Supplementary Note

Note on CD34+ HSPC editing protocol: We note that our CD34+ cell editing protocol was performed under research conditions that deviate substantially from those that are expected to be employed for therapeutic editing of autologous HSCs, including the use of an electroporation system for use in small-scale research-based applications only and different cell culture methods. Our study examined CD34+ cells immediately after editing, while therapeutic protocols generally require cryopreservation of edited CD34+ cells in order to quantify the editing efficiency and evaluate other product release criteria prior to transplantation. Hypothetically, different editing and cell culture methods might alter the rates of genotoxicities and selection pressures against deleterious mutations. Such differences might affect the frequency at which micronucleated cells and/or those with chromothripsis proliferate into cell clones.

Note on Live-cell imaging: For live-cell imaging experiments to investigate bridge formation, cell division, and micronucleus formation, doxycycline-inducible Cas9 expressing RPE-1 cells expressing eGFP-BAF, RFP-H2B, and the guide targeting chr5q were plated on ibiTreat 24-well µ-Plates (ibidi) and placed on a Nikon inverted microscope (Ti-E or Ti2) with Perfect Focus for widefield microscopy. The microscope was equipped with an environmental chamber to maintain cells at 37 ºC and with humidified 5 % CO_2. Imaging was performed in 20 min intervals using a 20x/0.75 NA Plan Apochromat Lambda objective (Nikon) and Z-stacks of three images with a 2 µm step size using NIS-Elements 5.11.02 or 5.11.03 AR software (Nikon). Images were acquired using a Zyla 4.2 sCMOS camera (Andor) for up to ~50 h. Cell division frequency measurements, in the text include some cells whose previous mitosis

was not observed. For the data shown in Fig. 4b, we only include lifetime profiles of cells where we viewed a complete cell cycle, starting with the previous mitosis.

Note on Look-Seq (Live-cell imaging followed by single cell isolation and single cell whole genome sequencing): Briefly, single cells were seeded by flow sorting in 384-well µClear plates (Greiner), or were seeded in bulk on a 35-mm gridded ibiTreat dish (ibidi) and eGFP-H2B and TDRFP-NLS were imaged by widefield fluorescence imaging in intervals of 10-15 min, as above.

After sufficient time for micronucleus-containing cells to divide, daughter cells of interest were separated (~40 h after micronucleus formation). Cells were considered to have reincorporated their micronuclei if no fragments of GFP-H2B were detected in the cytoplasm after division. Cells were separated by trypsinization and limited dilution of daughters into a new 384-well plate[28], or for cells on ibiTreat dishes, the dish was transferred to another Nikon inverted microscope equipped with a CellEctor single-cell isolation system (Molecular Machines and Industries) and cell adhesion was loosened by exchanging the culture medium with a PBS-based non-enzymatic dissociation reagent (Sigma). Within ~30 min of applying dissociation reagent, cells of interest from live-cell imaging were identified and directly picked from the imaging dish using a robotically controlled glass capillary with an inner diameter of 40 µm that aspirated 80 nL of volume (Molecular Machines & Industries). This volume containing the cell was then deposited into a 5 µL droplet of PBS contained in a PCR-tube cap.

Whole genome amplification was performed using the REPLI-g Single Cell kit (QIAGEN), with initial lysis steps being performed in the PCR-tube lids, and amplification

terminated after 80 min. Amplified DNA was purified and sheared by sonication (Covaris) into ~500 bp fragments. Sheared DNA was processed by a Library Preparation Kit (KAPA) for multiplexed next-generation sequencing as previously described[28].

Note on structural variant calling: Because the Phi29 polymerase produces artificial chimeras between loci within close proximity due to template switching, we excluded shortrange intra-chromosomal discordant reads (distance between fragments < 10 kb). Because of this limitation, we do not detect short-range SV events, including insertion/deletion events at the Cas9 cut sites and local fold-back events that are expected to be generated between fused sister chromatids. We previously reported that false structural variants due to chimeric DNA generated by single-cell whole-genome amplification are enriched between loci separated by ≤ 150 kb[28]. By contrast, most *de novo* rearrangements resulting from DNA damage from micronuclei are formed between loci separated by 1 Mb. To exclude false SV events due to artificial chimeras with a stringent threshold for intrachromosomal SVs, we only considered long-range events with breakpoints separated by ≥ 1 Mb in this study.

In addition to the above described short-range chimeric DNA fragments occurring within amplicons, whole-genome amplification also generates random chimeric fragments between amplified DNA. As such chimeras are generated between random amplified DNA rather than from the original DNA template, the allele fraction of random chimeric fragments should be lower than the allele fraction of reads supporting true structural variants. Due to variation in the sequence coverage, we filtered low allele fraction variants using sample-specific read-depth cutoffs determined from the average allelic coverage. The average allelic coverage at different read-depth cutoffs was determined from the allelic depths at heterozygous sites in the parental

RPE-1 line[28]. In each sample, we determined the allelic depth at which the average allelic coverage (detection sensitivity for a genetic variant on a single chromosome) is approximately 50% and used this value as the minimum read-depth cutoff if it is above three. To further filter artificial chimeras, we additionally required at least one of the supporting reads to be a split alignment and one to be a discordant read pair mapping to call interchromosomal events, which occur less frequently than intrachromosomal rearrangements. We only included SVs that aligned to autosomes or sex chromosomes. Interchromosomal rearrangements were not included in rearrangement plots.

We note that the conclusions regarding statistical enrichment of structural variants within each sample are not dependent on the read-depth cutoff, but the choice of sample-specific read depth cutoffs allows consistent estimation and comparison of the frequency of DNA breaks across different samples with varying uniformity and sequencing depth.

Notes pertaining to Extended Data Figure 6: Methods: We electroporated healthy donor CD34+ HSPCs with Cas9 or Cas9/gRNA RNP targeting the erythroid-specific *BCL11A* enhancer. After 24 hours, when dividing cells could have formed micronuclei, we sorted live lineage negative cells (CD3, CD14, CD16, CD19, CD20, CD56 negative and DAPI-negative) into 8 x 96-well plates for RNP treated cells and 4 x 96-well plates for Cas9-only cells. The cells were cultured for 14 days in phase 1 erythroid differentiation medium, and colonies of any size were collected for DNA extraction. We note that we harvested all possible colonies because micronucleated cells and/or those with chromothripsis might have a fitness disadvantage resulting in smaller colonies. To determine which SNPs might be useful markers of

heterozygosity and therefore copy number, we performed a global diversity Infinium array (Illumina) on DNA from the donor used in the experiment. Heterozygous SNPs were chosen within intergenic regions, based on the expectation that copy number alterations around noncoding SNPs would more likely to have a neutral impact on fitness. We chose 8 SNPs to assay: 4 telomeric of the cut-site, 2 centromeric of the cut-site, and 2 on the uncut chromosome arm of the targeted chromosome. Each SNP in each clone was amplified by PCR, subjected to next-generation sequencing, and the fraction of sequencing reads containing the reference or alternate sequence was measured. In total we harvested DNA from 254 Cas9 control colonies and 415 RNP colonies.

Results: From this assay we did not detect a statistically significant difference at individual SNPs in the distribution of allele-frequencies between Cas9 only control and Cas9/gRNA electroporated samples. We noted high variability in measured allele frequencies at all SNPs even in Cas9 only controls and at SNP sites centromeric of the cut site. This variability improved significantly for samples with high read counts, likely reflecting larger colonies and more input DNA [lower segments of the heatmap in panel (c)]. Of the samples with >100 reads for each SNP, we did not observe evidence suggesting either arm-level LOH or arm-level copy number gains. Although arm-level copy number alterations were not detected after clonal expansion in this experiment, the detection sensitivity of the experiment is limited by its small sample size. Notably, the sample size in this experiment is orders of magnitude lower than would be relevant for therapeutic genome editing.