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Received: March 6, 2025.

Accepted: June 26, 2025.

Citation: Ruiqing Chen, Qiongye Dong, Lihong Zhou, Hanqing He, Yingxue Du, Qianwen Sun, Toshio Suda, Tao Cheng and Jianwei Wang. Aging-declined RNA exportation impairs hematopoietic stem cells by inducing R-loop.

Haematologica. 2025 July 3. doi: 10.3324/haematol.2025.287765 [Epub ahead of print]

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Aging-declined RNA exportation impairs hematopoietic stem cells by inducing R-loop

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Running title: DNA damage with aging stems from R-loop

Disclosures

No conflicts of interest to disclose.

Author Contributions

Conceptualization, J.W.; Methodology, C.R.; D.Q., Z.L.; Investigation, C.R., D.Q., Z.L., H.H., D.Y., S.Q., T.S., T.C., J.W.; Formal Analysis, J.W.; Resources, J.W.; Writing, T.C., J.W.; Funding Acquisition, D.Q., J.W.; Supervision, T. C., J.W.

Data sharing statement

For original data, please contact Dr. Jianwei Wang (wangjianwei@ihcams.ac.cn). RNA-seq, cytoplasmic and nuclear RNA sequencing, ssDRIP-seq data are available at GEO under accession number GSE239301. Whole Genome sequencing data is available at SRA under accession number PRJNA1000120.

Acknowledgments

We thank Dr. Hong Cheng (Institute of Biochemistry and Cell Biology, University of Chinese Academy of Sciences) for providing constructive suggestions for this project.

Funding

This work was supported by grant numbers 2024-I2M-ZD-010, W2441024, 82250002 and 92249305 to J.W.W. from CAMS Innovation Fund for Medical Sciences (CIFMS) and the National Natural Science Foundation of China; and by 62103397 to Q.Y. D from National Natural Scientific Foundation of China.

Additional Information

Online Supplemental data includes five figures and seven tables.

Abstract

Aging-related accumulation of DNA damage adversely affects hematopoietic stem cell (HSC). However, the mechanisms underlying this accumulation and strategies for its elimination to rejuvenate aged HSCs remain largely obscure. This study uncovers a notable surge in R-Loop presence within aged HSCs, notably co-localized with γ H2AX and RPA (Replication Protein A), and correlated with RNA residency in the nucleus. Targeted induction of R-Loop impairs the function of HSCs. Mechanistically, RNA exportation is compromised in aged HSCs due to a decline in Alyref, the primary constituent of the TREX (transcription-export complex). Specifically, Alyref dysfunction results in RNA retention within the nucleus, mimicking the functional characteristics of aged HSCs. The nuclear accumulation of RNA leads to the formation of RNA:DNA hybrids, known as R-Loop structures, consequently inducing replication stress and DNA damage. Introducing a quantitative boost of Alyref in aged HSCs notably reinstates RNA transportation, diminishes R-Loop formation and replication stress, and ultimately enhances the performance of aged HSCs. Taken together, our research demonstrates the initial revelation that aging-triggered replication stress stems from abnormal RNA transportation-propelled R-Loop configurations, hinting at the potential of quantitatively modulating RNA transportation to mitigate the physiological drawbacks of aging on HSCs.

Introduction

The diminished regenerative capability of tissue-specific stem cells due to aging contributes significantly to disruptions in tissue equilibrium during the aging process¹⁻³. Hematopoietic stem cells (HSCs) are responsible for life-span cell production⁴, and experience a decline in reconstitution capacity with aging⁵. Moreover, aged HSCs display a bias towards myeloid lineage differentiation⁶⁻⁸, posing a risk factor for various hematopoietic malignancies⁹. Therefore, elucidating the molecular mechanisms underlying HSC aging and devising strategies to anti-aging intervention for aged HSCs function will unquestionably benefit the elderly and individuals afflicted by hematopoietic system degenerative changes.

We and others have previously shown that DNA damage accumulation, characterized by γ H2AX, has been considered one of the driving forces of HSC aging¹⁰⁻¹⁵. Faithful DNA replication is pivotal for the maintenance of genome stability. Any molecular event interfering with DNA replication, collectively named replication stress, makes DNA molecules more vulnerable to damage, mutations, or genomic instability¹⁶⁻¹⁸. A previous study has shown that replication stress is a potent driver of functional decline in aged HSCs¹⁰. While, the critical cellular signaling leading to replication stress in aged HSCs has not been clearly elucidated. In this study, we observed that R-Loop, a three-stranded nucleic acid structure consisting of a DNA:RNA hybrid and a displaced strand of DNA, which is generated during DNA transcription^{19, 20}, accumulated in aged HSCs, which impairs HSCs by inducing replication stress and DNA damage. Mechanistically, the retention of RNA in nucleus of aged HSCs, induced by the aging-declined RNA exportation, results in the increase of R-Loop.

Method

Mice

Alyref^{flox/flox} mice were generated in Cyagen Biosciences Inc. (Guangzhou, China). Alyref^{flox/flox} mice (C57BL/6N) were generated by inserting loxP sites spanning the third exon of Alyref via homologous recombination. To achieve

hematopoietic-specifically knockout mice, Alyref^{flox/flox} were crossed to Mx1-Cre mice. To induce Cre expression in Mx1-Cre⁺; Alyref^{flox/flox} mice were intraperitoneally injected with Poly I:C (25 mg/kg) every other day for 14 days. All genotyping primers are listed in *Online Supplementary Table S1*.

The recipients used in the competitive transplantation assays were CD45.1/2 that were the first generation of C57BL/6 (CD45.2) and B6.SJL (CD45.1) mice. All mice were housed in specific-pathogen-free, and all procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Tsinghua University.

RNA fluorescent in situ hybridization

RNA FISH was conducted using a Ribo fluorescence in situ hybridization kit (RiboBio, C10910) in accordance with the manufacturer's directions. For the R-Loop and mRNA co-localization assay, cells were performed for RNA FISH after cells incubated by Anti-DNA-RNA Hybrid, clone S9.6 and secondary antibodies.

Cytoplasmic and nuclear RNA fractionation and sequencing

The cytoplasmic and nuclear fractions derived from CD34⁺LSK cells were used to generate cDNA libraries, which were sequenced with 150-bp paired-end reads on an Illumina instrument by Novogene.

R-Loop peak calling

Strand-specific peaks were called by MACS3 (v3 3.0.0b1) with R-Loop and input samples. Peaks with q values less than 0.01 and enrichment score more than 10, and located outside the blacklisted region were defined as R-Loop peaks. We annotate peaks with genomic regions by R package ChIP seeker.

ssDRIP-seq and ssDRIP-qPCR

ssDRIP-seq library construction was performed according to published procedures²¹. Freshly isolated CD34⁺ LSK cells were lysed in DNA lysis buffer and incubated at 37°C. Genomic DNA was fragmented using restriction enzymes and subjected to DRIP with the S9.6 antibody. DRIP DNA was validated by qPCR or sonicated for ssDRIP-seq library construction, followed by sequencing on an Illumina NovaSeq system.

dCas9 coupled RNaseH1 D209N-mediated site-specific R-Loop

dCas9 coupled RNaseH1 D209N-mediated site-specific R-Loop was performed according to published reference ²². In brief, dCas9 infused with RNaseH1 D209N was co-expressed with gRNA targeting the selected R-Loop sites in NIH-3T3 cells. mCherry⁺ cells were purified for ssDRIP-qPCR and Immunofluorescence staining. The gRNA and qPCR primers are listed in *Online Supplementary Table S6*.

RNA-seq

In brief, 50 HSCs were sorted directed into lysis buffer. Then the RNA-seq library was prepared using the method of Smart-seq2. After the library construction, assess the insertion size by Agilent Bioanalyzer 4200 system (Agilent Technologies), and quantify the accurate insertion size by Taqman fluorescence probe of AB Step One Plus Real-Time PCR system. Perform the clustering of the index-coded samples using HiSeq PE Cluster Kit v4-cBot-HS (Illumina) following the manufacturer's instructions. The libraries were sequenced by an Illumina Hiseq platform with 150-bp paired-end. The transcriptome sequencing was performed by ANNOROAD Gene Technology Company.

Statistical Analysis

Data are shown as mean $\pm$ SD. Student's *t* test (Two-tailed unpaired) were used for comparisons (GraphPad Prism v.7.0). ns, not significant, **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001. All experiments were repeated 2 or 3 times independently.

Result

R-Loop is increased in aged hematopoietic stem cells

We conducted immunofluorescence for R-Loop using the S9.6 antibody ²³, and observed accumulated R-Loop foci in aged HSCs (Figure 1 A-D), with RNase H, but not RNase III, serving as a positive control to dissolve R-Loops ^{24,25}.

We then profiled genome-wide R-Loop at single-nucleotide resolution in young and aged HSCs to systemically examine the correlation of R-Loops with aging by conducting ssDRIP-seq according to a recently developed method ²¹ (*Online Supplementary Figure S1A*). As expected, R-Loops correlate with high G/C skewing

in both young and aged HSCs (Figure 1E), indicating accurate mapping of R-Loops by ssDRIP-seq data. We then examined the global distribution of age-associated changes in R-Loops across the mouse genome. The results show that most R-Loops are located in the intergenic region (~50%) and gene body region (~40%), which includes the 5'UTR, 3'UTR, exons, and introns, with no significant differences observed between young and aged HSCs (Figure 1F). However, 6.6% of R-Loop peaks are located in the promoter region of young HSCs, and this significantly increases to 10% with aging (Fisher's exact test, odd ratio = 1.5, p value = 1.97e-10) (Figure 1F). To further characterize the increase of R-Loops with aging, we performed a genome browser inspection and observed a significant increase in R-Loop signals from 2 kb upstream of the TSS to downstream of the TES in aged HSCs (*Online Supplementary Figure S1B*). Additionally, we noted increased R-Loop coverage in aged HSCs (*Online Supplementary Figure S1C*), which aligns with previous reports²⁶. We quantified the peak width in aged and young samples. Consistent with these observations, the results show that the width of R-Loop peaks exhibit significant increase in aged HSCs (*Online Supplementary Figure S1D*). To delve deeper into the molecular characteristics influenced by aging-associated increases in R-Loops, we conducted Gene Set Enrichment Analysis (GSEA) of genes ranked based on their differential R-Loop signals in the gene body region between aged and young HSCs. The results indicate that the augmented presence of R-Loops is enriched with genes associated with cell aging and myeloid functions, which is a classical feature of aged HSCs (*Online Supplementary Figure S1E, Online Supplementary Table S7*).

Accumulated R-Loop induces DNA damage and impairs the function of hematopoietic stem cells

Previous studies have demonstrated that aged HSCs accumulate DNA damage, as evidenced by increased γ H2AX foci¹¹. To investigate this further, we conducted whole-genome sequencing of young and aged HSPCs to identify such damage between them. Surprisingly, we observed no significant increase in Insertions and Deletions (InDels) in aged HSPCs, suggesting that physiological DNA breaks are not elevated with aging (Figure 2A). Subsequently, we performed immunofluorescence

staining for γ H2AX and R-Loops in aged HSCs. The results revealed co-localization between them (Figure 2B). Since γ H2AX is also indicative of replication stress¹⁰, we further investigated immunofluorescence staining for RPA, a marker of replication stress^{10, 17, 27, 28}. Remarkably, R-Loops co-localized with RPA foci in aged HSCs (*Online Supplementary Figure S2A*), indicating an association between R-Loops and replication stress during aging.

To further explore the causal relationship between R-Loops and DNA damage, we focused on Nfe2l2, which modulates the migration and retention of HSCs²⁹ and is enriched with R-Loop in aged HSCs (Figure 2C). Upon introducing an R-Loop in the intron of Nfe2l2 (*Online Supplementary Figure S2B* and Figure 2D, refer to the Methods section for details), we observed the accumulation of RPA and γ H2AX foci (Figure 2E, F), suggesting that the elevation of R-Loops indeed triggers DNA damage in HSCs.

To further investigate the causation between R-Loop and the functional decline of HSCs, we generated two efficient shRNAs against RNaseH1 and RNaseH2A (*Online Supplementary Figure S2C*), the key enzymes responsible for dissolving R-Loops³⁰⁻³². By conducting a competitive transplantation assay, we observed that knockdown of these enzymes impairs the reconstitution capacity of HSCs and promotes a myeloid differentiation bias (*Online Supplementary Figure S2D, E*), which are key features of aged HSCs. Bone marrow analysis exhibited compromised reconstitution capacity and a reduction of HSCs upon knockdown of these enzymes (*Online Supplementary Figure S2F, G*). Moreover, we purified GFP⁺ hematopoietic stem and progenitor cells from the recipients at the end of the third month and examined the level of R-Loop. The results show that the level of R-Loop is significantly increased in cells carrying shRNaseH1 and shRNaseH2A (*Online Supplementary Figure S2H, I*). It is notable that these cells exhibit significant increased γ H2AX foci (*Online Supplementary Figure S2J, K*).

In brief, the above data suggest that the accumulation of R-Loops co-localizes with DNA damage signal and results in the functional decline of HSCs.

Aging-increased R-Loop correlates with the lingering RNA in the nucleus of

hematopoietic stem cells

Given that the R-Loop is a microstructure of chromosomes composed of DNA:RNA strands, we then hypothesize whether the increase in R-Loops correlates with the retention of RNA in the nucleus of aged HSCs. To explore this hypothesis, we categorized the genes into four groups based on the fold change of R-Loop signals between aged and young HSCs: Category-1 having the greatest loss of R-Loops and Category-4 with the greatest gain of R-Loops. The results reveal that genes showing increased R-Loop formation display a higher ratio of nuclear to cytoplasmic RNA with aging (Figure 3A). This suggests that the accumulation of RNA in the nucleus of aged HSCs might contribute to the formation of R-Loops.

To corroborate this observation, we focused on two genes: *Nfe2l2* and *Adgrg1*, which play crucial role in regulating HSCs^{29, 33}. Our observations indicate that the mRNAs of *Nfe2l2* and *Adgrg1* accumulate in the nucleus of aged HSCs (Figure 3B, C). Moreover, the R-Loop signal across the *Nfe2l2* and *Adgrg1* loci increases with aging (Figure 2C, 3D). RNA fluorescence *in situ* hybridization (FISH) and Immunofluorescence staining reveal the co-localization of their RNA and the accumulated R-Loops within them (Figure 3E).

In summary, these findings suggest that R-Loops are heightened in aged HSCs and are associated with the accumulation of RNA in nucleus during aging.

RNA exportation is impaired in aged hematopoietic stem cells

We then conducted an RNA FISH assay to assess the bulk poly(A)⁺RNA distribution between the nucleus and cytoplasm of both young and aged HSCs, aiming to scrutinize RNA distribution patterns associated with aging. Our findings indicate a notable accumulation of mRNA within the nucleus of aged HSCs compared to their younger counterparts (Figure 3F, *Online Supplementary Figure S3A*). To corroborate this observation, we analyzed the profiles of nuclear and cytoplasmic RNA from both young and aged HSCs. The results unequivocally demonstrate a significant increase in mRNA accumulation within the nucleus of aged HSCs (*Online Supplementary Figure S3B*).

To unravel the molecular mechanisms underlying this phenomenon, we initiated

an integrative analysis of five previously published datasets (RNA-sequencing data of young and aged HSCs³⁴⁻³⁸). Our examination revealed a significant enrichment of Gene Ontologies related to RNA localization with aging (*Online Supplementary Figure S3C*). Delving deeper, we found a notable impairment in the RNA transportation pathway within aged HSCs (*Online Supplementary Figure S3D*), with Alyref emerging as the most significantly downregulated gene (*Online Supplementary Figure S3E*). Subsequently, we embarked on validating the alteration of Alyref expression between young and aged HSCs, confirming a marked decrease in Alyref at both the mRNA and protein levels with aging (*Online Supplementary Figure S3F, G*).

Targeted dysfunction of Alyref induces R-Loop and disrupts hematopoietic homeostasis

To probe the functional implications of Alyref in HSC aging, we generated Alyref^{flox/flox} mice (*Online Supplementary Figure S4A*), and bred them with Mx1-Cre mice to produce Mx1-Cre; Alyref^{flox/flox} and Mx1-Cre; Alyref^{flox/+} offspring. Upon poly I:C treatment for 14 days, Alyref is uniformly and heterozygously deleted in hematopoietic cells yielding Alyref^{-/-} and Alyref^{+/-} mice, respectively (*Online Supplementary Figure S4B, C*). We observed increased and co-localized signals among R-Loops, γ H2AX and RPA in Alyref^{-/-} and Alyref^{+/-} HSCs (Figure 4A, B, *Online Supplementary Figure S4D, E*). Additionally, replication fork assays exhibited increased replication stress in Alyref^{+/-} (Figure 4C), Alyref^{-/-} HSCs (Figure 4D) and aged HSCs (Figure 4E). Collectively, these data indicate that dysfunction of Alyref lead to an increase in R-Loops, DNA damage and replication stress in HSCs.

We then sought out to examine the hematopoietic phenotype of Alyref^{+/-} and Alyref^{-/-} mice. Complete blood count assay revealed a notable decrease in white blood cell (WBC), neutrophil (NEUT), lymphocyte (LYM), basophilic granulocyte (BAS) and platelet (PLT) counts in Alyref^{-/-} mice, contrasting with Alyref^{+/-} counterparts (Figure 4F). However, both strains exhibited a drop in red blood cell (RBC), hemoglobin (HGB) and hematocrit (HCT) levels (Figure 4F and *Online Supplementary Figure S4F*). The bone marrow cellularity of Alyref^{-/-} mice exhibited a significant decrease compared to their littermate controls (*Online Supplementary*

Figure S4G). Subsequently, we delved into investigating the lineage composition in the peripheral blood (PB) and bone marrow (BM) of both *Alyref*^{+/-} and *Alyref*^{-/-} mice, focusing on T cells, B cells, and myeloid cells. Our analysis unveiled a significant increase in T lineage cells and a decrease in B and myeloid lineages in both PB and BM of *Alyref*^{-/-} mice, contrasting with *Alyref*^{+/-} mice (*Online Supplementary Figure S4H, I*). Moving forward, we scrutinized the hematopoietic stem and progenitor cells of these mice, encompassing common myeloid progenitor (CMP), granulocyte-macrophage progenitor (GMP), megakaryocyte-erythroid progenitor (MEP), common lymphoid progenitor (CLP), multipotent progenitor cell (MPP), short-term hematopoietic stem cell (ST-HSC) and Long-term hematopoietic stem cell (LT-HSC). Strikingly, our findings demonstrated a dramatic decrease in all populations within *Alyref*^{-/-} mice, while no such effect was observed in *Alyref*^{+/-} mice (*Online Supplementary Figure S4J-L*). These findings suggest that short-term heterozygous deletion of *Alyref* does not significantly impact hematopoietic homeostasis. To further explore the long-term effects, we retained *Alyref*^{+/-} mice for 10 months and observed an increase in RNA accumulation within the nucleus of HSCs (*Online Supplementary Figure S4M, N*). Notably, complete blood count assay revealed evident decreases in WBC, LYM, HGB and HCT levels (*Online Supplementary Figure S4O*). Moreover, the BM cellularity of *Alyref*^{+/-} mice displayed a mild decrease compared to their littermate controls (*Online Supplementary Figure S4P*). Further analysis of lineage composition unveiled a significant decrease in B lineage cells and a mild increase in myeloid lineage cells in the PB, but not BM, of *Alyref*^{+/-} mice (*Online Supplementary Figure S4Q, R*). Additionally, hematopoietic stem and progenitor cells (LSK), ST-HSC, GMP and CLP exhibited a significant decrease in *Alyref*^{+/-} mice (*Online Supplementary Figure S4S, T*).

In summary, these data collectively indicate that *Alyref* plays a crucial and dosage-dependent role in maintaining hematopoietic homeostasis.

Targeted dysfunction of *Alyref* impairs hematopoietic stem cells

Subsequently, we conducted a competitive transplantation assay to assess the reconstitution capacity of *Alyref*^{+/-} and *Alyref*^{-/-} HSCs (*Online Supplementary Figure*

S4U). The findings revealed compromised reconstitution capacity in both Alyref^{+/-} and Alyref^{-/-} HSCs (*Online Supplementary Figure S4V, W*). Notably, the reconstitution capacity of Alyref^{-/-} HSCs was significantly lower than that of Alyref^{+/-} HSCs, suggesting a dosage-dependent effect of Alyref on HSC function. To further evaluate the self-renewal capacity of Alyref^{+/-} and Alyref^{-/-} HSCs, we conducted a secondary transplantation assay using BM cells from primary recipients (*Online Supplementary Figure S4U*). The results demonstrated a significant impairment in the self-renewal capacity of both Alyref^{+/-} and Alyref^{-/-} HSCs (*Online Supplementary Figure S4V*). Additionally, both groups exhibited a myeloid-biased potential (*Online Supplementary Figure S4X*), characteristic of aged HSCs.

Further exploration of the molecular distinctions among Alyref^{+/-}, Alyref^{-/-} and WT HSCs, involved RNA-sequencing analysis of these cells, revealing distinct groupings by Principal component analysis (PCA) analysis (*Online Supplementary Figure S4Y*). Gene Set Enrichment Analysis (GSEA) unveiled the loss of HSC fingerprint genes and activation of cell aging-related genes in both Alyref^{-/-} and Alyref^{+/-} HSCs (*Online Supplementary Figure S4Z, Online Supplementary Table S7*).

In summary, these data underscore that targeted dysfunction of Alyref recapitulates the characteristics of aged HSCs, and demonstrates a dosage-dependent effect.

Dysfunction of RNA transportation impairs hematopoietic stem cells

The aforementioned results strongly suggest a potential role for Alyref-mediated RNA transportation in HSC aging. To further investigate this hypothesis, we sought to determine whether the dysfunction of other RNA transporters affects the reconstitution capacity of HSCs. To this end, we developed two efficient shRNAs targeting Thoc1 and Thoc5 (Figure 5A), essential components of the RNA transportation complexes TREX and AREX³⁹, respectively. RNA FISH analysis revealed that knockdown of these genes resulted in the accumulation of Poly(A)⁺RNA in the nucleus of hematopoietic stem and progenitor cells (Figure 5B). Subsequently, we conducted competitive transplantation assays to assess the functional impact of their knockdown on HSCs. The results demonstrated that knockdown of Thoc1 and

Thoc5 severely impaired the reconstitution capacity of HSCs (Figure 5C, D).

Bulk mRNA nuclear export relies on the RNA export receptor Nxf1, which interacts with FG-repeat containing nucleoporins to facilitate RNA translocation^{40, 41}. We subsequently designed and tested two highly effective shRNAs targeting Nxf1 (*Online Supplementary Figure S5A*). Upon performing the same assay, we observed that Nxf1 knockdown leads to the accumulation of Poly(A)⁺RNA in the nucleus (Figure 5E) and significantly diminishes the reconstitution capacity of HSCs (Figure 5F).

Taken together, these findings demonstrate that dysfunction of RNA transportation genes causes RNA buildup in the nucleus of HSCs, thereby compromising their function.

Quantitative refill of Alyref rescues the function of aged hematopoietic stem cells by dampening R-Loop and replication stress

We then explored whether overexpression of Alyref could rescue the function of aged HSCs. To test this hypothesis, we cloned the cDNA of mouse Alyref into a lentiviral vector⁴², which resulted in efficient overexpression of ALYREF (*Online Supplementary Figure S6A*). Subsequently, we conducted competitive transplantation assays to assess the functional impact of enforced Alyref on HSCs. Surprisingly, it was found that overexpression of Alyref impaired the reconstitution capacity of aged HSCs (Figure 6A). A previous study has indicated that overexpression of Alyref leads to the accumulation of RNA in nucleus⁴³. To investigate this further, we performed RNA fluorescence in situ hybridization (FISH) assays to examine the RNA distribution in Alyref-overexpressed HSCs. The results confirmed that RNA is indeed accumulated in the nucleus upon Alyref overexpression (Figure 6B).

The earlier results have demonstrated that Alyref modulates the function of HSCs in a dosage-dependent manner (*Online Supplementary Figure S4V*). Subsequently, we quantified the protein level of ALYREF in young and aged HSCs, revealing a 50% decrease in ALYREF levels in aged HSC (*Online Supplementary Figure S3G, 6C, left histogram*). However, lentiviral-mediated ALYREF overexpression resulted in a 45-fold increase (Figure 6C, right histogram), explaining

the cytotoxic effect of Alyref overexpression on aged HSCs. To precisely replenish ALYREF levels in aged HSCs, we developed a quantitative gene expression system (QGES) to compensate for the drop of ALYREF. In this system, QGES-2^{Alyref} exhibits a 2-fold increase (Figure 6D). We conducted competitive transplantation assays to assess the functional role of quantitative refill of ALYREF on aged HSCs using QGES-2^{Alyref} system. LSK cells isolated from 18 months old WT mice were infected with QGES-2^{Alyref} and empty control lentivirus. After 72 hours, 4000 CD48⁻ Sca1⁺ GFP⁺ cells were FACS-purified and transplanted into lethally irradiated recipients along with 3×10⁵ competitor cells, which were isolated from 2- to 3-month-old CD45.1 mice (*Online Supplementary Figure S6B*). Donor-derived chimerism was examined every 4 weeks until the 16th week. The results showed a mild, though not significant (p value is 0.18), increase in the QGES-2^{Alyref} group (Figure 6E). Subsequently, we performed secondary transplantation from primary recipients, and the results revealed that aged HSCs with quantitative expression of Alyref significantly outcompeted the control group (Figure 6E), indicating that precise compensation of ALYREF in aged HSCs significantly rescues their self-renewal capacity.

By conducting RNA FISH, we observed that the aberrant RNA accumulation in the nucleus of aged HSC is rescued by precise refill of ALYREF (Figure 6F). Moreover, we observed dramatic reduction in R-Loop (*Online Supplementary Figure S6C, D*), γ H2AX (*Online Supplementary Figure S6E, F*) and RPA (*Online Supplementary Figure S6G, H*) foci in HSCs carrying the QGES-2^{Alyref}- construct. The replication fork assay showed a decrease in replication stress in the QGES-2^{Alyref} group (*Online Supplementary Figure S6I, J*).

Collectively, these data indicate that precise refill of ALYREF in aged HSCs rescues the self-renewal capacity of aged HSCs by diminishing DNA damage and replication stress.

Discussion

Our study demonstrates that the imbalance of RNA distribution between the nucleus and cytoplasm, mediated by compromised RNA transportation, contributes to HSC

aging by inducing R-Loops and subsequent replication stress (*Online Supplementary Figure S6K*). Furthermore, quantitative refill of ALYREF ameliorates the function of aged HSCs. This study delves into the functional role of R-Loop in HSC aging and unveils a novel mechanism driving HSC aging.

RNA imbalance promotes hematopoietic stem cell aging

The homeostasis of RNA distribution between nucleus and cytoplasm is vital for cellular function, wherein RNA transportation playing a central role. In this study, we observed that the RNA distribution between the nucleus and cytoplasm of aged HSCs became unbalanced, stemming from the decline of Alyref. Targeted dysfunction of Alyref recapitulated the phenotype of aged HSCs, while precise refill of it rejuvenated the function of aged HSCs by restoring RNA shuttling and dampening R-Loops. Since stable expression of Alyref is crucial for hematopoietic homeostasis and HSC function, investigating the molecular mechanisms compromising Alyref during aging is worthy of further study. A previous study has reported that Alyref specifically interacts with m⁵C RNA ⁴⁴. It is intriguing to explore the specificity of RNA transported by Alyref, whether it is mediated by m⁵C or other RNA modifications.

DNA damage vs aging and aging-related diseases

Aging is the highest risk factor for many diseases. It has been reported that aged blood induces cell and tissue senescence and *vice versa*, young blood revitalizes aged organism ⁴⁵⁻⁴⁹. Aged HSCs compromise the function of blood system. Therefore, rejuvenating aged HSCs will benefit the function of aged organism.

On the basis of R-Loop findings of this study, we open a new aspect in the aging study. Firstly, we demonstrated that aging-accumulated R-loop is the resource of replication stress. Accumulated R-Loop results in more displaced ssDNA, which is vulnerable to damage ²⁰. In addition, accumulated R-Loop interfere with proper DNA repair machinery, especially the homologous recombination pathway. Secondly, R-Loop is a multi-faced player in modulating transcription by influencing promoter activity or chromatin structure, altering the local chromatin environment, promoting epigenetic modifications like DNA methylation or histone modifications ²⁰. Hence, in addition to R-Loop-induced replication stress, whether other aforementioned molecular events,

such as those involving long non-coding RNAs (lncRNAs) and DNA damage response RNAs (DDRNs)⁵⁰, participate in regulating hematopoietic stem cell aging is worthy of further exploration.

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Figure legends

Figure 1. R-Loop is increased in aged hematopoietic stem cells.

(A) Representative images depicting immunofluorescence staining of S9.6 in both young and aged HSCs. Freshly isolated HSCs (CD34⁺ Flt3⁺ LSK) from young (2 months) and aged (24-30 months) mice were subjected to immunofluorescence staining. The cells were either left untreated (-RNase H) or were instead treated with RNase H (+RNase H). The panels are shown at the same exposure. The cell nuclei were stained with DAPI.

(B) Representative images depicting immunofluorescence staining of S9.6 in both young and aged HSCs. Freshly isolated HSCs from young (2 months) and aged (24-30 months) mice were subjected to immunofluorescence staining. The cells were either left untreated (-RNase III) or were instead treated with RNase III (+RNase III). The panels are shown at the same exposure. The cell nuclei were stained with DAPI.

(C-D) S9.6 foci distribution in both young and aged HSCs treated with RNase H (C) or RNase III (D).

(E) Metaplot showing G/C skews, A/T skews, G/C contents and base compositions in 500 bp upstream and downstream regions of R-Loop peak summits in young and aged HSCs.

(F) Barplot showing the genomic distribution of R-Loop strand-specific peaks (forward and reverse strand separately) in both young and aged HSCs.

Figure 2. Accumulated R-Loop induces DNA damage and impairs the function of hematopoietic stem cells.

(A) The absolute number of insertions and deletions (InDels) in young (2 months) and aged (24-30 months) HSPCs. Freshly isolated LSK cells from young and aged mice were subjected to Whole Genome Sequencing.

(B) Representative fluorescence images depicting the co-localization of the R-Loop with γ H2AX in aged HSCs.

(C) Representative tracks showing aged HSCs-specific R-Loop peaks (red, Aged HSCs; blue, Young HSCs) in the region comprising Nfe2l2.

(D) ssDRIP-qPCR monitoring of the R-Loop levels at specific regions. Data are presented as mean $\pm$ SD.

(E-F) Representative fluorescence images depicting the co-localization of the R-Loop with RPA (E) and γ H2AX (F) in NIH-3T3 cells.

Figure 3. Aging-increased R-Loop correlates with the lingering RNA in the nucleus of hematopoietic stem cells.

(A) Boxplot of the relative Nucleus/Cytoplasm ratio in four groups of genes with different R-Loop signal changes that occurred during aging. DESeq2 was performed to measure the difference in ssDRIP reads in the gene body and its regulatory region in both young and aged HSCs. The genes were divided into four groups according to the quantiles of the DESeq2 test statistic. Genes belonging to level 1 have the highest R-Loop loss, while genes in level 4 have the highest R-Loop gain. The Y-axis indicates the Nucleus/Cytoplasm ratio of aged samples divided by the same ratio in young samples (one sided Wilcoxon signed rank test).

(B) Histograms depicting the nuclear and cytoplasmic mRNA ratio of Nfe2l2 and Adgrg1 in both young and aged HSCs. The data were obtained from nuclear and cytoplasmic RNA-seq.

(C) Representative images depicting the Nfe2l2 and Adgrg1 mRNA in aged HSCs. Freshly isolated HSCs from young (2 months old) and aged (24-27 months old) mice were subjected to the assay. The panels are shown at the same exposure. The cell nuclei were stained with DAPI.

(D) Representative tracks showing aged HSCs-specific R-Loop peaks (red, Aged HSCs; blue, Young HSCs) in the region comprising Adgrg1.

(E) Representative images depicting the co-localization of the R-Loop with Nfe2l2 and Adgrg1 mRNA in aged HSCs. Freshly isolated HSCs from young (2 months old) and aged (24-27 months old) mice were subjected to the co-localization assay. The panels are shown at the same exposure. The cell nuclei were stained with DAPI.

(F) Representative images showing the distribution of Poly(A)⁺RNA in young and aged HSCs. These images were obtained by subjecting freshly isolated HSCs (CD34⁺

Flt3⁻ LSK) from young (2 months) and aged (24-30 months) mice to RNA fluorescence in situ hybridization (FISH) using a Cy3-labelled oligo(dT)₅₀ probe to visualize Poly(A)⁺RNA. Nuclei were stained with DAPI. The panels are shown at the same exposure for accurate comparison.

Figure 4. Targeted dysfunction of Alyref induces R-Loop and disrupts hematopoietic homeostasis.

(A-B) Representative fluorescence images depicting the co-localization of the R-Loop with RPA (A) and γ H2AX (B) in Alyref^{+/-} HSCs.

(C, left representative image) Representative images depicting the CldU/IdU-labelled DNA replication tracks in WT and Alyref^{+/-} HSCs (12 months).

(C, right dot plot) Dot plots depicting the DNA replication fork symmetry in WT and Alyref^{+/-} HSCs. The dot plot of IdU to CldU track length ratios for individual replication forks. Data are represented as mean $\pm$ SD. A minimum of 493 molecules were analyzed in each group.

(D, left representative image) Representative images depicting the CldU/IdU-labelled DNA replication tracks in WT and Alyref^{+/-} HSCs (2-4 months).

(D, right dot plot) Dot plots depicting the DNA replication fork symmetry in WT and Alyref^{+/-} HSCs. The dot plot of IdU to CldU track length ratios for individual replication forks. Data are represented as mean $\pm$ SD. A minimum of 358 molecules were analyzed in each group.

(E, left representative image) Representative images depicting the CldU/IdU-labelled DNA replication tracks in Young (3 months) and Aged HSCs (24 months).

(E, right dot plot) Dot plots depicting the DNA replication fork symmetry in Young and Aged HSCs. The dot plot of IdU to CldU track length ratios for individual replication forks. Data are represented as mean $\pm$ SD. A minimum of 423 molecules were analyzed in each group.

(F) The dot plot displays the counts of white blood cells (WBC), neutrophils (NEUT), lymphocytes (LYM), red blood cells (RBC), basophilic granulocytes (BAS) and

platelets (PLT) in WT, Alyref^{+/-} and Alyref^{-/-} mice. The data are based on at least 7 mice per group and are presented as mean $\pm$ SD.

Figure 5. Dysfunction of RNA transportation impairs hematopoietic stem cells.

(A) RT-PCR analysis of the knockdown efficiency of shRNA against Thoc1 and Thoc5 using freshly isolated LSK cells.

(B, left representative image) Representative images depicting the distribution of Poly(A)⁺RNA in shNT, shThoc1 and shThoc5 HSPCs. Freshly isolated LSK cells from young mice (2 months) were infected with lentivirus carrying Thoc1 shRNA, Thoc5 shRNA or control shRNA. After three days, GFP⁺ cells were purified for Poly(A)⁺RNA FISH. The panels are shown at the same exposure. Poly(A)⁺RNA was detected using a Cy3-labelled oligo(dT)₅₀ probe. The cell nuclei were stained with DAPI.

(B, right dot plot) The dot plots show the ratio of nuclear to cytoplasmic Poly(A)⁺RNA signals ratio in shNT, shThoc1 and shThoc5 HSPCs. Data are presented as mean $\pm$ SD. N and C indicate nuclear and cytoplasmic FISH signals, respectively. N/C ratios were determined for at least 33 cells in each experiment.

(C) Experimental design of the competitive transplantation assay. Freshly isolated LSK cells from young mice (2 months old) were infected by lentivirus carrying Thoc1 shRNA, Thoc5 shRNA or control shRNA, labelled by GFP fluorescence. Three days later, 2000 GFP⁺Sca1⁺CD48⁻ cells were purified and transplanted into lethally irradiated recipients (CD45.2) along with 3 $\times$ 10⁵ competitor cells (CD45.1). Chimerism in peripheral blood was evaluated every 4 weeks until the 12th week.

(D) These line plots depict the percentage of donor-derived cells (Overall, B cells, T cells, myeloid cells) in the peripheral blood of recipients. n=4-7 recipients per group, data are presented as mean $\pm$ SD.

(E, left representative image) Representative images depicting the distribution of Poly(A)⁺RNA in both shNT and shNxf1 HSPCs. Freshly isolated LSK cells

from young mice (2 months old) were infected with lentivirus carrying Nxf1 shRNA or control shRNA. After three days, GFP⁺ cells were purified for Poly(A)⁺RNA FISH. The panels are shown at the same exposure. Poly(A)⁺RNA was detected using a Cy3-labelled oligo(dT)₅₀ probe. The cell nuclei were stained with DAPI.

(E, right dot plot) The dot plot shows the ratio of nuclear to cytoplasmic Poly(A)⁺RNA signals ratio in shNT and shNxf1 HSPCs. Data are presented as mean $\pm$ SD. N and C indicate nuclear and cytoplasmic FISH signals, respectively. N/C ratios were determined for at least 78 cells in each experiment.

(F) These line plots depict the percentage of donor-derived cells (Overall, B cells, T cells, myeloid cells) in the peripheral blood of recipients. n=6-7 recipients per group, data are shown as mean $\pm$ SD.

Figure 6. Quantitative refill of Alyref rescues the function of aged hematopoietic stem cells by dampening R-Loop and replication stress.

(A) Line plots depicting the proportion of donor-derived cells (Overall, T cells, B cells, myeloid cells) in the peripheral blood of recipients. n=5-7 recipients per group. Data are represented as mean $\pm$ SD.

(B, left representative image) Representative images depicting the distribution of Poly(A)⁺RNA in EV and Alyref OE HSCs. The panels are shown at the same exposure. Poly(A)⁺RNA was detected using a Cy3-labelled oligo(dT)₅₀ probe. The cell nuclei were stained with DAPI.

(B, right dot plot) Dot plots depicting the nuclear and cytoplasmic Poly(A)⁺RNA signals ratio in both EV and Alyref OE HSCs. Data are represented as mean $\pm$ SD. N and C indicate nuclear and cytoplasmic FISH signals, respectively. N/C ratios were determined for a minimum of 73 cells in each experiment.

(C) Histograms illustrate the quantitative analysis of ALYREF protein levels in young/aged HSCs and EV/Alyref OE HSPCs. Data were analyzed using image J.

(D, left representative western blot) Representative western blot showing the

expression of ALYREF. NIH-3T3 cells were infected with QGES-1^{Allyref}, QGES-2^{Allyref}, KC^{Allyref} and control lentivirus (KC). After three days, GFP⁺ cells were purified for western blot.

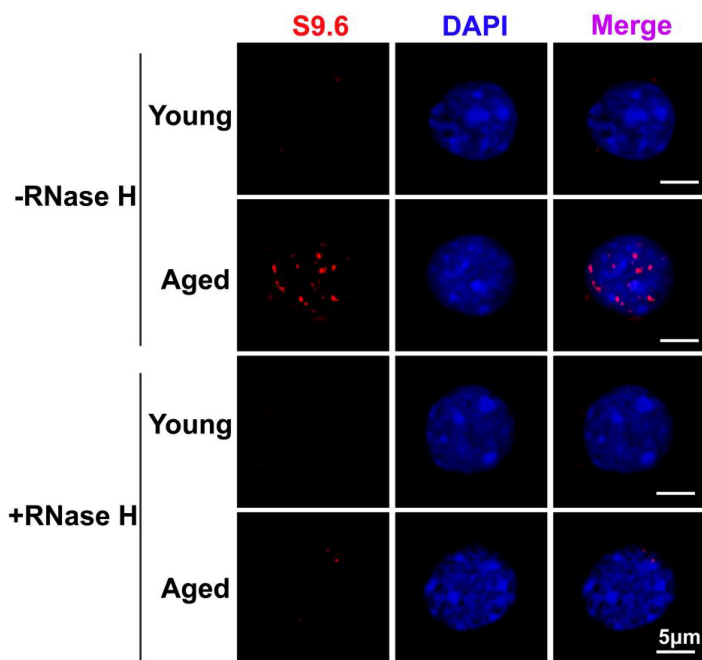
(D, right histogram) Histograms depicting the quantitative ALYREF protein in QGES-1^{Allyref}, QGES-2^{Allyref}, KC^{Allyref} and KC (Figure 6D, left representative western blot). Data were analyzed using image J.

(E) Line plots depicting the proportion of donor-derived cells (Overall, B cells, T cells, myeloid cells) in the peripheral blood of recipients. n=6-7 recipients per group. Data are represented as mean $\pm$ SD.

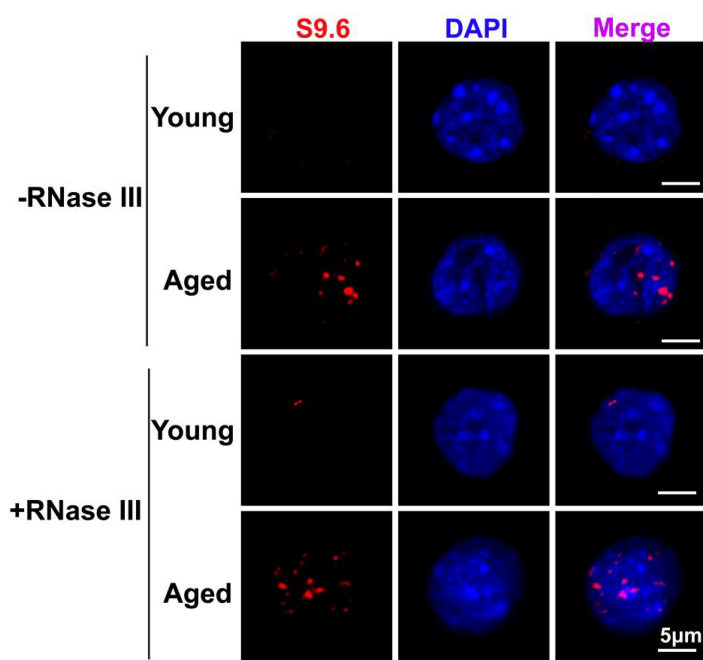
(F, left representative image) Representative images depicting the distribution of Poly(A)⁺RNA in QGES-2^{Allyref} and KC HSPCs using freshly isolated GFP⁺ LSK cells from the recipients of (*Online Supplementary Figure S6B*) at the end of the 16th week after transplantation. The panels are shown at the same exposure. Poly(A)⁺RNA was detected using a Cy3-labelled oligo(dT)₅₀ probe. The cell nuclei were stained with DAPI.

(F, right dot plot) Dot plots depicting the nuclear and cytoplasmic Poly(A)⁺RNA signals ratio in KC and QGES-2^{Allyref} HSPCs. Data are represented as mean $\pm$ SD. N and C indicate nuclear and cytoplasmic FISH signals, respectively. N/C ratios were determined for a minimum of 108 cells in each experiment.

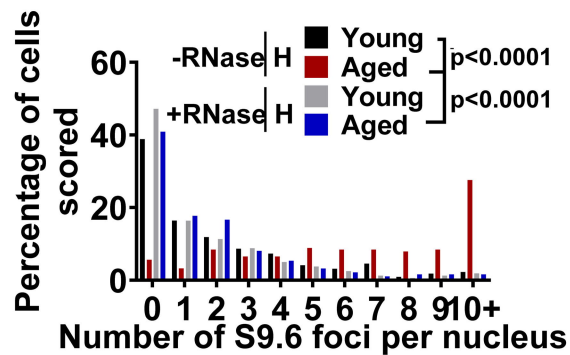
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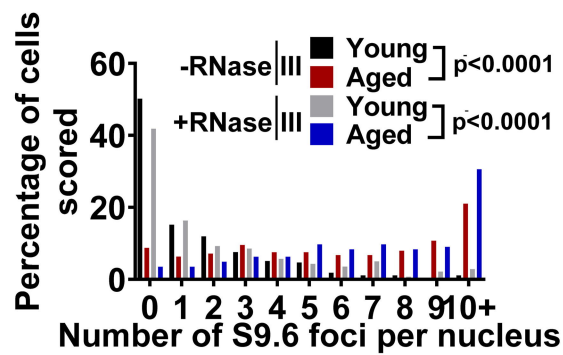
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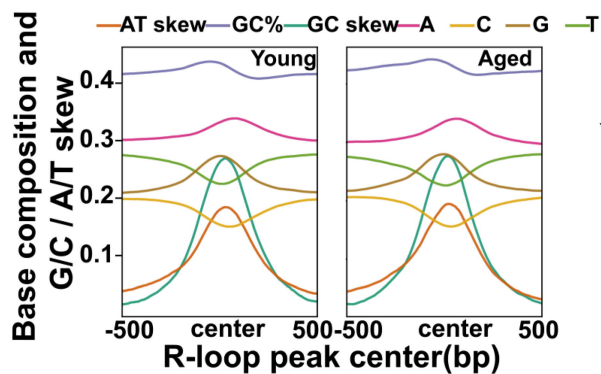
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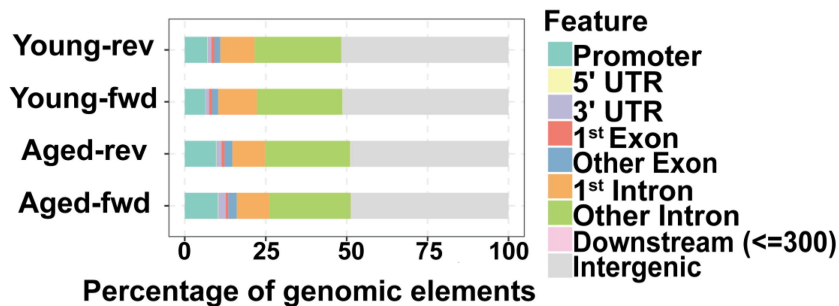
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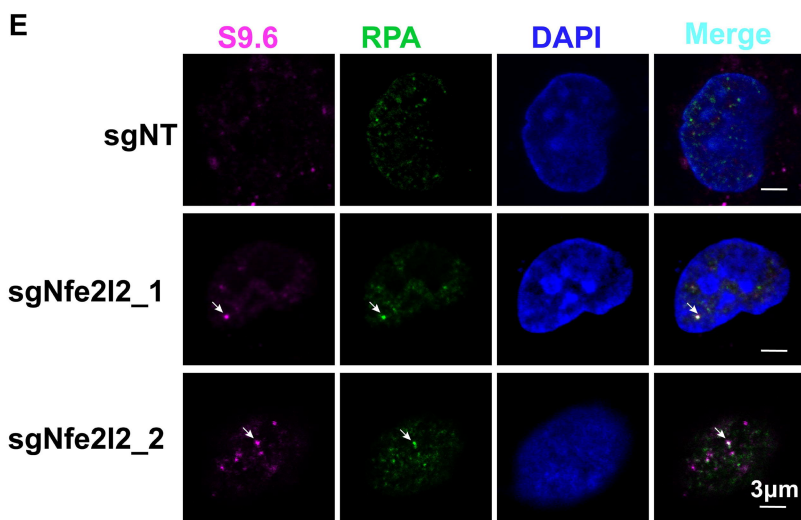
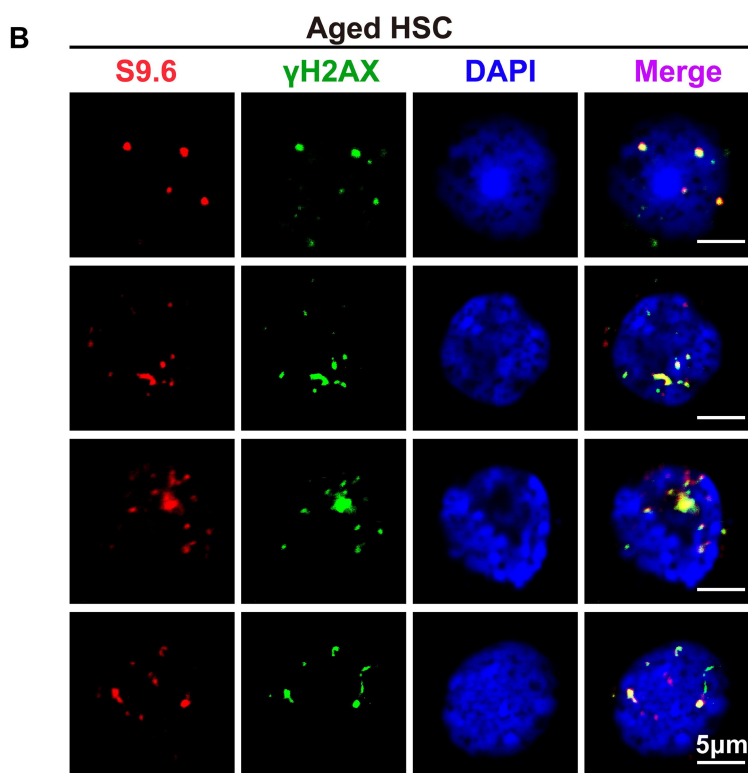
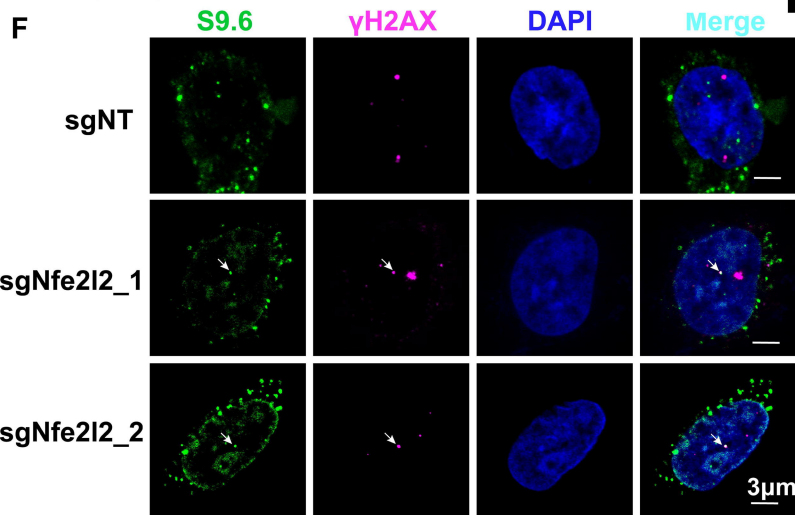
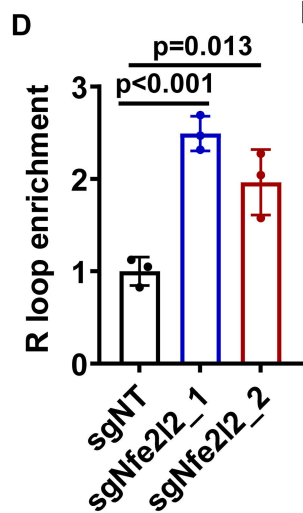
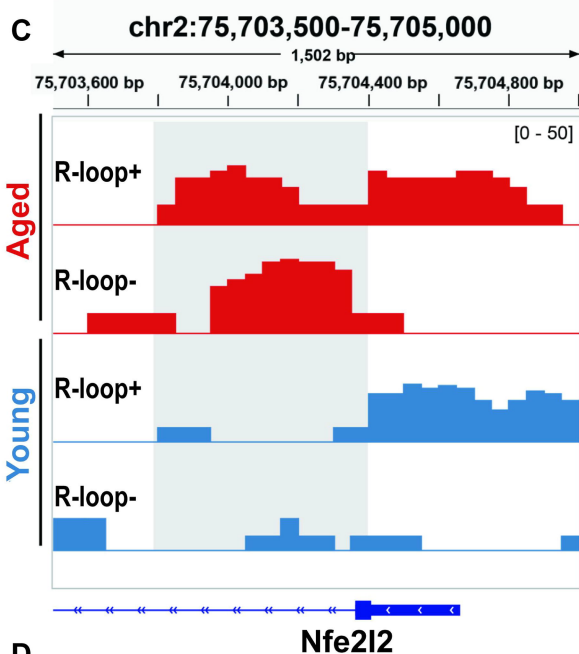
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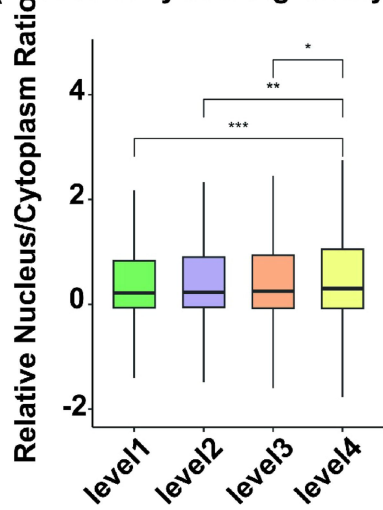
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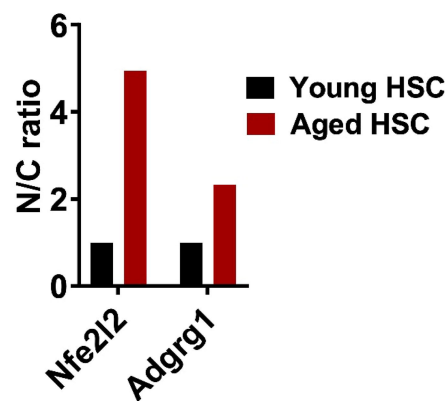
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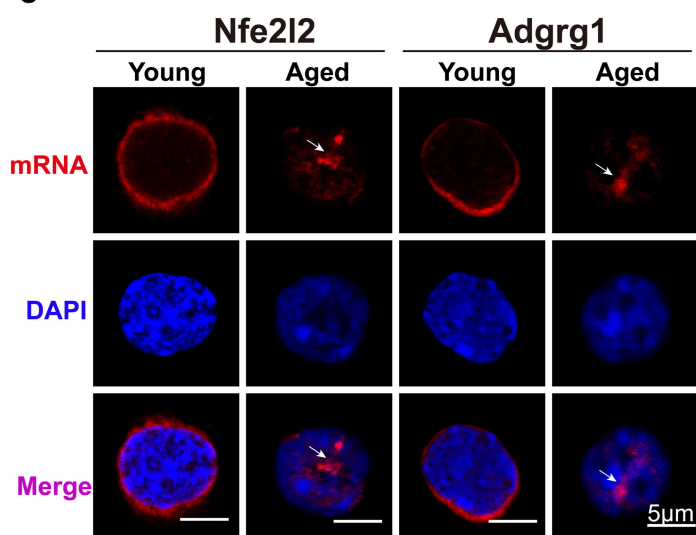
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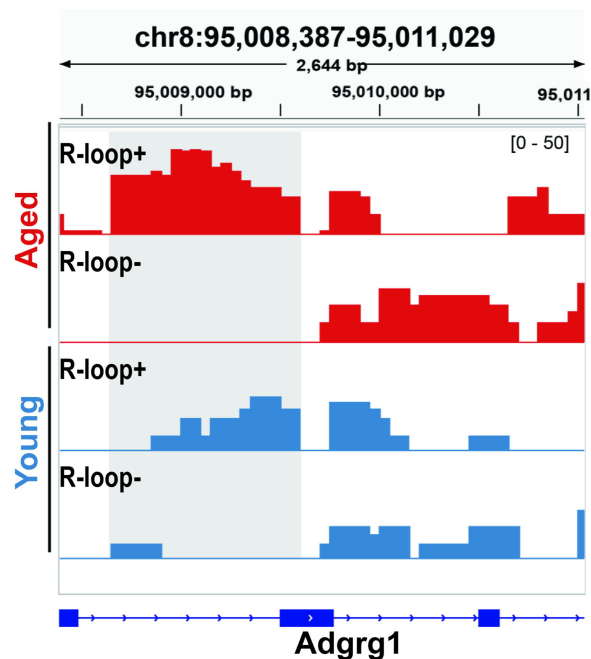
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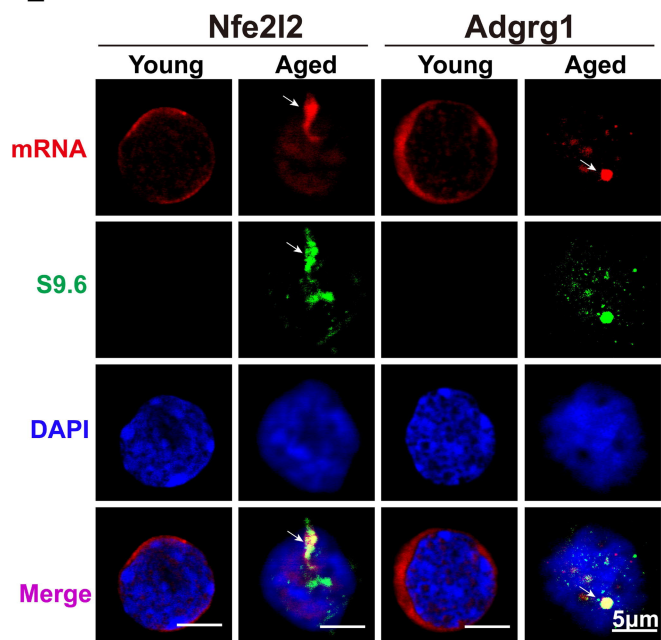
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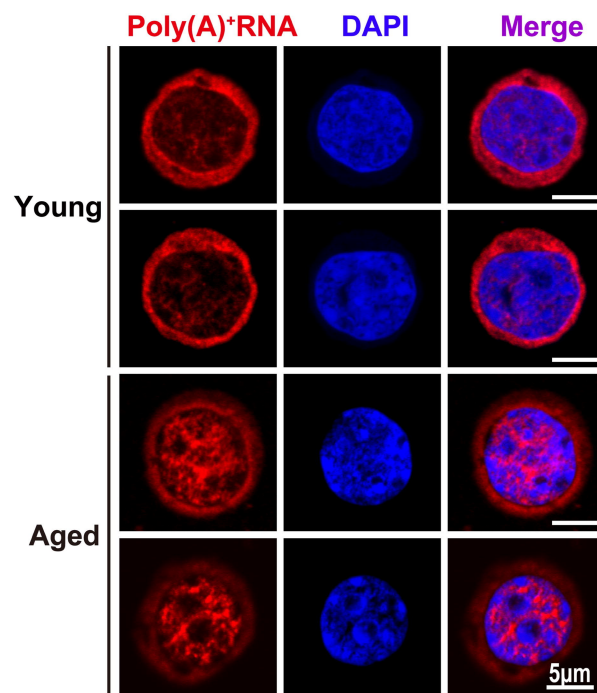
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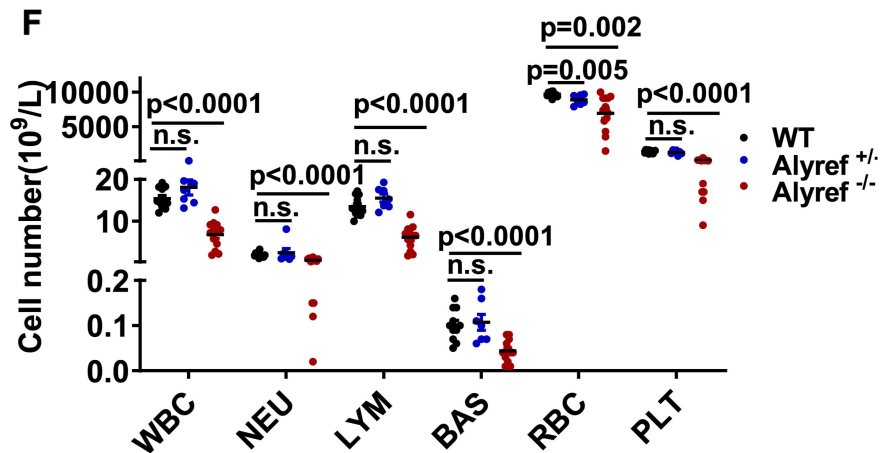
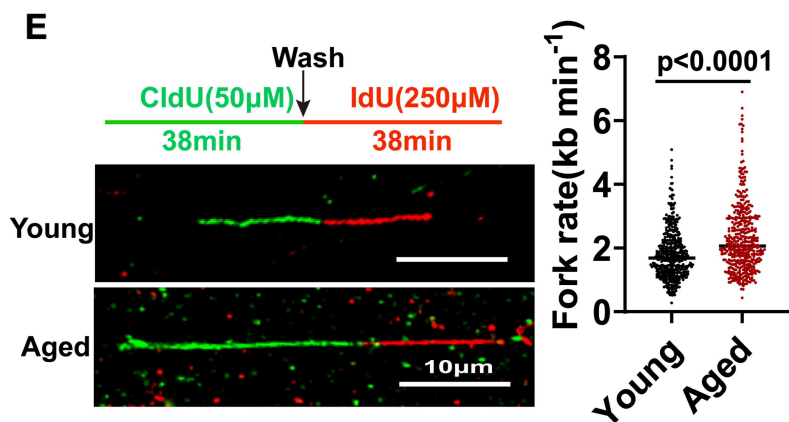
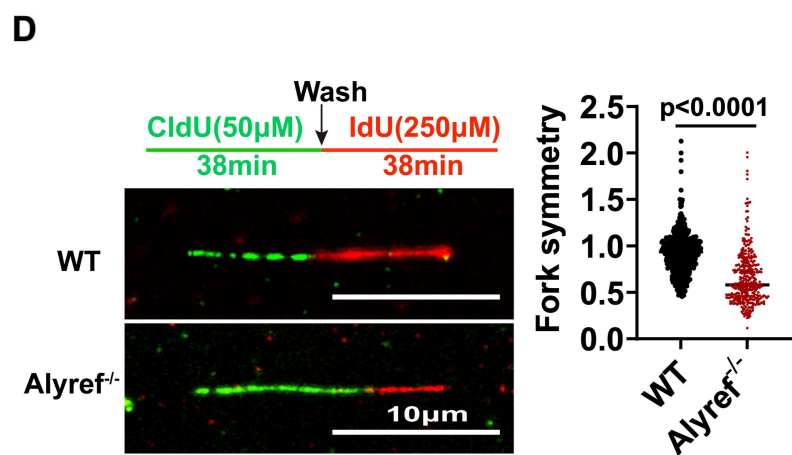
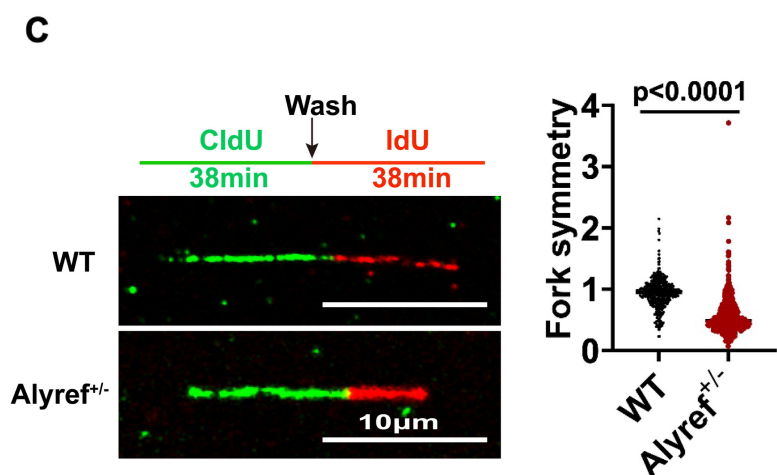
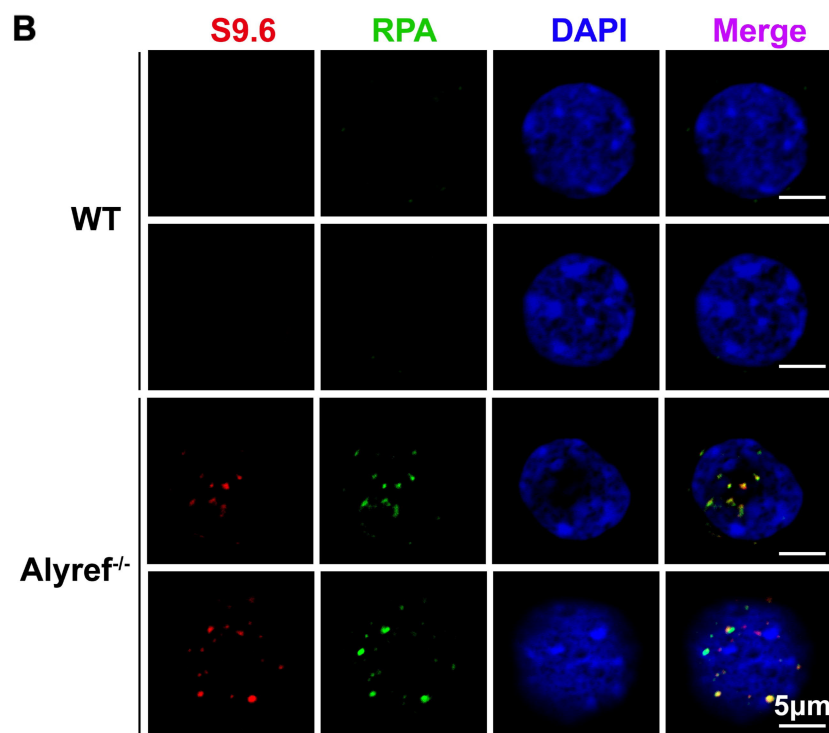
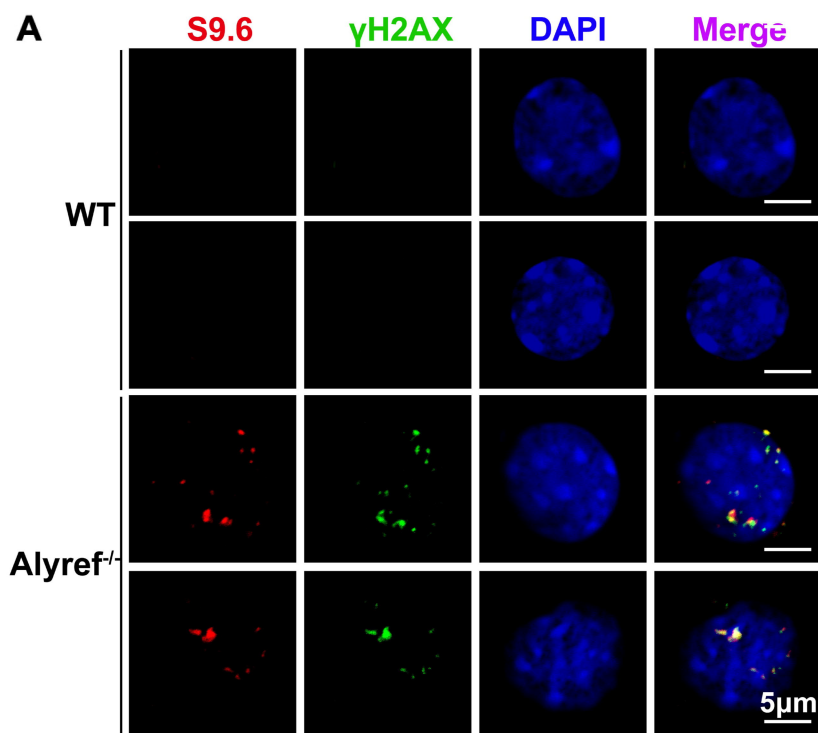


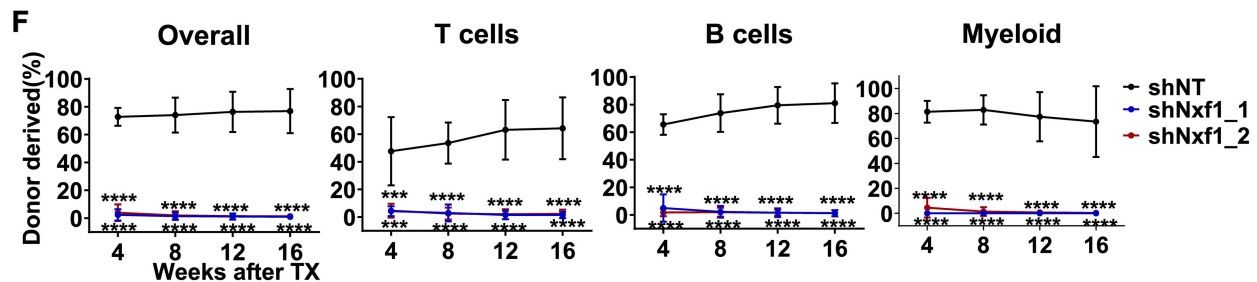
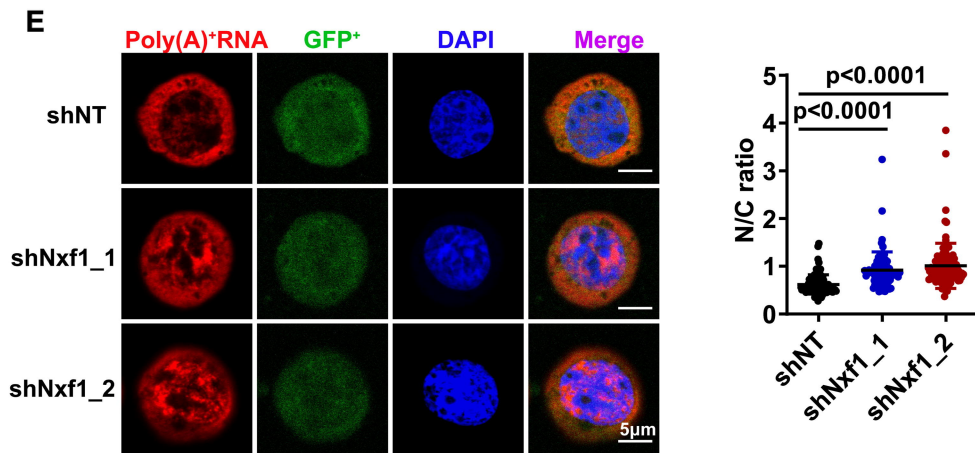
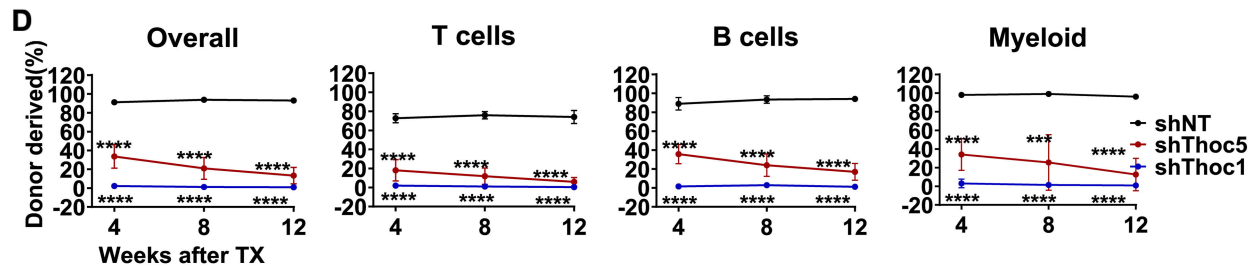
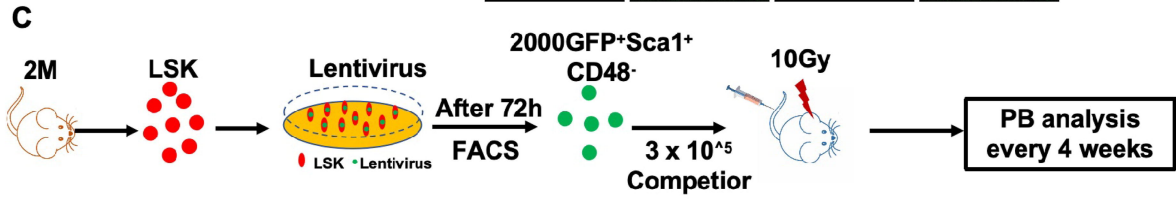
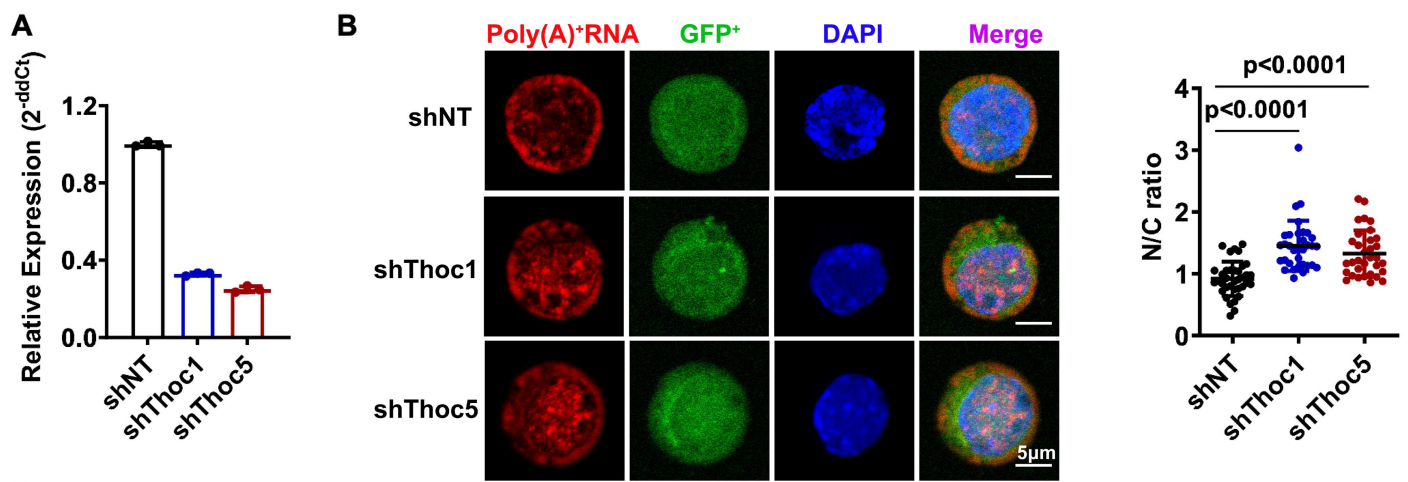
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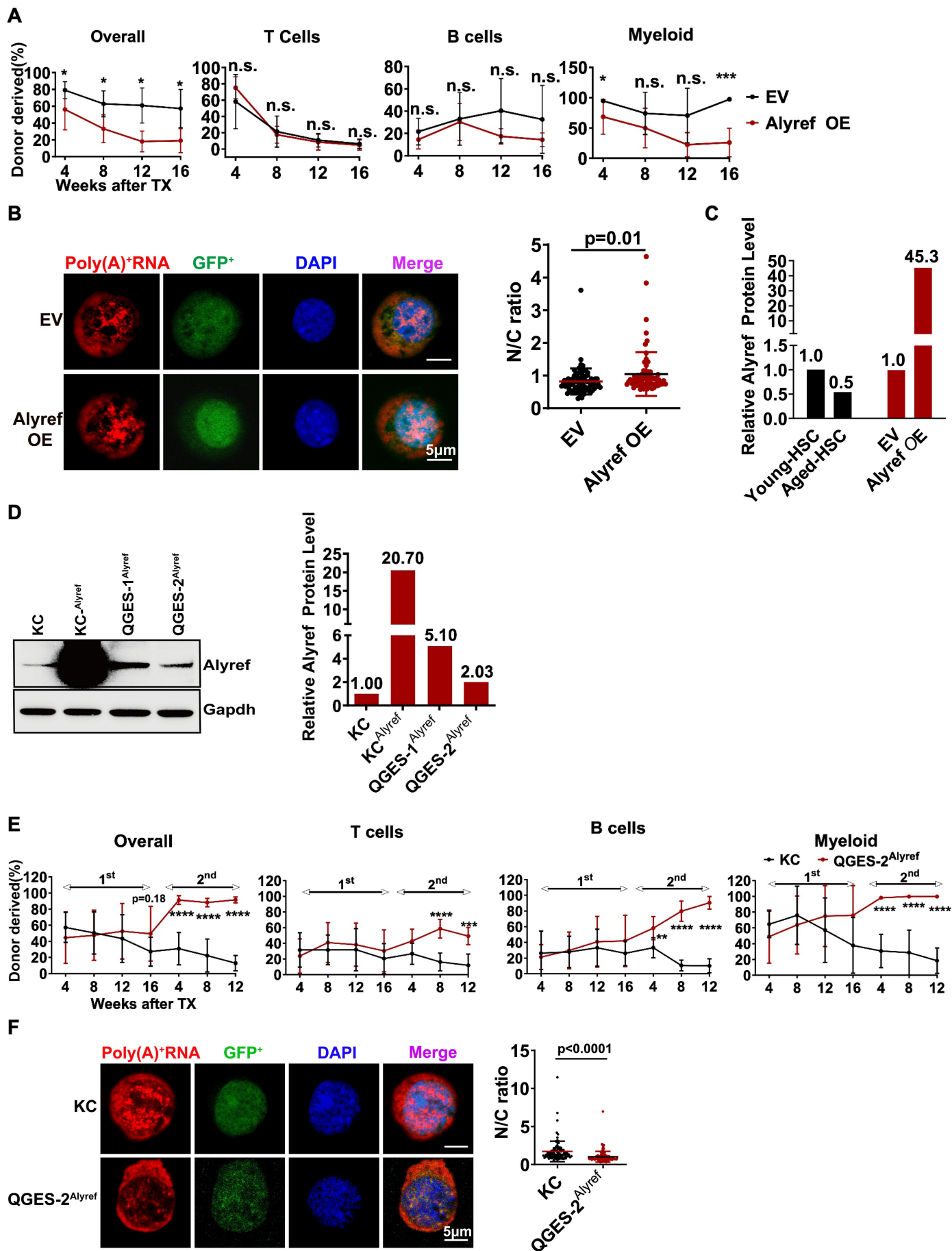


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Online Supplemental Methods

Mice

Alyref^{flox/flox} mice were generated in Cyagen Biosciences Inc. (Guangzhou, China). Alyref^{flox/flox} mice (C57BL/6N) were generated by inserting loxP sites spanning the third exon of Alyref via homologous recombination. To achieve hematopoietic-specifically knockout mice, Alyref^{flox/flox} were crossed to Mx1-Cre mice. To induce Cre expression in Mx1-Cre⁺; Alyref^{flox/flox} mice were intraperitoneally injected with Poly I:C (25 mg/kg) every other day for 14 days. All genotyping primers are listed in *Online Supplementary Table S1*.

C57BL/6 mice (CD45.2) and C57BL/6-SJL (CD45.1) mice were from the Jackson Laboratory. All of these strains were maintained on C57BL/6 background. The recipients used in the competitive transplantation assays were CD45.1/2 that were the first generation of C57BL/6 (CD45.2) and B6.SJL (CD45.1) mice. All mice were housed in specific-pathogen-free, and all procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Tsinghua University.

Hematological cell counts

PB after tail bleeding was analyzed by Auto Hematology Analyzer BC-5000 (MINDRAY). BM cells were harvested from one femur and suspended in HBSS⁺ buffer on ice before counting by Vi-CELL Cell Counter (Beckman).

Lentivirus production and transduction

The cDNA was cloned into SF-LV-cDNA-EGFP vector and the shRNA was cloned into pLKD-CMV-G&PR-U6-shRNA vector. Lentivirus was produced in 293T cells and was concentrated according to standard procedures¹. For lentivirus transduction, 1×10^5 Lineage⁻ c-Kit⁺ Sca1⁺ (LSK) cells were sorted and plated in 96 well plate added 100 μ L SFEM medium (Stem Cell Technology, 09650) with 20 μ g/mL mSCF, 20 μ g/mL mTPO and 1 % penicillin/streptomycin. All shRNA sequences and clone primers are listed in *Online Supplementary Table S2*.

Transplantations and peripheral blood analysis

For the competitive HSC transplantations assay. Freshly isolated 100 HSCs (CD45.2) were injected into lethally irradiated recipient mice (CD45.1/2) together with 3×10^5

whole BM cells (CD45.1) as competitor. Recipient mice were irradiated a lethal dose of radiation (10 Gy) using X-ray irradiator (RS-2000, Rad Source Technologies) before transplantation. Regarding the secondary transplantation, 2×10^6 whole BM cells from the primary recipients were injected into the secondary recipient mice. Recipient mice were analyzed for donor-derived chimaerism (including T cells, B cells, myeloid cells) every 4 weeks. The antibodies combination (CD3, B220, CD11b, CD45.1 and CD45.2) were used to analyze PB.

For the competitive HSPC transplantations assay. 2000 GFP⁺ Sca1⁺ CD48⁻ HSPC (CD45.2) were injected into lethally irradiated recipient mice (CD45.2) together with 3×10^5 whole bone marrow cells (CD45.1) as competitor. Regarding the secondary transplantation, 2×10^6 whole BM cells from the primary recipients were injected into the secondary recipient mice. Recipient mice were analyzed for donor-derived chimaerism (including T cells, B cells, myeloid cells) every 4 weeks. The antibodies combination (CD3, B220, CD11b, CD45.1) were used to analyze PB.

Flow cytometric analysis and cell sorting

Cells were suspended in HBSS⁺ buffer and then stained for the fluorophore coupled antibodies. Then flow cytometry analysis data were collected from BD LSRFortessa SORP flow cytometer (BD Biosciences), and then analysed by FlowJo software. Cell sorting was performed by BD Influx (BD Biosciences) and cells were sorted into HBSS⁺ buffer. A detailed list of antibodies used for flow cytometric analysis and cell sorting are provided in the *Online Supplementary Table S3*.

Immunofluorescence staining

Cells were centrifuged onto poly-lysine coated coverslips by Thermo Scientific Shandon Cytospin 4 (1000 rpm, 5 min), fixed with 4% PFA for 20 min at room temperature, washed with PBS for 3 times. Then cells were permeabilized in 0.5% TritonX-100 for 30 min at room temperature and blocked in 5% BSA/PBS for 1 h. Coverslips were then incubated overnight in 4°C in 5% BSA/PBS with the following antibodies alone or in combination: anti-phospho-H2AX (Ser139) (Millipore, 05-636), anti-RPA32 (CST, 2208s), Anti-DNA-RNA Hybrid, clone S9.6 (Millipore, MABE1095). Coverslips were washed 3 times in PBST and incubated for 1 h at room

temperature in 5% BSA/PBS with appropriate secondary antibodies (Invitrogen). Coverslips were then washed 3 times in PBST and stained with DAPI (1 μ g/mL) for 10 min, washed with PBS for 3 times. Cells were observed under a FV1200 confocal microscope (Olympus), using a 100 $\times$ objective.

For in vitro RNase H or RNase III treatment, cells were treated with RNase H (New England Biolabs, M0297S) for 2 h or ShortCut RNase III (New England Biolabs, M0245L) for 20 minutes at 37°C after 0.5% TritonX-100 permeabilization before 5% BSA blocking. A detailed list of antibodies used for Immunofluorescence staining are provided in the *Online Supplementary Table S4*.

RNA fluorescent in situ hybridization

RNA FISH was conducted using a Ribo fluorescence in situ hybridization kit (RiboBio, C10910) in accordance with the manufacturer's directions. In brief, cells were centrifuged onto poly-lysine coated coverslips by Thermo Scientific Shandon Cytospin 4 (1000 rpm, 5min), fixed with 4% PFA for 10 min at room temperature, washed with PBS for 3 times. Then cells were permeabilized in 0.5% TritonX-100 for 30 min at 4°C, washed with PBS for 3 times. Then Pre-hybridization buffer was added at 37°C for 30 min. Hybridization was carried out with a Cy3-labelled oligo (dT)₅₀ probe at 37°C in the dark overnight. The coverslips were washed 3 times with Wash Buffer I, once each with Wash Buffer II and Wash Buffer III at 42°C in the dark and once with PBS for 5 min at room temperature. Then cells were stained with DAPI (1 μ g/mL) in the dark for 10 min, washed with PBS for 3 times. Cells were observed under a FV1200 confocal microscope (Olympus), using a 100 $\times$ objective.

For the R-Loop and mRNA co-localization assay, cells were performed for RNA FISH after cells incubated by Anti-DNA-RNA Hybrid, clone S9.6 and secondary antibodies.

Quantitative real-time PCR

Total RNA was extracted using TRIzol (Invitrogen, 15596018) according to the manufacturer's Instructions. Total RNA was subjected to reverse transcription using PrimeScriptTMRT reagent Kit with gDNA Eraser (Takara, RR047A). Acquired cDNA was analyzed by PowerUp SYBR Green mix (Applied Biosystems, A25780) on

QuantStudio-3 Real-time PCR System (Applied Biosystems). The primer information is listed in *Online Supplementary Table S5*.

Western blot

Freshly isolated CD34⁺ LSK cells were lysed in sodium dodecyl sulfate (SDS) loading buffer, lysis was completed by sonication and denatured by boiling. Samples were resolved by 10% SDS-PAGE. PVDF membranes were blocked by 5% skim milk in Tris-buffered saline with Tween-20 buffer and then incubated with indicated primary and second antibodies. A detailed list of antibodies used for Western blot are provided in the *Online Supplementary Table S4*.

DNA fiber assay

Freshly isolated CD34⁺ Flt3⁺ LSK cells were cultured for 36 h, then pulsed for 38 min with 50 μ M CIdU (TargetMol, T19151), washed twice with PBS, and then pulsed for 38 min with 250 μ M IdU (TargetMol, T0863). Labeled cells were resuspended in ice-cold PBS at 1000 cells per microlitre. 2.5 μ L cell resuspension was spotted onto a glass slide and lysed with 7.5 μ L of spreading buffer (0.5% SDS in 200 mM Tris-HCl pH 7.4, 50 mM EDTA). Slides were tilted at an angle of 15° to 30° to allow the droplet run down slowly and at a constant speed. Then the slides were air-dried for 15 min, fixed in 3:1 volume absolute methanol: glacial acetic acid for 20 min and air-dried. DNA was denatured with 2.5 M HCl overnight at 4°C, washed twice with PBS and blocked with 1%BSA in PBS for 1h at room temperature and incubated with rat anti-CIdU/BrdU (Abcam, ab6326) and mouse anti-IdU/BrdU (Becton Dickinson, 347580) antibodies for 3h at room temperature to detect CIdU and IdU respectively. Then slides were washed with PBS and incubed for 2 h at room temperature with A488-conjugated goat anti-rat (Invitrogen, A-11006) and A594-conjugated goat anti-mouse (Invitrogen, A-11005) secondary antibodies. Images were observed under a FV1200 confocal microscope (Olympus), using a 60 $\times$ objective. The length of CIdU and IdU tracks was measured using ImageJ software.

Cytoplasmic and nuclear RNA fractionation and sequencing

Freshly isolated CD34⁺ LSK cells were lysed in 20 μ L cold cytoplasmic lysis buffer (0.15% NP-40, 10 mM Tris-HCl pH 7.5, 150 mM NaCl). The lysate was layered onto

50 μ L cold sucrose buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 24% sucrose), and centrifuged in microfuge tubes at $16000 \times g$ for 10 min. The supernatant from this spin represented the cytoplasmic fraction, and the pellet represented the nuclear fraction. Then, $3.5 \times$ volume TRIzol was added to the supernatant for cytoplasmic RNA purification, and an equal volume of TRIzol was added to the pellet for nuclear RNA purification. The cytoplasmic and nuclear fractions were used to generate cDNA libraries sequenced with 150-bp paired-end reads on an Illumina instrument by Novogene.

ssDRIP-seq and ssDRIP-qPCR

ssDRIP-seq library construction was performed according to published procedures ¹. Briefly, freshly isolated CD34⁺ LSK cells were resuspended in DNA lysis buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 0.5% SDS, 0.1 mg/mL Proteinase K), incubated at 37°C in a shaker with 180~240 rpm for 4~6 h. The extracted genomic DNA was fragmented using a cocktail of restriction enzymes (MseI, DdeI, AluI and MboI, New England Biolabs). The negative control was treated with RNase H (New England Biolabs, M0279S). DRIP was performed with the commercial S9.6 antibody (Millipore, MABE1095). Then the DRIPed DNA was validated by qPCR or sonicated into the size of 250 bp using Covaris S220 with 10% duty factor, 175 W, 200 cycles per burst, and 2 min treatment time. The ssDRIP-seq libraries were constructed from the sonicated DNA using VAHTS[®] ssDNA Library Prep Kit for Illumina (Vazyme, ND620-C), following instructions from the manufacturer. The libraries were checked on a fragment analyzer, followed by sequencing on an Illumina NovaSeq system.

dCas9 coupled RNaseH1 D209N-mediated site-specific R-Loop

dCas9 coupled RNaseH1 D209N-mediated site-specific R-Loop was performed according to published reference ². In brief, dCas9 infused with RNaseH1 D209N was co-expressed with gRNA targeting the selected R-Loop sites in NIH-3T3 cells. mCherry⁺ cells were purified for ssDRIP-qPCR and Immunofluorescence staining. The gRNA and qPCR primers are listed in *Online Supplementary Table S6*.

RNA-seq

In brief, 50 HSCs were sorted directed into lysis buffer. Then the RNA-seq library

was prepared using the method of Smart-seq2. After the library construction, assess the insertion size by Agilent Bioanalyzer 4200 system (Agilent Technologies), and quantify the accurate insertion size by Taqman fluorescence probe of AB Step One Plus Real-Time PCR system. Perform the clustering of the index-coded samples using HiSeq PE Cluster Kit v4-cBot-HS (Illumina) following the manufacturer's instructions. The libraries were sequenced by an Illumina Hiseq platform with 150-bp paired-end. The transcriptome sequencing was performed by ANNOROAD Gene Technology Company.

RNA-seq data processing

TrimGalore software (v0.6.6) (<https://github.com/FelixKrueger/TrimGalore>) was performed to remove adaptor, low quality base for raw reads and FastQC (v0.11.9) was used to do quality control check. Trimmed reads were then mapped to the mouse reference genome (Ensemble GRCm38) by STAR (v2.7.10 b), raw read counts and TPM (Transcripts Per Million mapped reads) of genes were quantified with RSEM (v1.3.1). R-package DESeq2 was performed to normalize gene counts and infer the differential expression levels of samples between two different groups.

Public RNA-seq data meta-analysis

Five public RNA-seq data sets were collected from GEO and ArrayExpress database. GSE70657 and GSE87631 were scRNA-seq (single cell) data of HSCs, raw read counts of which were normalized by using ZINB-WaVE. The other three datasets were normalized through DESeq2 method. For each dataset, DESeq2 was performed to get the differential expression levels between young and aged HSCs. 6073 genes expressed in all the five datasets were left in the following meta-analysis. Fisher's combined p-value and median of log2 fold change were obtained for each gene. 763 genes with Bonferroni corrected p-value < 0.05 and median (log2 FC) < 0 were identified as down-expressed genes in aged HSCs.

RNA-seq data analysis

RNA-seq reads were processed as described above. Samples of Alyref^{+/-} and Alyref^{-/-} HSCs were compared to WT HSCs through DESeq2, separately. The statistics value from DESeq2 was used as input to perform the pre-ranked GSEA.

Cytoplasmic and nuclear RNA-seq data analysis

Raw sequencing reads were processed as described above and TPM for each gene was obtained. Average log2-transformed TPM of replicates was calculated for each fraction and sample. Nucleus/Cytoplasm (Nuc/Cyto) ratio was used to quantify as RNA localization for young or aged sample.

ssDRIP-seq data processing

TrimGalore (v0.6.6) was conducted to remove adaptor sequences and to trim 10 bases on both ends for all files of raw reads. Reads with less than 50 bases were filtered out. Then, the trimmed reads were aligned to the mouse reference genome (ENSEMBL GRCm38) using Bowtie2, with “--local” parameters. Duplicated reads were removed by Picard tools (v2.26.3) (<https://broadinstitute.github.io/picard>). Reads with mapping quality more than 10 were remained and sorted, then was separated into forward (Watson strand) and reverse strand (Crick strand) using SAMtools. Reads of replicates for each kind of samples were merged for the subsequent analysis.

RPGC (Reads Per Genome Coverage)-normalized signal across whole genome were obtained, and log-transformed ratio of RPGC-normalized R-Loop signal and input were calculated by deepTools (v3.5.1). We draw metaplot of comparative normalized R-Loop signal of aged and young samples on the genebody region and peak summits.

R-Loop peak calling

Strand-specific peaks were called by MACS3 (v3 3.0.0b1) with R-Loop and input samples. Peaks with q values less than 0.01 and enrichment score more than 10, and located outside the blacklisted region were defined as R-Loop peaks. We annotate peaks with genomic regions by R package ChIP seeker.

Differential analysis of R-Loop signals in genebody and its regulatory region

In our study, genebody and its regulatory region was defined as from 2000 bp upstream of TSS (transcription start site) to 2000 bp downstream of TES (transcription end site), and genebody region as from the TSS to TES. For each gene, read coverage on these two regions of ssDRIP-seq reads were counted by deepTools (v3.5.1) in young and aged samples. DESeq2 R package was used to normalize the

raw reads count and estimate the difference of R-Loop signal between young and aged samples. DESeq2 statistics of R-Loop reads on the genebody region was used as input to perform pre-ranked GSEA. According to the quantiles of DESeq2 statistics of R-Loop reads on the genebody and its regulatory region, genes were grouped into four levels with different R-Loop difference (level 1 < -0.53583 < level 2 < 0.01050 < level 3 < 0.56268 < level 4). To investigate the relationships of RNA localization and R-Loop signals of genes, we compared the ratios of RNA localization (Nuc/Cyto ratio) in young and aged samples of genes in different levels defined above.

Whole-genome sequencing and data processing

Freshly isolated 1×10^6 LSK cells were extracted using the HiPure Blood DNA Mini Kit (Magen, D3111-03), according to the manufacturer's instructions. After DNA libraries construction and quality control, sequencing was performed using BGISEQ sequencing platform. Raw reads were trimmed using TrimGalore (v0.6.6). Then, cleaned reads were aligned to the mouse reference genome (ENSEMBL GRCm38) by BWA, low mapping quality reads and duplicates were removed by Picard tools (v2.26.3). The InDels of young and aged samples were called by HaplotypeCaller in GATK tools (v4.0.5.1). The whole-genome sequencing and data analysis were performed by BGI.

Gene ontology enrichment analysis and gene set enrichment analysis

R package ClusterProfiler was used to make enrichment analysis on BP (Biological Process) GO. Pre-ranked GSEA was conducted on collected gene sets to make the gene set enrichment analysis.

Statistical Analysis

Statistical analysis was made using the Prism software (Prism, GraphPad Software Inc., San Diego, CA USA). Data are shown as mean $\pm$ SD. Student's *t* test (Two-tailed unpaired) were used for comparisons (GraphPad Prism v.7.0). ns, not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. All experiments were repeated 2 or 3 times independently.

Data availability

RNA-seq, cytoplasmic and nuclear RNA sequencing, ssDRIP-seq data have been

deposited in the GEO (Gene Expression Omnibus). The accession code is GSE239301. Whole Genome sequencing data have been submitted to SRA (Sequence Read Archive), and the BioProject accession is PRJNA1000120. Further information and requests for resources and reagents should be directed to and will be fulfilled by the Contact, Dr. Jianwei Wang (wangjianwei@ihcams.ac.cn).

1. Xu W, Xu H, Li K, et al. The R-loop is a common chromatin feature of the Arabidopsis genome. *Nat Plants*. 2017;3(9):704-714.
2. Li Y, Song Y, Xu W, et al. R-loops coordinate with SOX2 in regulating reprogramming to pluripotency. *Sci Adv*. 2020;6(24):eaba0777.

Online Supplementary figures

Online Supplementary Figure S1. R-Loop is increased in aged hematopoietic stem cells, related to Figure 1.

- (A) Experimental design of ssDRIP-seq. Freshly isolated 5×10^4 HSCs (CD34⁻ LSK) from young (2 months old) and aged mice were subjected to ssDRIP-seq.
- (B) Metaplot of RPGC-normalized R-Loop signals located between 2 kb upstream of the transcription start site (TSS) and the downstream region of the transcription end site (TES) for all genes with R-Loop reads.
- (C) Metaplot of RPGC-normalized R-Loop signal located between 1 kb upstream and 1 kb downstream of the peak summits.
- (D) Boxplot of the width of the R-Loop peaks separately obtained in young and aged HSCs (one-sided Wilcoxon signed rank test).
- (E) Pre-RANKED GSEA of cell aging-related genes and myeloid cell differentiation-related genes with R-Loop signal differences between young and aged HSCs. The genes were ranked according to the DESeq2 statistics after comparing ssDRIP reads in the region corresponding to the gene body in aged versus young samples. NES, normalized enrichment score; FDR q-values are provided. $|\text{NES}| > 0.3$ and $q < 0.05$ represent statistically significant differences.

Online Supplementary Figure S2. Accumulated R-Loop induces DNA damage and impairs the function of hematopoietic stem cells, related to Figure 2.

(A) Representative fluorescence images depicting the co-localization of the R-Loop with RPA in aged HSCs.

(B) Schematic of the strategy employed to introduce R-Loop to specific regions.

(C) Representative western blot showing the knockdown efficiency of shRNA against RNaseH1 and RNaseH2A using freshly isolated LSK cells.

(D) Line plots depicting the proportion of donor-derived cells (Overall, B cells, T cells, myeloid cells) in the peripheral blood of recipients. n=6 recipients per group. The data are presented as mean $\pm$ SD.

(E) Dot plots depicting the lineage distribution of donor-derived peripheral blood cells at the 12th week of recipients. n=6 recipients per group. Data are presented as mean $\pm$ SD.

(F) The dot plot displays the percentage of donor-derived cells (Overall, T cells, B cells, myeloid cells) in the bone marrow of recipients at the conclusion of the 12th week. Each group comprises n=6 recipients, and the data are presented as mean $\pm$ SD.

(G) The dot plot demonstrates both the number and the percentage of HSCs in the whole bone marrow of recipients at the conclusion of the 16th week. Each group comprises n=6 recipients, and the data are presented as mean $\pm$ SD.

(H) Representative images depicting the immunofluorescence staining of S9.6 in shNT, shRNaseH1 and shRNaseH2A HSPCs using freshly isolated GFP⁺ LSK cells from the recipients of (Figure 3I) at the end of the 12th week after transplantation. The panels are shown at the same exposure. The cell nuclei were stained with DAPI.

(I) S9.6 foci distribution in shNT, shRNaseH1 and shRNaseH2A HSPCs.

(J) Representative images depicting the immunofluorescence staining of γ H2AX in shNT, shRNaseH1 and RNaseH2A HSPCs using freshly isolated GFP⁺ LSK cells from the recipients of (Figure 3I) at the end of the 12th week after transplantation. The panels are shown at the same exposure. The cell nuclei were stained with DAPI.

(K) γ H2AX foci distribution in shNT, shRNaseH1 and shRNaseH2A HSPCs.

Online Supplementary Figure S3. Aging-increased R-Loop correlates with the lingering RNA in the nucleus of hematopoietic stem cells, related to Figure 3.

(A) The dot plots show the ratio of nuclear to cytoplasmic Poly(A)⁺RNA signals in young and aged HSCs. The data are presented as mean $\pm$ SD. In the histograms, “N” and “C” represent nuclear and cytoplasmic FISH signals, respectively. N/C ratios were determined for at least 250 cells in each experiment.

(B) This heatmap illustrates the genome-wide subcellular localization of Poly(A)⁺RNA, represented by normalized Nucleus/Cytoplasm values in aged HSC samples compared to young ones. The color intensity reflects local point density. Spearman’s R and p values are indicated. Each dot represents a gene, and the black dashed line represents equality between aged and young HSC samples ($X = Y$).

(C) This visualization displays the top 10 level-four Biological Process (BP) Gene Ontology (GO) terms significantly enriched with down-expressed genes in aged hematopoietic stem cells (HSCs). The size of each circle corresponds to the amount of overlap between the input gene list and the gene set associated with the GO term. The color of the circle indicates the FDR-adjusted p-value. The x-axis represents the gene ratio of the overlapping gene set to the input gene list.

(D) Enhanced child nodes of the GO term “RNA Localization” with genes exhibiting downregulation in aged HSCs.

(E) The heatmap displays the differential expression levels between aged and young HSCs for genes within the “RNA export from nucleus” GO term across five public RNA-seq datasets. In the heatmap, blue indicates down-regulated genes in aged HSCs, while red indicates up-regulated genes.

(F) The histogram illustrates the relative expression of Alyref in young and aged HSCs. HSCs (CD34⁺ LSK) , freshly isolated from young (2 months) and aged (24-30 months) mice, are subjected to RT-PCR. The data are presented as mean $\pm$ SD.

(G) The western blot image displays the expression of ALYREF in young and aged HSCs. Freshly isolated HSCs (CD34⁺ LSK) from young (2 months) and aged (24-30 months) mice were subjected to a western blot assay to detect ALYREF.

Online Supplementary Figure S4. Targeted dysfunction of Alyref induces R-Loop and disrupts hematopoietic homeostasis, related to Figure 4.

- (A) This diagram displays the scheme to generate Alyref^{flox/flox} mice.
- (B) Experimental design of Western blot assay for WT, Alyref^{+/-} and Alyref^{-/-} HSPCs. Mx1-Cre⁻; Alyref^{flox/flox}, Mx1-Cre⁺; Alyref^{flox/+} and Mx1-cre⁺; Alyref^{flox/flox} mice (abbreviated as WT, Alyref^{+/-} and Alyref^{-/-} in the figure) were treated with Poly I:C every other day for 14 days. Lineage-cells were sorted at 7 days post last Poly I:C injection for Western blot.
- (C) Representative Western blot showing the expression of ALYREF in WT, Alyref^{+/-} and Alyref^{-/-} HSPC.
- (D-E) Representative fluorescence images depicting the co-localization of R-Loop with γ H2AX (D) and RPA (E) in Alyref^{+/-} HSCs.
- (F) The dot plot displays the counts of hemoglobin (HGB) and hematocrit (HCT) in WT, Alyref^{+/-} and Alyref^{-/-} mice. The data are based on at least 7 mice per group and are presented as mean $\pm$ SD.
- (G) The absolute cell number per femur of WT, Alyref^{+/-} and Alyref^{-/-} mice was determined based on samples from 6 mice per group.
- (H-I) WT, Alyref^{+/-} and Alyref^{-/-} mice were analyzed for T cells, B cells and myeloid cells. The histogram illustrates the frequency of T cells (CD3⁺), B cells (B220⁺) and myeloid cells (CD11b⁺) in the peripheral blood (H) and bone marrow (I) of WT, Alyref^{+/-} and Alyref^{-/-} mice. Data from at least 6 mice per group, are presented as mean $\pm$ SD.
- (J-K) Alyref^{+/-}, Alyref^{-/-} and age-matched WT mice, aged 2-3 months were analyzed for hematopoietic stem cells (HSCs) and progenitors. Histograms illustrate the frequency (J) and absolute number (K) of HSC and progenitors in the bone marrow of WT, Alyref^{+/-} and Alyref^{-/-} mice. Data are based on samples from 6 mice per group.
- (L) Illustrated plots demonstrate the gating strategies employed for the analysis of various hematopoietic stem and progenitor cell populations, including LT-HSC (Lineage⁻ Sca1⁺ c-Kit⁺ CD34⁻ CD135⁻), ST-HSC (Lineage⁻ Sca1⁺ c-Kit⁺ CD34⁺ CD135⁻), MPP (Lineage⁻ Sca1⁺ cKit⁺ CD34⁺ CD135⁺), CMP (Lineage⁻ Sca1⁻ c-Kit⁺

CD16/32⁻ CD34⁺), GMP (Lineage⁻ Sca1⁻ cKit⁺ CD16/32⁺ CD34⁺), MEP (Lineage⁻ Sca1⁻ cKit⁺ CD16/32⁺ CD34⁻), CLP (Lineage⁻ Sca1^{low} c-Kit^{low} CD135⁺ CD127⁺) cells analysis in the bone marrow of WT, Alyref^{+/-} and Alyref^{-/-} mice.

(M) Experimental design of complete blood count and Poly(A)⁺RNA FISH assay for WT and Alyref^{+/-} mice. Mx1-Cre⁻; Alyref^{fllox/+} and Mx1-Cre⁺; Alyref^{fllox/+} mice (2 months old) were treated with Poly I:C every other day for 14 days. WT and Alyref^{+/-} mice were subjected to complete blood count and Poly(A)⁺RNA FISH assay at 10 months after the last Poly I:C injection.

(N, left illustrative images) Illustrative images displaying the distribution of Poly(A)⁺RNA in both WT and Alyref^{+/-} HSCs are presented. All panels are displayed at identical exposure levels. Detection of Poly(A)⁺RNA utilized a Cy3-labelled oligo(dT)₅₀ probe, while nuclei were visualized with DAPI staining.

(N, right dot plot) Dot plots illustrate the ratio of nuclear to cytoplasmic Poly(A)⁺RNA signals in WT and Alyref^{+/-} HSCs. Data are represented as mean ± SD. “N” and “C” denote nuclear and cytoplasmic FISH signals, respectively. N/C ratios were determined for a minimum of 360 cells in each experiment set.

(O) The dot plot displays the quantification of various blood cell types, including white blood cells (WBC), neutrophils (NEUT), lymphocytes (LYM), red blood cells (RBC), basophilic granulocytes (BAS), platelets (PLT), hemoglobin (HGB) and hematocrit (HCT) in both WT and Alyref^{+/-} mice. Each group consists of 13-14 mice, and all data are presented as mean ± SD.

(P) The absolute cell count per femur in both WT and Alyref^{+/-} mice is presented. Each group consists of 6 mice, and the data are expressed as mean ± SD.

(Q-R) Dot plots illustrate the frequency of T cells (CD3⁺), B cells (B220⁺) and myeloid cells (CD11b⁺) in the peripheral blood (Q) and bone marrow (R) of WT and Alyref^{+/-} mice. A minimum of 6 mice per group were analyzed, and all data are presented as mean ± SD.

(S-T) Analysis of HSCs and progenitors was conducted in WT and Alyref^{+/-} mice. Histograms display both the absolute number (S) and frequency (T) of HSCs and progenitors. Each group includes n = 6 mice and the data are presented as mean ± SD.

(U) The experimental design for the competitive transplantation assay involved the use of wild-type (WT), Alyref^{+/-} and Alyref^{-/-} hematopoietic stem cells (HSCs). A total of 100 freshly isolated HSCs were obtained from Mx1-Cre⁻; Alyref^{flox/flox}, Mx1-Cre⁺; Alyref^{flox/+} and Mx1-Cre⁺; Alyref^{flox/flox} mice (aged 2-3 months). These HSCs were transplanted into lethally irradiated recipients (CD45.1/2) along with 3×10⁵ competitor cells (CD45.1). Peripheral blood (PB) chimerism was assessed 4 weeks post- transplantation. Subsequently, recipients were treated with Poly I:C (12.5 mg/kg) every other day for 14 days. PB chimerism was monitored every 4 weeks up to the 12th week post last Poly I:C injection. Then, freshly isolated 2×10⁶ whole bone marrow cells from the primary recipients were transplanted into lethally irradiated recipients (CD45.1/2). The chimerism in the PB of the secondary recipients was examined every 4 weeks until the 12th week.

(V) These line plots show the percentage of donor-derived cells (Overall, B cells, T cells, myeloid cells) in the PB of recipients. n=6-9 recipients per group, data are presented as mean ± SD.

(W) The gating strategy is outlined for assessing the percentage of donor-derived lineage cells (T cells, B cells and myeloid cells) and the lineage distribution of donor-derived T, B and myeloid cells in the peripheral.

(X) The dot plot illustrates the lineage distribution of donor-derived PB cells at the 12th week in secondary recipients. n=6-9 recipients per group, data are presented as mean ± SD.

(Y) A dimensionality reduction plot illustrates WT, Alyref^{+/-} and Alyref^{-/-} HSCs using normalized RNA-seq data of all mouse genes, generated through the PCA (principal component analysis) algorithm.

(Z) These figures present the Gene Set Enrichment Analysis (GSEA) results of cell aging-related genes and HSC fingerprints-related genes in comparisons between WT HSCs vs Alyref^{-/-} HSCs, or WT HSCs vs Alyref^{+/-} HSCs. The normalized enrichment score (NES) and false discovery rate (FDR) q values are provided. A significant difference is indicated by |NES|>0.3 and q<0.05.

Online Supplementary Figure S5. Dysfunction of RNA transportation impairs hematopoietic stem cells, related to Figure 5.

(A) Representative western blot showing the knockdown efficiency of shRNA against Nxf1 using freshly isolated LSK cells.

Online Supplementary Figure S6. Quantitative refill of Alyref rescues the function of aged hematopoietic stem cells by dampening R-Loop and replication stress, related to Figure 6.

(A) Representative western blot depicting the expression of in Alyref-overexpressed LSK cells. Freshly isolated LSK cells from aged (26 months) mice were infected with Alyref-carrying lentivirus (Alyref OE) and empty vector (EV). After three days, GFP⁺ cells were purified for western blot assay with indicated antibodies.

(B) The experimental design of the competitive transplantation assay is described. LSK cells freshly isolated from aged mice (18 months old) were infected with KC and QGES-2^{Alyref} lentivirus, labelled by GFP fluorescence. Three days postinfection, 4000 GFP⁺Sca1⁺CD48⁻ cells were purified and transplanted into lethally irradiated recipients (CD45.2) alongside 3×10⁵ competitor cells (CD45.1). Chimerism in the peripheral blood was evaluated every 4 weeks until the 16th week. Subsequently, freshly isolated 2×10⁶ whole bone marrow cells from the primary recipients were transplanted to lethally irradiated secondary recipients (CD45.2) and chimerism in peripheral blood of the secondary recipients was examined every 4 weeks until the 12th week.

(C-F) Representative images depicting immunofluorescence staining of S9.6 (C) and γH2AX (E) in KC and QGES-2^{Alyref} HSPCs using freshly isolated GFP⁺ LSK cells from the primary recipients (*Online Supplementary Figure S6B*) at the end of the 16th week after transplantation. The panels are shown at the same exposure. The cell nuclei were stained with DAPI. (D, F) Distribution of S9.6 (D) and γH2AX (F) foci in KC and QGES-2^{Alyref} HSPCs.

(G) Representative images depicting immunofluorescence staining of RPA in KC and QGES-2^{Alyref} HSPCs. Freshly isolated LSK cells from aged mice (24-30 months) were infected with KC and QGES-2^{Alyref} lentivirus, which is labelled by GFP fluorescence. After three days, GFP⁺ cells were purified for immunofluorescence staining. The panels are shown at the same exposure. The cell nuclei were stained with DAPI.

(H) Dot plots depicting the mean fluorescence intensity of RPA in KC and QGES-2^{Alyref} HSPCs. Data are represented as mean ± SD, A minimum of 110 cells

were analyzed in each group.

(I) Representative images depicting the CldU/IdU-labelled DNA replication tracks in KC and QGES-2^{Alyref} HSCs. Freshly isolated HSCs from aged mice (24-30 months) were infected with KC and QGES-2^{Alyref} lentivirus. After three days, GFP⁺ cells were purified for DNA fiber assay.

(J) Dot plots depicting the DNA replication fork symmetry in KC and QGES-2^{Alyref} HSCs. The dot plot of IdU to CldU track length ratios for individual replication forks. Data are represented as mean $\pm$ SD. A minimum of 517 molecules were analyzed in each group.

(K) This chart illustrates the proposed model of Alyref $\rightarrow$ RNA exportation $\rightarrow$ R-Loop $\rightarrow$ DNA replication stress promotes hematopoietic stem cell aging.

Online Supplementary Table S1. Geneotyping primer

Online Supplementary Table S2. shRNA and clone primer

Online Supplementary Table S3. Flow cytometric analysis and cell sorting antibody

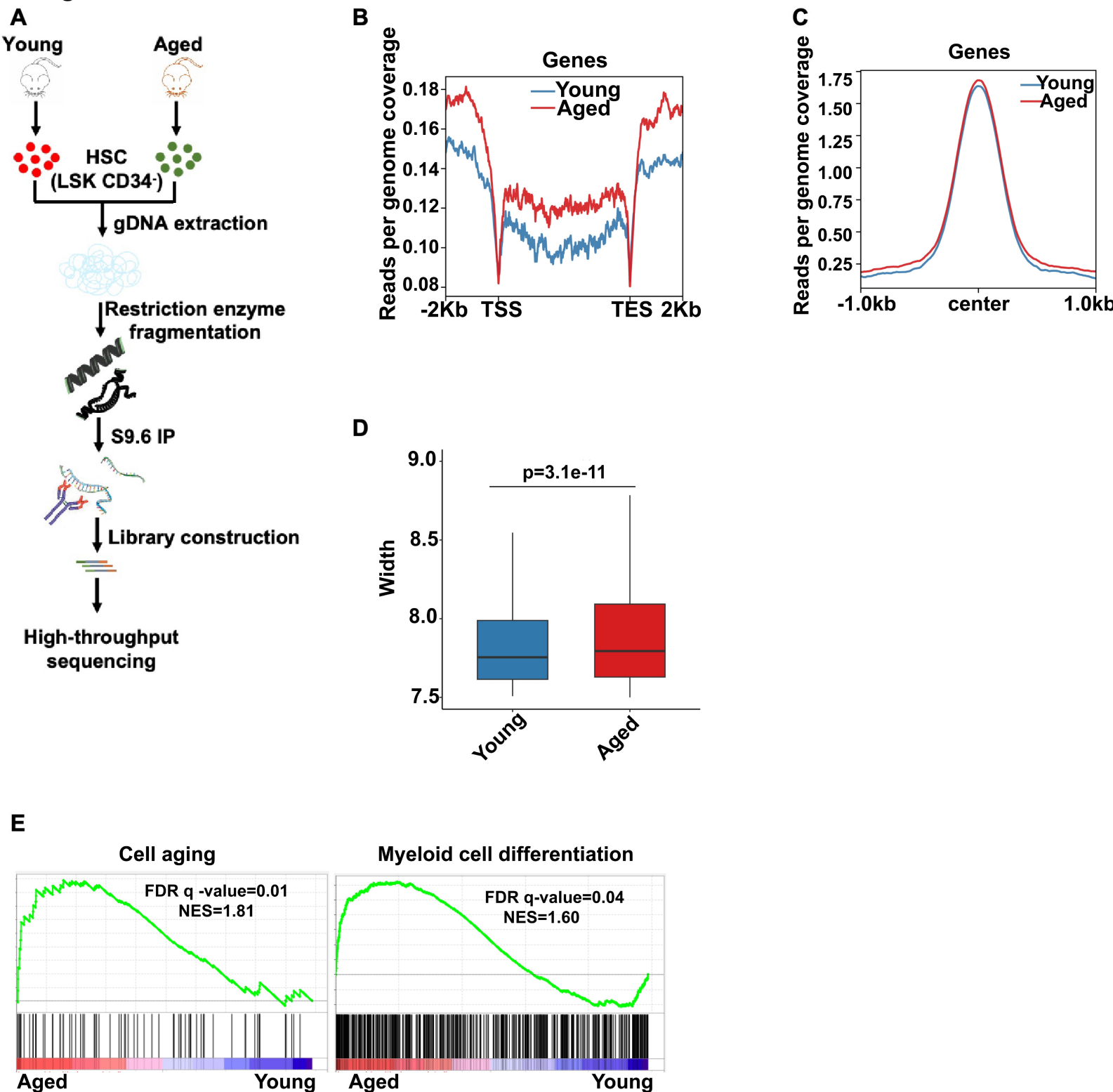
Online Supplementary Table S4. Western blot and Immunofluorescence staining antibody

Online Supplementary Table S5. RT-PCR primer list

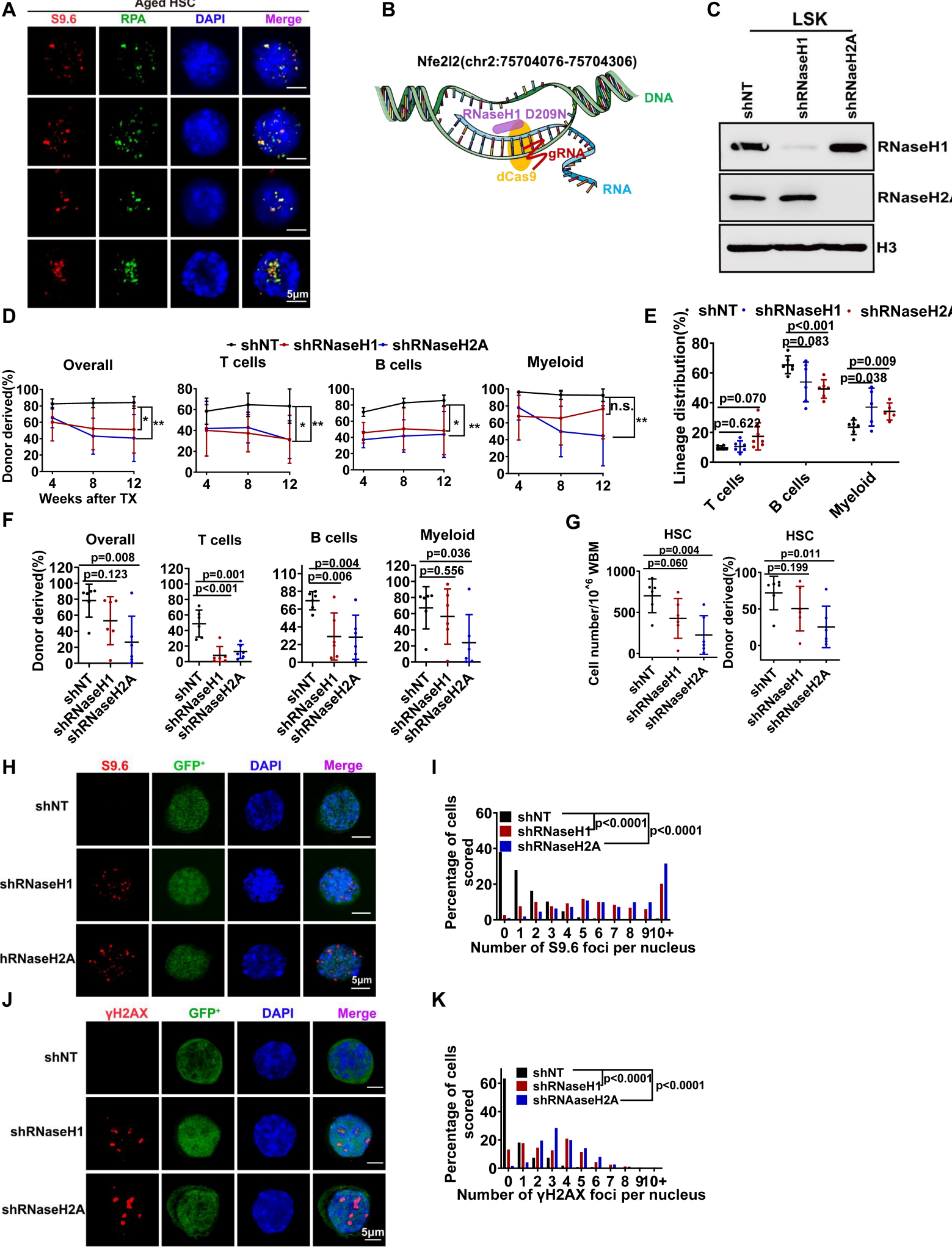
Online Supplementary Table S6. gRNA primer

Online Supplementary Table S7. HSC fingerprint gene, Cell aging and Myeloid cell differentiation

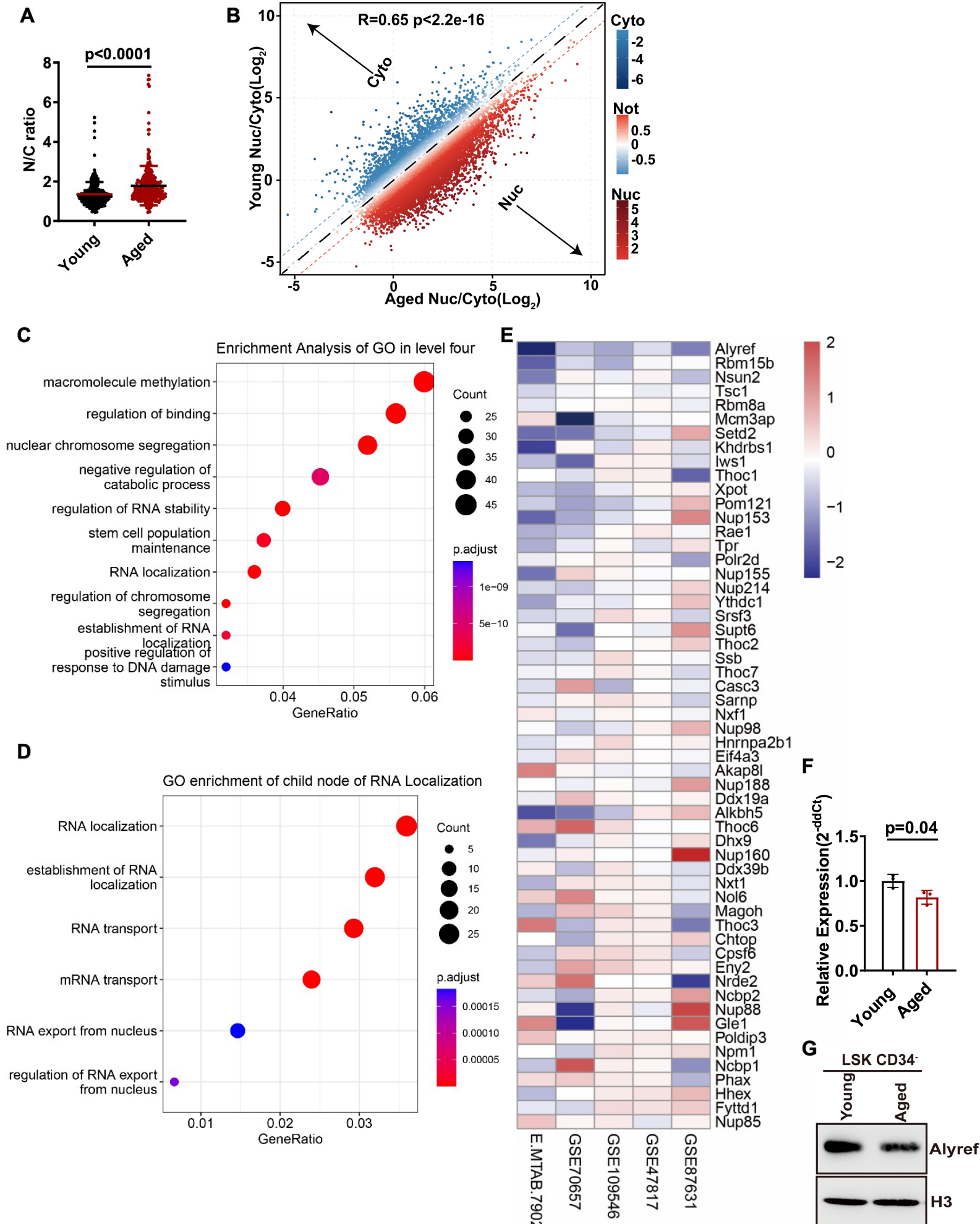
Online Supplementary Figure S1. R-Loop is increased in aged hematopoietic stem cells, related to Figure 1.



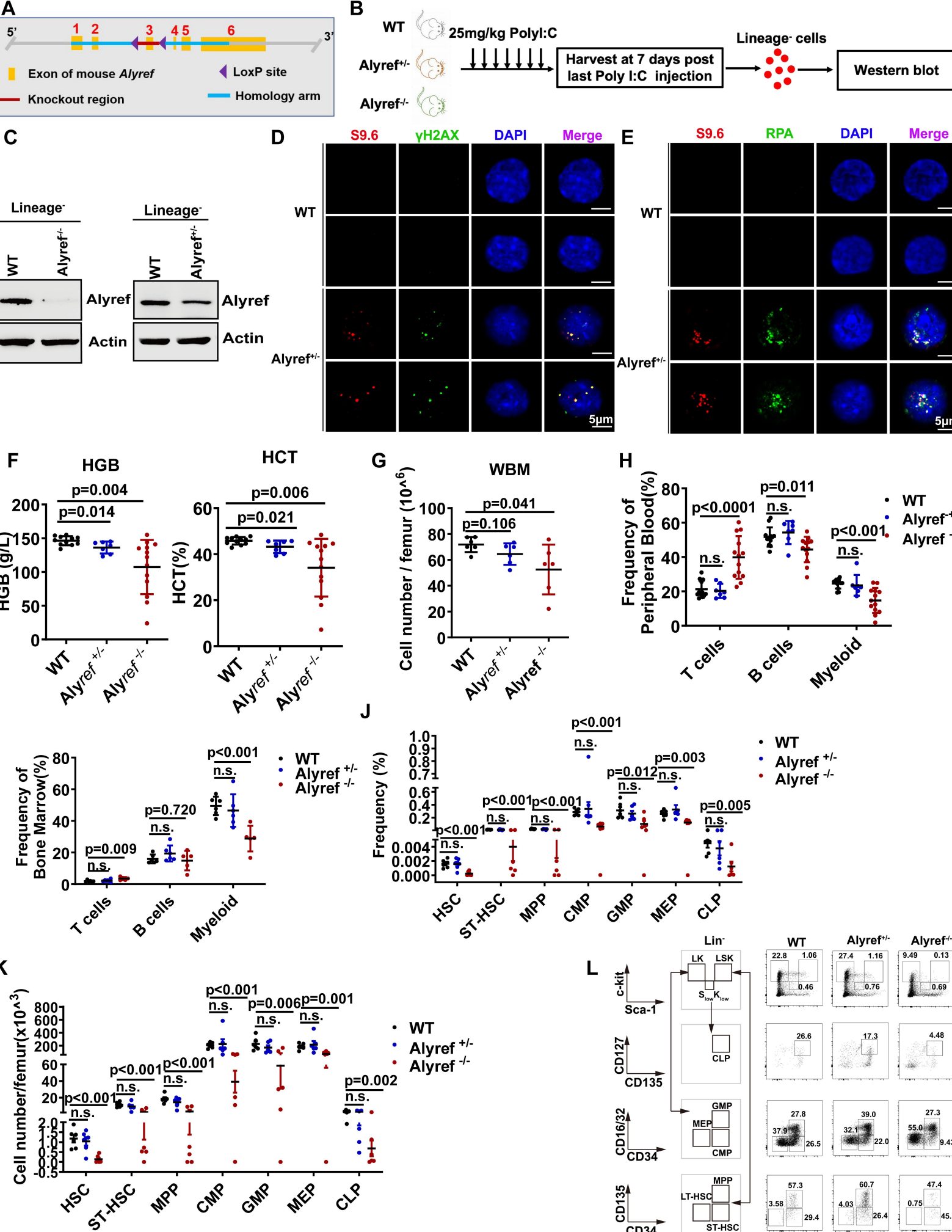
Online Supplementary Figure S2. Accumulated R-Loop induces DNA damage and impairs the function of hematopoietic stem cells, related to Figure 2.



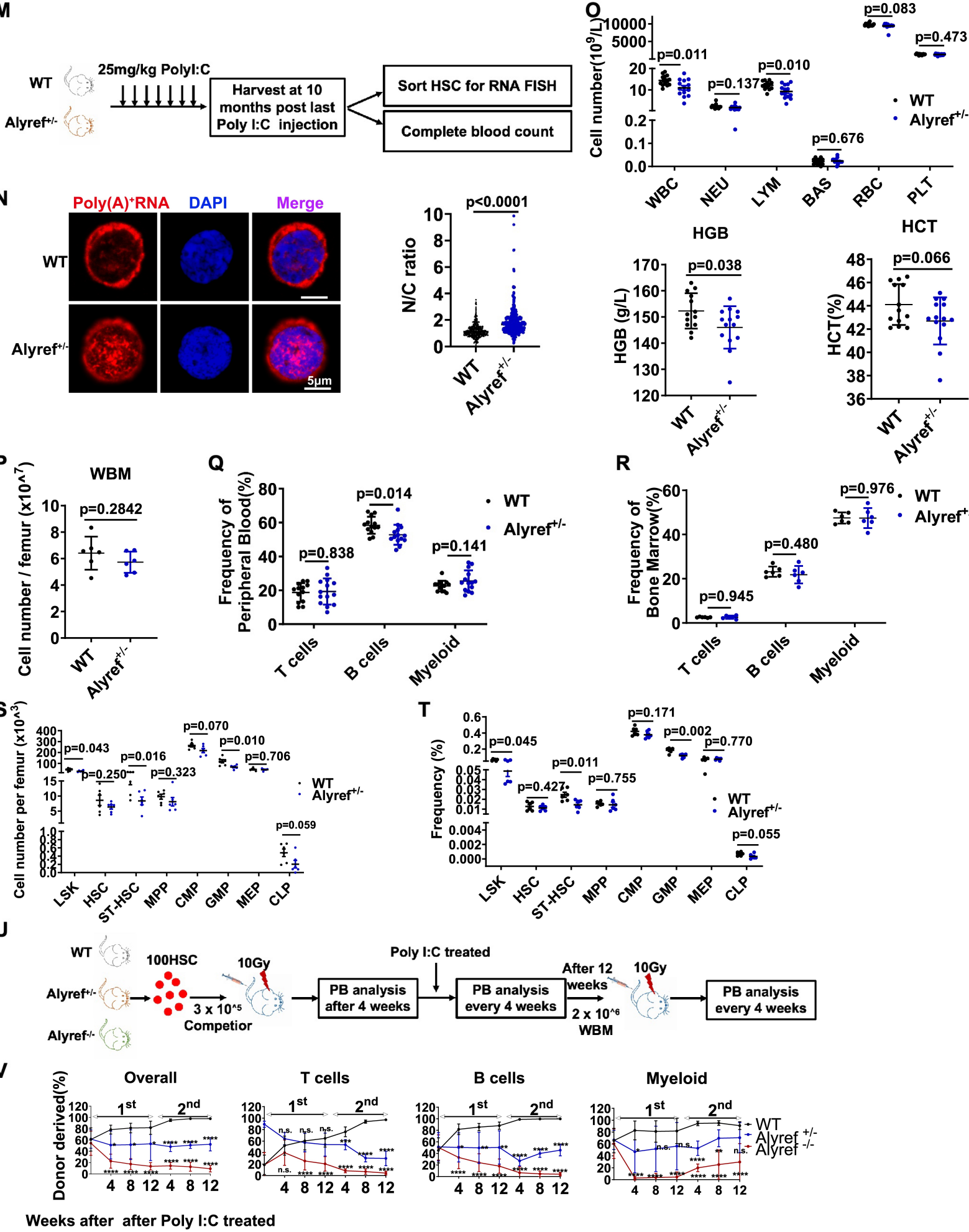
Online Supplementary Figure S3. Aging-increased R-Loop correlates with the lingering RNA in the nucleus of hematopoietic stem cells, related to Figure 3.



Online Supplementary Figure S4. Targeted dysfunction of Alyref induces R-Loop and disrupts hematopoietic homeostasis, related to Figure 4.

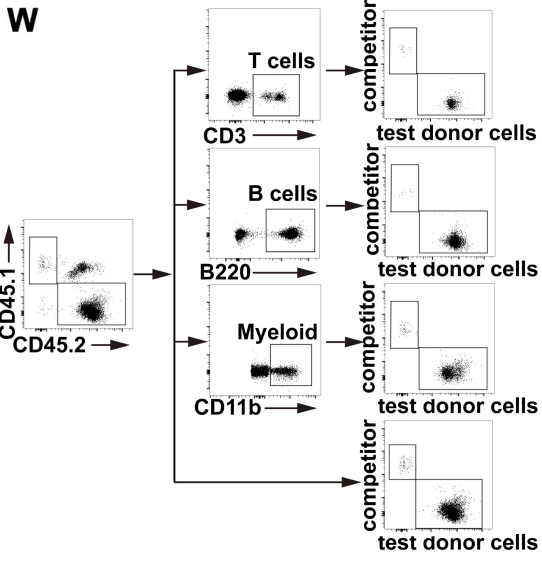


Online Supplementary Figure S4. Targeted dysfunction of Alyref induces R-Loop and disrupts hematopoietic homeostasis, related to Figure 4.

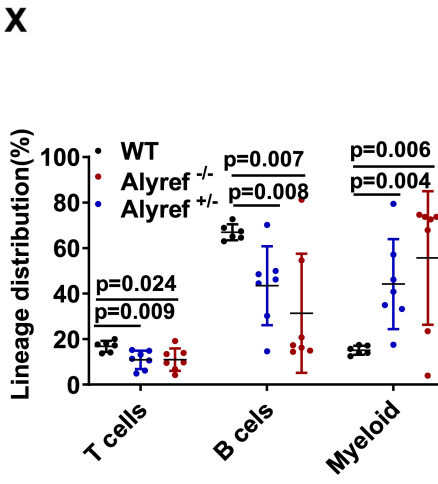


Online Supplementary Figure S4. Targeted dysfunction of Alyref induces R-Loop and disrupts hematopoietic homeostasis, related to Figure 4.

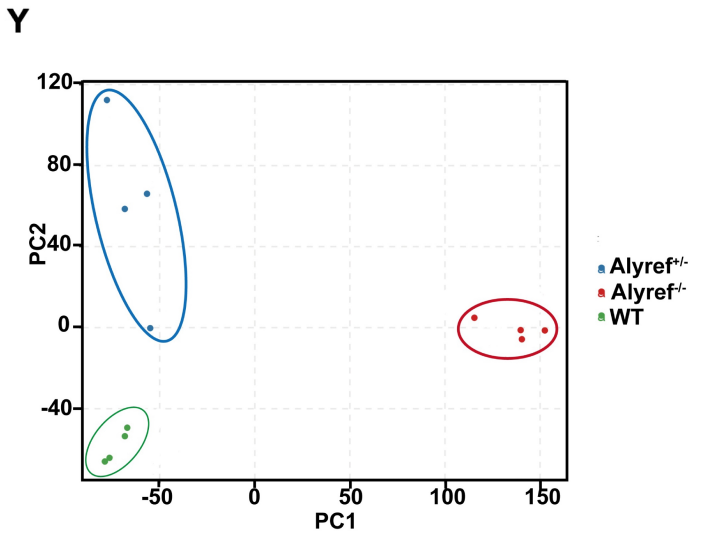
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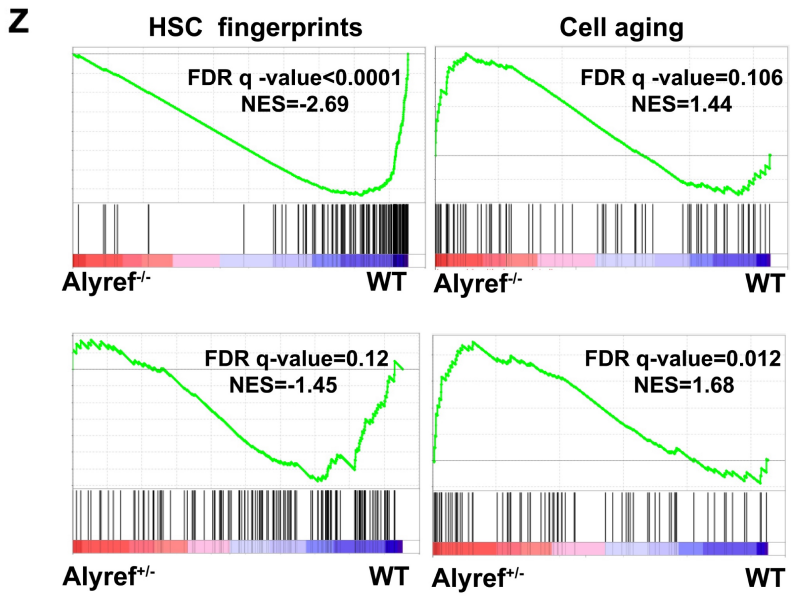
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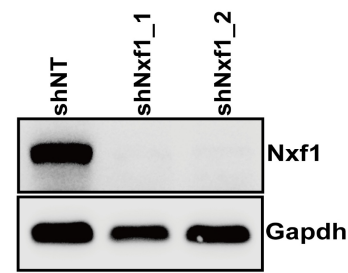


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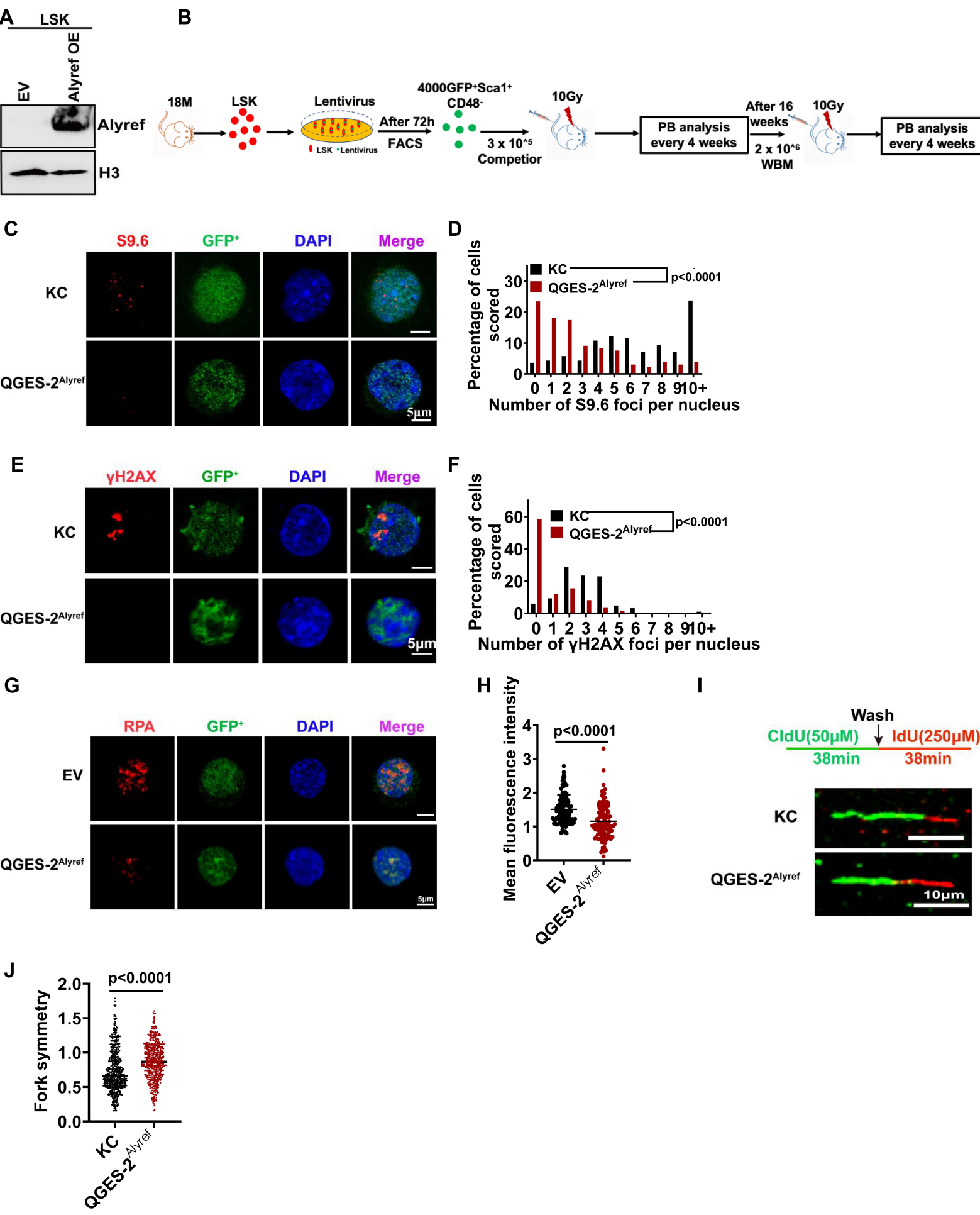


Online Supplementary Figure S5. Dysfunction of RNA transportation impairs hematopoietic stem cells, related to Figure 5.

A

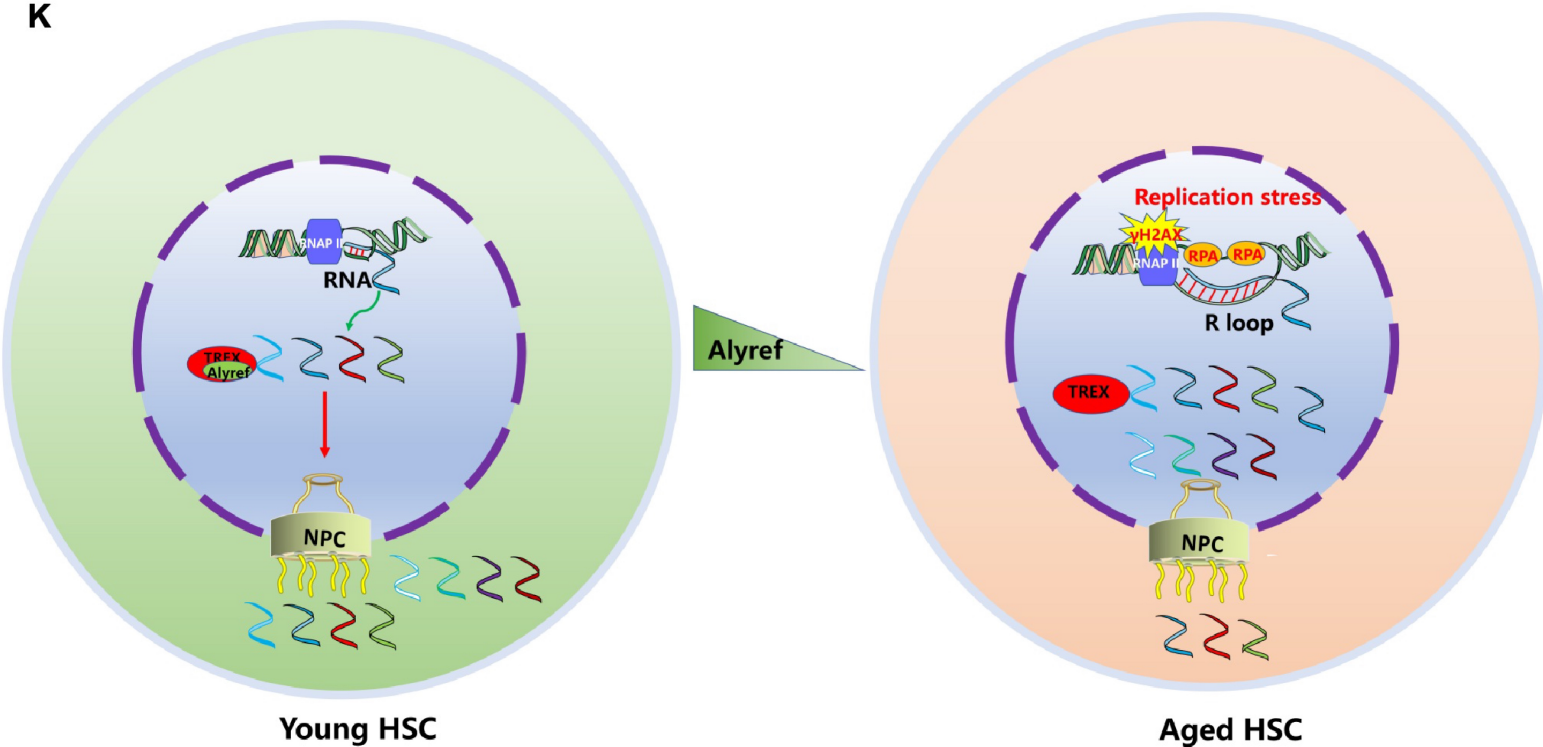


Online Supplementary Figure S6. Quantitative refill of Alyref rescues the function of aged hematopoietic stem cells by dampening R-Loop and replication stress, related to Figure 6.



Online Supplementary Figure S6. Quantitative refill of Alyref rescues the function of aged hematopoietic stem cells by dampening R-Loop and replication stress, related to Figure 6.

K



Online Supplementary Table S1. Geneotyping primer

	Forward (5'-3')	Reverse(5'-3')
Alyref ^{fl/f}	ATTTCACTTCAGACTTGGCAGC AG	CCAGAGGCCAATAGTAAACTGT C
	GCGGTCTGGCAGTAAAACTAT C (F1)	GTGAAACAGCATTGCTGTCACTT (R1)
Mx1-cre	CTAGGCCACAGAATTGAAAGA TCT (F2)	GTAGGTGGAAATTCTAGCATCAT CC (R2)

Online Supplementary Table S2. shRNA and clone primer

Gene	Sequence
Thoc1	ACAGATTGAGTGTGACAGTGAA
Thoc5	ATGGCAGAGACTATGAGTTGTA
Nxf1_1	AAAGGATATCTATCATCATCAA
Nxf1_2	CCGCGAACGATTTCCCAAGTTA
RNaseH1	AAGACAGCATGTTCAACATCAA
RNaseH2A	ACCAGGAATAAAAGCTGTTCAA
shRNA	ACTTCGACTTCTTAACCCAACAGAAGGCTCGAGAAGGTATATTGCT
clone	GTTGACAGTGAGCG (Forward, 5'-3')
primer	GTTTTAGTAAACAAGATAATTGCTCGAATTCTAGCCCCTTGAAGTC
	CGAGGCAGTAGGCA (Reverse, 5'-3')

Online Supplementary Table S3. Flow cytometric analysis and cell sorting antibody

Name	Manufacturer	Cat#	RRID	Clone
Biotin B220 anti-mouse	Biologend	103204	AB_312989	RA3-6B2
Biotin Ly-6G and Ly-6C anti-mouse	Biologend	108404	AB_313369	RB6-8C5
Biotin CD8a anti-mouse	Biologend	100704	AB_312743	53-6.7
Biotin CD3ε anti-mouse	Biologend	100304	AB_312669	145-2C11
Biotin CD4 anti-mouse	Biologend	100508	AB_312711	RM4-5
Biotin CD11b anti-mouse	Biologend	101204	AB_312787	M1/70
Biotin Ter-119 anti-mouse	Biologend	116204	AB_313705	TER-119
FITC CD45.2 anti-mouse	Biologend	109806	AB_313443	104
Pacific Blue™ B220 anti-mouse	Biologend	103227	AB_492876	RA3-6B2
APC CD3ε anti-mouse	Biologend	100312	AB_312677	145-2C11
PerCP-Cy™5.5 CD11b anti-mouse	Biologend	101228	AB_893232	M1/70
Alexa Fluor® 700 Gr1 anti-mouse	Biologend	108422	AB_2137487	RB6-8C5
PE CD45.1 anti-mouse	Biologend	110708	AB_313497	A20
FITC CD8a anti-mouse	Biologend	100706	AB_312745	53-6.7
PE CD4 anti-mouse	Biologend	100512	AB_312715	RM4-5
Streptavidin APC-eFluor780	Invitrogen	47-4317-82	AB_10366688	
PerCP/Cyanine5.5 CD127 anti-mouse	Biologend	135022	AB_1937273	A7R34
PE CD150 anti-mouse	Biologend	115904	AB_313683	TC15-12F12.2
APC CD117 anti-mouse	Invitrogen	17-1171-83	AB_469431	2B8

Online Supplementary Table S3 (Continued).

Name	Manufacturer	Cat#	RRID	Clone
PE/Cyanine7 CD150 anti-mouse	Biologend	115914	AB_439797	TC15-12F12.2
PE/Cyanine7 CD16/32 anti-mouse	Biologend	101318	AB_2104156	93
PE-Cy TM 7 Sca-1 anti-mouse	Invitrogen	25-5981-82	AB_469669	D7
BV421 CD127 anti-mouse	Biologend	135024	AB_11218800	A7R34
FITC CD16/CD32 anti-mouse	Biologend	101306	AB_312805	93
PE-CF594 CD135 anti-mouse	BD Biosciences	562537	AB_2737639	A2F10.1
FITC CD34 anti-mouse	Invitrogen	11-0341-85	AB_465022	RAM34
Alexa Fluor® 700 CD34 anti-mouse	Invitrogen	56-0341-82	AB_493998	RAM34

Online Supplementary Table S4. Western blot and Immunofluorescence staining antibody

Name	Manufacturer	Cat#	RRID	Clone
Rabbit anti-Histone H3	Cell Signaling Technology	4499	AB_10544537	D1H2
Rabbit anti- β -Actin	Cell Signaling Technology	4970	AB_2223172	
Rabbit anti-Gapdh	Abclonal	AC001	AB_2619673	
Rabbit Anti-Mouse IgG (HRP Conjugate)	Cell Signaling Technology	58802	AB_2799549	D3V2A
Mouse Anti-Rabbit IgG (HRP Conjugate)	Cell Signaling Technology	93702	AB_2800208	D4W3E
Anti-Mouse IgG Alexa Fluor® 594	Invitrogen	A-11005	AB_2534073	
Anti-Rabbit IgG Alexa Fluor® 488	Invitrogen	A-11008	AB_143165	
Anti-Rat IgG Alexa Fluor® 488	Invitrogen	A-11006	AB_2534074	
Anti-Rabbit IgG Alexa Fluor® 647	Invitrogen	A-21245		
Anti-Mouse IgG Alexa Fluor® 647	Abcam	ab150115		
Mouse γ H2AX	Millipore	05-636		
Rabbit -Nxf1	Abclonal	A5907		
Rat anti-RPA	Cell Signaling Technology	2208s		
Rabbit -RNaseH1	Abclonal	A9116		

Online Supplementary Table S4 (Continued).

Name	Manufacturer	Cat#	RRID	Clone
Rabbit -RNaseH2A	Abclonal	A15132		
Rat anti-CIdU	Abcam	ab6326		
Mouse anti-IdU	Becton Dickinson	347580		
Mouse anti-S9.6	Millipore	MABE1095		
Rabbit -Alyref	Abcam	ab202894		
Rabbit -Alyref	Cell Signaling Technology	12655		
Rabbit - γ H2AX	Cell Signaling Technology	9718		

Online Supplementary Table S5. RT-PCR primer list

Gene	Forward(5'-3')	Reverse(5'-3')
Actin	GTGACGTTGACATCCGTAAAGA	GCCGGACTCATCGTACTCC
Alyref	ACCGAAACAACCTCCCGACAA	CACATCTGCTGTCCCTAAACTT
Thoc1	GTGACCGAAAGTGTTTGTACTGC	CTGAGGAGATCATTGCACATACG
Thoc5	GGACCTGAAGAGTAAGGGCAG	CTGATGTGGGCTAATCGGTTAAG

Online Supplementary Table S6. gRNA primer

Gene	Sequence
Nfe2l2	CTGCGCCTCCCACCCAGC
	TTGCCCTCCCAGCTCGGC
ssDRIP-qPCR primer	
Forward(5'-3')	GCGAGGTGGTGGTGTCTC
Reverse(5'-3')	AACGGAGGATGTTGGGGC

Online Supplementary Table S7.

HSC fingerprint gene

Column1	Column2
ENSMUSG00000073293	Nudt10
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ENSMUSG00000070822	Zscan18
ENSMUSG00000068270	Shroom4
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ENSMUSG00000061762	Tac1
ENSMUSG00000058135	Gstm1
ENSMUSG00000057614	Gnai1
ENSMUSG00000056758	Hmga2
ENSMUSG00000055799	Tcf7l1
ENSMUSG00000055737	Ghr
ENSMUSG00000055692	Tmem191
ENSMUSG00000054252	Fgfr3
ENSMUSG00000053080	Zfta
ENSMUSG00000052155	Acvr2a
ENSMUSG00000050953	Gja1
ENSMUSG00000049791	Fzd4
ENSMUSG00000049536	Tceal1
ENSMUSG00000049382	Krt8
ENSMUSG00000048960	Prex2
ENSMUSG00000048537	Phldb1
ENSMUSG00000048489	Depp1
ENSMUSG00000048388	Fam171b
ENSMUSG00000046402	Rbp1
ENSMUSG00000045954	Cavin2
ENSMUSG00000045103	Dmd
ENSMUSG00000044393	Dsg2
ENSMUSG00000043518	Rai2
ENSMUSG00000042826	Fgf11
ENSMUSG00000042340	Ctf1
ENSMUSG00000040891	Foxa3
ENSMUSG00000040537	Adam22
ENSMUSG00000040289	Hey1
ENSMUSG00000039943	Plcb4
ENSMUSG00000039765	Cc2d2a
ENSMUSG00000039084	Chad
ENSMUSG00000038700	Hoxb5

ENSMUSG00000038235	F11r
ENSMUSG00000037440	Vnn1
ENSMUSG00000037169	Mycn
ENSMUSG00000036564	Ndrg4
ENSMUSG00000036356	Csgalnact1
ENSMUSG00000036120	Rfxank
ENSMUSG00000035413	Tmem98
ENSMUSG00000034795	Ccdc122
ENSMUSG00000034771	Tle2
ENSMUSG00000034382	Al661453
ENSMUSG00000034037	Fgd5
ENSMUSG00000033590	Myo5c
ENSMUSG00000033585	Ndn
ENSMUSG00000033032	Afap111
ENSMUSG00000032968	Inha
ENSMUSG00000032717	Mdfi
ENSMUSG00000032547	Ryk
ENSMUSG00000032348	Gsta4
ENSMUSG00000032194	Kank2
ENSMUSG00000031997	Trpc6
ENSMUSG00000031870	Pgr
ENSMUSG00000031778	Cx3cl1
ENSMUSG00000031740	Mmp2
ENSMUSG00000031548	Sfrp1
ENSMUSG00000031503	Col4a2
ENSMUSG00000031502	Col4a1
ENSMUSG00000031375	Bgn
ENSMUSG00000031290	Lrch2
ENSMUSG00000030796	Tead2
ENSMUSG00000030711	Sult1a1
ENSMUSG00000030208	Emp1
ENSMUSG00000030088	Aldh1l1
ENSMUSG00000030022	Adamts9
ENSMUSG00000029661	Col1a2
ENSMUSG00000029469	Ift81
ENSMUSG00000029311	Hsd17b11
ENSMUSG00000029267	Mtf2
ENSMUSG00000028957	Per3
ENSMUSG00000028469	Npr2
ENSMUSG00000028463	Car9

ENSMUSG00000028402	Mpdz
ENSMUSG00000028023	Pitx2
ENSMUSG00000027954	Efna1
ENSMUSG00000027800	Tm4sf1
ENSMUSG00000027684	Mecom
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ENSMUSG00000027646	Src
ENSMUSG00000027559	Car3
ENSMUSG00000027499	Pkia
ENSMUSG00000027358	Bmp2
ENSMUSG00000027351	Spred1
ENSMUSG00000026994	Galnt3
ENSMUSG00000026826	Nr4a2
ENSMUSG00000026814	Eng
ENSMUSG00000026796	Niban2
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ENSMUSG00000026315	Serpinb8
ENSMUSG00000025887	Casp12
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ENSMUSG00000025094	Slc18a2
ENSMUSG00000024940	Ltbp3
ENSMUSG00000024924	Vldlr
ENSMUSG00000024486	Hbegf
ENSMUSG00000024420	Zfp521
ENSMUSG00000024268	Celf4
ENSMUSG00000024109	Nrxn1
ENSMUSG00000023828	Slc22a3
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ENSMUSG00000020176	Grb10
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ENSMUSG00000019768	Esr1
ENSMUSG00000018845	Unc45b
ENSMUSG00000018800	Abca5
ENSMUSG00000017390	Aldoc
ENSMUSG00000016494	Cd34
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ENSMUSG00000014932	Yes1
ENSMUSG00000014704	Hoxa2
ENSMUSG00000009378	Slc16a12
ENSMUSG00000008305	Tle1
ENSMUSG00000007989	Fzd3
ENSMUSG00000006389	Mpl
ENSMUSG00000006386	Tek
ENSMUSG00000004267	Eno2
ENSMUSG00000004044	Cavin1
ENSMUSG00000003949	Hlf
ENSMUSG00000003226	Ranbp2
ENSMUSG00000002799	Jag2
ENSMUSG00000002265	Peg3
ENSMUSG00000000938	Hoxa10
ENSMUSG00000000753	Serpinf1
ENSMUSG00000000058	Cav2
ENSMUSG00000000031	H19

Cell aging

Column1	Column2
ENSEMBL	SYMBOL
ENSMUSG00000030562	Nox4
ENSMUSG00000025499	Hras
ENSMUSG00000001517	Foxm1
ENSMUSG00000020364	Zfp354a
ENSMUSG00000054580	Pla2r1
ENSMUSG00000055917	Zfp277
ENSMUSG00000022982	Sod1
ENSMUSG00000023067	Cdkn1a
ENSMUSG00000027820	Mme
ENSMUSG00000024975	Pdcd4

ENSMUSG00000040274	Cdk6
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ENSMUSG00000028063	Lmna
ENSMUSG00000000093	Tbx2
ENSMUSG00000022510	Trp63
ENSMUSG00000022508	Bcl6
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ENSMUSG00000022672	Prkdc
ENSMUSG00000037405	Icam1
ENSMUSG00000034218	Atm
ENSMUSG00000046711	Hmga1
ENSMUSG00000055116	Arntl
ENSMUSG00000035799	Twist1
ENSMUSG00000030890	Ilk
ENSMUSG00000056758	Hmga2
ENSMUSG00000028991	Mtor
ENSMUSG00000026814	Eng
ENSMUSG00000004936	Map2k1
ENSMUSG00000020898	Ctc1
ENSMUSG00000035873	Pawr
ENSMUSG00000021796	Bmpr1a

Myeloid cell differentiation

Column1	Column2
ENSEMBL	SYMBOL
ENSMUSG00000066677	Ifi208
ENSMUSG00000035692	Isg15
ENSMUSG00000074417	Pira11
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ENSMUSG00000090272	Mndal

ENSMUSG00000074419	Pira6
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ENSMUSG00000032786	Alas1
ENSMUSG00000025270	Alas2
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ENSMUSG00000022892	App
ENSMUSG00000054428	Atpif1

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ENSMUSG00000036896	C1qc
ENSMUSG00000023964	Calcr
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ENSMUSG00000022952	Runx1
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ENSMUSG00000031885	Cbfb
ENSMUSG00000023274	Cd4
ENSMUSG00000037706	Cd81
ENSMUSG00000040274	Cdk6
ENSMUSG00000037664	Cdkn1c
ENSMUSG00000034957	Cebpa
ENSMUSG00000056501	Cebpb
ENSMUSG00000056216	Cebpg
ENSMUSG00000026031	Cflar
ENSMUSG00000025199	Chuk
ENSMUSG00000038037	Socs1
ENSMUSG00000025804	Ccr1
ENSMUSG00000064039	Ccr11l
ENSMUSG00000037944	Ccr7
ENSMUSG00000025958	Creb1
ENSMUSG00000014599	Csf1
ENSMUSG00000024621	Csf1r
ENSMUSG00000018916	Csf2
ENSMUSG00000038067	Csf3
ENSMUSG00000028859	Csf3r
ENSMUSG00000022150	Dab2
ENSMUSG00000007805	Twist2
ENSMUSG00000040856	Dlk1
ENSMUSG00000014773	Dll1
ENSMUSG00000003812	Dnase2a

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ENSMUSG000000030400	Ercc2
ENSMUSG000000006311	Etv2
ENSMUSG000000021474	Sfxn1
ENSMUSG000000021678	F2rl1
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ENSMUSG000000031077	Fadd
ENSMUSG000000024778	Fas
ENSMUSG000000025153	Fasn
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ENSMUSG000000058715	Fcer1g
ENSMUSG000000024588	Fech
ENSMUSG000000053158	Fes
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ENSMUSG000000053617	Sh3pxd2a
ENSMUSG000000016087	Fli1
ENSMUSG000000021250	Fos
ENSMUSG000000027120	Fshb
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ENSMUSG000000008976	Gabpa
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