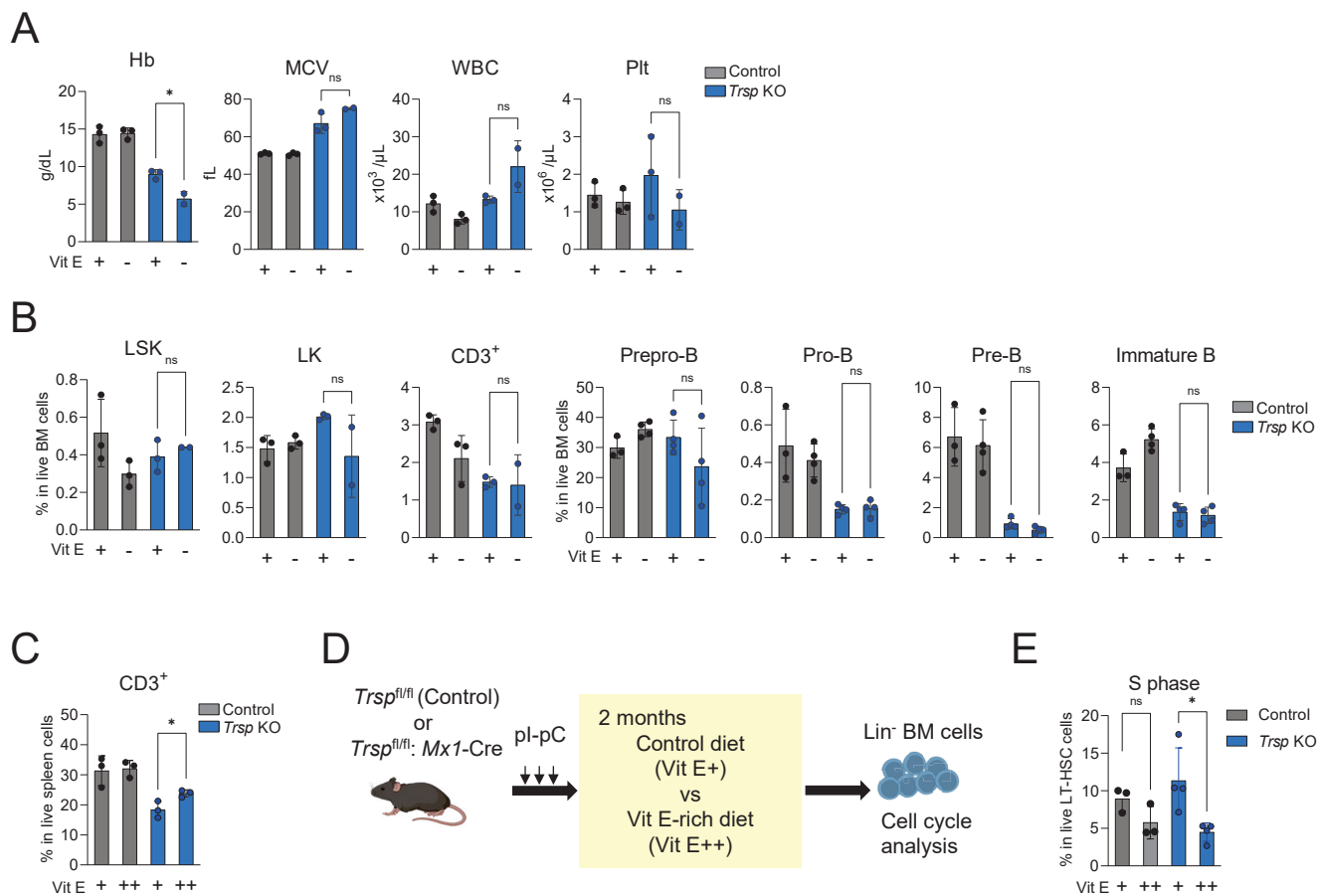
Supplemental Figure 7



Supplemental Figure 8

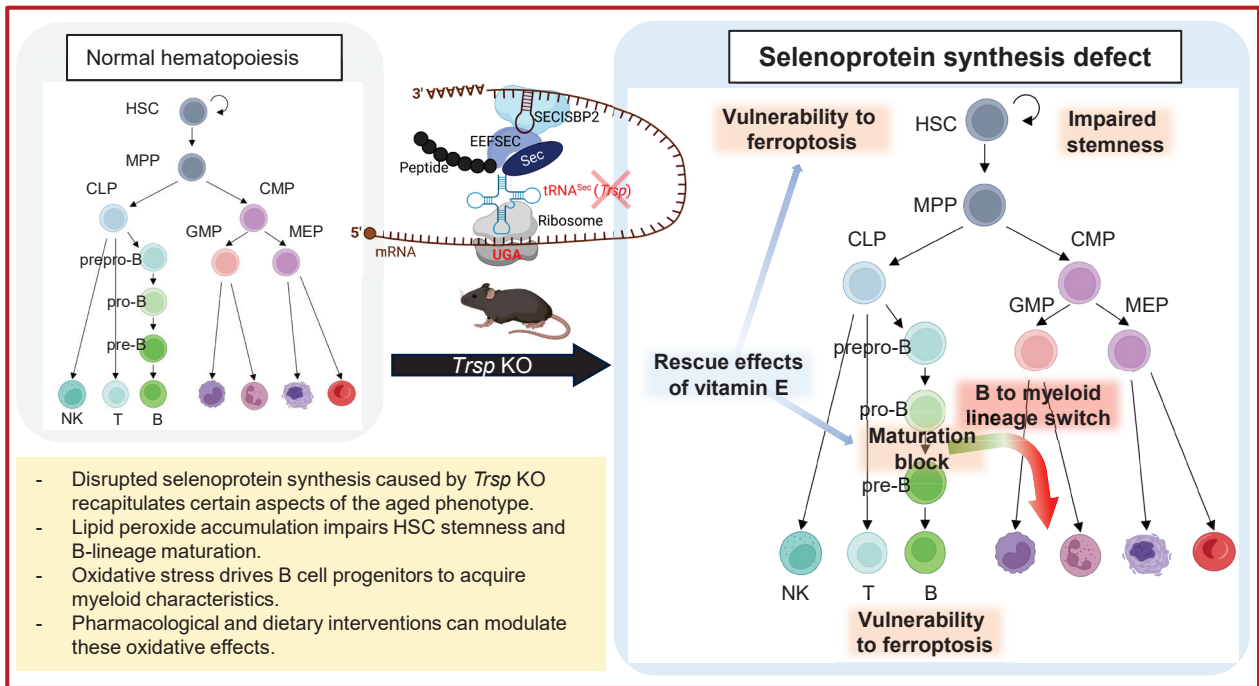


Supplemental Figure 9



Supplemental Figure 10

A



Supplemental Figure 11