**Supplemental Table 1. Nucleic acid sequences of single-guide RNAs, primers, and probes**

| Assay             | Name       | Sequences (5’ to 3’)                      |
|-------------------|------------|-------------------------------------------|
| sgRNAs            | AAVS1      | ACCCCACAGTGGGGCGACCAGA                  |
|                   | RPS19.1    | TACCCCGAGCTTTCCACAGCG                   |
|                   | RPS19.2    | TGTAAGAGCGTGGAACACCAG                   |
|                   | RPS19.3    | TACCCCGAGCTTTCCACAGCG                   |
|                   | TP53       | TACCCCGAGCTTTCCACAGCG                   |
| NGS primers       | AAVS1 forward | AGTCTTTTCCTCAACCGGGGCGGG                |
|                   | AAVS1 reverse | CTCCTTCCTCACCTCAACCGGGG                |
|                   | RPS19.1 and 3 forward | GACCTGCCTGCGGGGCTGCT                 |
|                   | RPS19.1 and 3 reverse | TCTGCTGCTGCGGGGCTGCT                 |
|                   | RPS19.2 forward | AGTCTTTTCCTCAACCGGGGCGGG                |
|                   | RPS19.2 reverse | CTCCTTCCTCACCTCAACCGGG                |
|                   | TP53 forward | AGTCTTTTCCTCAACCGGGGCGGG                |
|                   | TP53 reverse | CTCCTTCCTCACCTCAACCGGG                |
| ddPCR primers and probes | Psi forward | ACTTGAAAGCGAAGGCAAAC                  |
|                   | Psi reverse | CACCCATCTCTCTCTAAGCC                   |
|                   | Psi probe   | 5’FAM-AGCTCTCTCAGCGACTCGG               |
|                   | RPP30 forward | GACCTTGCTGACTACTGAGGT                 |
|                   | RPP30 reverse | GACCTTGCTGACTACTGAGGT                 |
|                   | RPP30 probe  | 5’HEX-AGCTCTCTCAGCGACTCGG               |
| Northern blot probe | ITS1 probe | CCTGCCCTCAGGGGCTGCTTAATGA               |
**Supplemental Table 2:** Reference sequence and top 10 most frequent edited reads for three different sgRNAs targeting RPS19. sgRNA-binding sequences are in red, insertions are in blue, and deletions are represented as dashes.

| Name                              | Sequence                                                                 |
|-----------------------------------|---------------------------------------------------------------------------|
| **Reference sequence (RPS19.1)**  | CACTACCCCAAGCTTCCACAGCGGCACCTGTACCTCC                                     |
| **Edited reads**                  |                                                                           |
|                                   | CACTACCCCAAGCTTCCACAGCGGCACCTGTACCTCC                                    |
|                                   |                                                                           |
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| Reference sequence (RPS19.2)      | TACTGTAAGAGCTGACACCAGCAGAGAGTTCGTCAGAGCT                                 |
| Edited reads                      |                                                                           |
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| Reference sequence (RPS19.3)      | GGTGGCTCCAAGCATGACCTGAGGAGTTCTCTGTCAGAGCT                                |
| Edited reads                      |                                                                           |
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### Supplemental Table 3. Media and cytokines

| Medium                        | Component                      | Manufacturer         | Catalog #        | Final Concentration |
|-------------------------------|-------------------------------|----------------------|------------------|--------------------|
| **HSPC maintenance**          | X-Vivo 10 (base)              | Lonza                | BEBP02-055Q      |                    |
|                               | Human stem cell factor        | R&D Systems          | 255-SC/CF        | 100 ng/mL          |
|                               | Thrombopoietin                | R&D Systems          | 288-TP/CF        | 100 ng/mL          |
|                               | FLT-3 ligand                  | R&D Systems          | 3088-FK/CF       | 100 ng/mL          |

**Erythroid differentiation**

| Medium                        | Component                      | Manufacturer         | Catalog #        | Final Concentration |
|-------------------------------|-------------------------------|----------------------|------------------|--------------------|
| IMDM (base)                   | Common to all phases          |                      |                  |                    |
|                               | Human male AB plasma          | SeraCare             | 1810-0001        | 2%                 |
|                               | Human AB serum                | Atlanta Biologicals  | S40110           | 3%                 |
|                               | Heparin                       | Sagent Pharmaceuticals| NDC 25021-401-02 | 3 IU/mL            |
|                               | EPO                           | Amgen                | NDC 55513-144-01 | 3 IU/mL            |
|                               | Penicillin–Streptomycin       | Thermo Fisher        | 15070063         | Penicillin 50 U/mL Streptomycin 50 µg/mL |

**Erythroid differentiation (Phase I)**

| Medium                        | Component                      | Manufacturer         | Catalog #        | Final Concentration |
|-------------------------------|-------------------------------|----------------------|------------------|--------------------|
|                               | Human holo-transferrin        | Millipore            | T0665            | 200 µg/mL          |
|                               | Human stem cell factor        | R&D Systems          | 255-SC/CF        | 10 ng/mL           |
|                               | Human IL-3                    | R&D Systems          | 203-IL/CF        | 1 ng/mL            |

**Erythroid differentiation (Phase II)**

| Medium                        | Component                      | Manufacturer         | Catalog #        | Final Concentration |
|-------------------------------|-------------------------------|----------------------|------------------|--------------------|
|                               | Human holo-transferrin        | Millipore            | T0665            | 200 µg/mL          |
|                               | Human stem cell factor        | R&D Systems          | 255-SC/CF        | 10 ng/mL           |
Supplemental Table 4. Antibodies used in flow cytometry panels and Western blots

| Panel                        | Antibody        | Clone     | Manufacturer | Catalog # |
|------------------------------|-----------------|-----------|--------------|-----------|
| Mouse bone marrow studies    | BV786 Anti-Mouse CD45 | 30-F11    | BD Biosciences | 564225    |
|                              | BV605 Anti-Human CD45 | HI30      | BD Biosciences | 564047    |
|                              | PE-Cy7 Anti-Human CD33 | P67.6     | BD Biosciences | 333946    |
|                              | PE Anti-Human CD19 | 4G7       | BD Biosciences | 349209    |
|                              | Alexa Fluor 700 Anti-Human CD34 | 581 | BD Biosciences | 561440    |
|                              | PerCP-Cy5.5 Anti-Mouse Ter119 | TER-119 | BD Biosciences | 560512    |
|                              | APC Anti-Human CD235 | GA-R2 (HIR2) | BD Biosciences | 551336    |
| Erythroid differentiation    | APC-Cy7 Anti-Human CD3 | SK7       | BD Biosciences | 557832    |
|                              | BV421 Anti-CD235a | GA-R2 (HIR2) | BD Biosciences | 562938    |
|                              | BV510 Anti-CD41a | HIP8      | BD Biosciences | 563250    |
|                              | PE Anti-CD117 | A3C6E2    | BioLegend     | 323408    |
|                              | PE-CF594 Anti-CD105 | 266      | BD Biosciences | 562380    |
|                              | PE-Cy7 Anti-IL3R | 6H6       | BioLegend     | 306010    |
|                              | APC Anti-CD34 | Clone 582 | BD Biosciences | 555824    |
|                              | APC-H7 Anti-CD71 | M-A712    | BD Biosciences | 563671    |
| Myeloid differentiation      |                 |           |              |           |
|                              | BV605 Anti-CD45 | HI30      | BD Biosciences | 564047    |
|                              | Alexa Fluor 488 Anti-CD15 | W6D3 | BioLegend     | 323010    |
|                              | PE-Cy7 Anti-CD33 | P67.6     | BioLegend     | 366618    |
| Western Blot                 |                 |           |              |           |
|                              | RPS19           | EPR10423  | Abcam        | 181365    |
|                              | TP53            | DO-1      | BD Biosciences | 554293    |
|                              | CDKN1A          | 12D1      | CST          | 2947      |
|                              | GFP             | N/A       | Abcam        | AB6556    |
Supplemental figure 1 (related to main Figure 1). Design of lentiviral vectors used in the study. (A) Diagram of third-generation self-inactivating LVs expressing GFP alone or RPS19 + GFP separated by auto-cleaving P2A sequence, driven by EF1α core promoter.
Supplemental Figure 2 (related to main Figure 1). *RPS19* indels in CD34+ hematopoietic stem and progenitor cells (HSPCs) 3 days after editing with Cas9 + *RPS19*.1 single-guide RNA (sgRNA) ribonucleoprotein (RNP). (A) *RPS19* reference sequence with complementary sgRNA *RPS19*.1 represented below. (B) Results of indel analysis performed on day 0 according to the scheme shown in main Figure 1B. The unedited wild-type (WT) sequence is shown at top. The percentage of each indel is shown at right, with the NGS read counts in parentheses.
Supplemental Figure 3 (related to main Figure 1). Reduced RPS19 protein and rescue by LV-derived RPS19 in RPS19-disrupted CD34\(^+\) HSPCs. Cells were edited with RPS19.1 RNP, transduced with or without LV, and analyzed 3 days after editing. (A) Representative Western blot showing RPS19 protein expression. (B) Relative RPS19 protein levels (normalized to actin) after editing with AAVS1 or RPS19.1 RNP. The bar chart shows the mean ± SD of 3 independent experiments (unpaired, 2-tailed Student t-test). (C) Western blot showing RPS19, GFP and actin loading control.
Supplemental Figure 4 (related to main Figure 2). Transduction with RPS19 lentiviral vector (LV) partially rescues the erythropoietic defect of RPS19<sup>+</sup>− HSPCs. (A) CD34<sup>+</sup> HSPCs were edited with the indicated concentrations of RPS19 RNP on day −3. On day 0, cells were switched to erythroid differentiation medium and indel frequencies were determined serially. Bar chart shows mean ± SD, with each symbol representing different HSPC donors. (B) RPS19 RNP-treated cells, ± RPS19 LV transduction, were generated as shown in main Figure 1B then grown in culture in erythroid medium. The graph shows the RPS19 indel frequency versus time. The data points are the mean ± SD of 6 biological replicates performed using 3 different CD34<sup>+</sup> HSPC donors (2 experiments per donor), represented by different symbols (Mixed model-effects analysis). (C) Cells per BFU-E colony generated by AAVS1 or RPS19-targeted CD34<sup>+</sup> HSPCs. Each symbol represents data from a different CD34<sup>+</sup> HSPC donor, with the bar chart showing the mean ± SD (unpaired, 2-tailed Student t-test).
Supplemental Figure 5 (related to main Figure 3). *RPS19*\+/- HSPCs exhibit erythroid maturation defect. (A) Representative flow cytometry plots of normal CD34+ HSPC in vitro erythroid differentiation showing the gating strategy for BFU-E (erythroid progenitor [EP] 1) and CFU-E (EP 2–4). (B) Effects of *RPS19* disruption on terminal erythroid maturation of CD34+ HSPCs. Cells were analyzed at day 14 of erythroid culture and gated as shown in Figure 3B. The bar chart shows the mean ± SD. Each symbol represents data from a different CD34+ HSPC donor (unpaired, 2-tailed Student t-test). (C) May–Grunwald and Giemsa–stained erythroblasts at days 7 and 14 of erythroid differentiation. Images were obtained with a Nikon Eclipse NI microscope, using a Nikon DS Qi2 camera.
Supplemental Figure 6 (related to main Figure 3). *RPS19* disruption does not impair in vitro myeloid differentiation. CD34+ HSPCs were treated with AAVS1 or RPS19 RNP as described in Figure 1B, grown in myeloid differentiation medium for 14 days, then analyzed for maturation markers. (A) Representative flow cytometry plots after staining with antibodies against myeloid surface markers. (B) Summary of multiple experiments performed as described above, using CD34+ HSPCs from 3 different donors. The bar chart shows the mean ± SD (unpaired, 2-tailed Student *t*-test).
Supplemental Figure 7 (related to main Figure 4). Multiplex editing of RPS19 and TP53 in CD34+ HSPCs. CD34+ HSPCs were edited with AAVS1 or RPS19.1 ± TP53 RNPs according to the protocol in Figure 1B. (A) Indel frequency corresponding to each targeting RNP at 3 days after electroporation. Each symbol represents data from different CD34+ cell donors. (B) Western blot showing TP53, CDKN1A and actin loading control. (C, D) Genotype distributions in BFU-E colonies generated from CD34+ HSPCs treated with RNPs targeting RPS19 ± TP53. n = total colonies analyzed from biological replicate experiments using 2 different CD34+ HSPC donors. All bar charts show the mean ± SD.
Supplemental Figure 8 (related to main Figure 5). RPS19 expressing LV partially rescues bone marrow repopulation defect of RPS19+/- HSPCs. Normal donor HSPCs were treated with RPS19.1 RNP ± RPS19-GFP LV then transplanted into NSGW mice, which underwent necropsy 16 weeks later, according to the protocol in Figure 4A. (A) Representative flow cytometry plots showing the gating strategy used to assess human HSPC repopulation. Asterisks indicate populations that were analyzed for RPS19 indels shown in main Figures 4D and 5C. (B) RPS19-GFP LV copy number per diploid genome in input HSPCs and after xenotransplantation. (C) LV-transduced (%GFP*) cells in input CD34* HSPCs and 16 weeks after xenotransplantation. (D) Percentages of human CD34* HSPCs and their progeny in recipient mouse bone marrow at 16 weeks. All bar charts show the mean ± SD, with each symbol representing data from a different CD34* cell donor.
Supplemental Figure 9 (related to main Figure 6). Engraftment of RPS19/TP53 multiplex-edited human cells after xenotransplantation. Normal human HSPCs were edited and transplanted into NSGW mice, as described in main Figure 4A. Necropsy was performed at 16 weeks, and recipient bone marrow populations were analyzed by flow cytometry. (A) Percentage of human CD45+ cells in recipient bone marrow at 16 weeks post transplant. Data were analyzed by ANOVA test and pairwise testing was performed with Tuckey's adjustment for multiple comparison. (B) Percentage of human CD34+ HSPCs and their differentiated progeny in recipient bone marrow. The corresponding RPS19 indel frequencies in each population are shown in main Figure 6C. (C) CDKN1A mRNA fold change over time, relative to the level in unedited cells. Each data point represents the mean ± SD of 3 biological replicate experiments using CD34+ cells from different donors. Linear mixed-effects model approach was used to test for statistical significance. Bar charts show the mean ± SD of the data, with each symbol representing data from a different CD34+ cell donor.