SUPPLEMENTAL MATERIAL

Target-enriched nanopore sequencing and *de novo* assembly reveals co-occurrences of complex genomic on-target rearrangements induced by CRISPR-Cas9 in human cells

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SUPPLEMENTARY FIGURES

SUPPLEMENTARY TABLES

SUPPLEMENTARY FILES

1. HAP1 Δt72 deletion clone Sanger Sequencing result: HAP1_t72.ab1
2. HepG2 Δt15 deletion clone Sanger Sequencing result: hepg2_t15.ab1
3. HAP1 Δt72 deletion clone assembled contig: HAP1_t72_ref_direc.fasta
4. HepG2 Δt15 deletion clone assembled contig: HepG2_t15_ref_direc_cor_210706.fasta
5. HepG2 Δt8 deletion clone assembled contig1: HepG2_t8_contig_pos_211225_contig1.fa
6. HepG2 Δt8 deletion clone assembled contig2: HepG2_t8_contig_pos_211225_contig2.fa
7. hTERT-RPE1 ΔR14 deletion clone Sanger Sequencing result: RNF_R14_c8_ext.ab1
8. Multiple sequence alignment output files comparing Sanger Sequencing results from HAP1 Δt72 and HepG2 Δt15 for the referee: CLUSTAL W HAP1_t72_HepG2_t15_ref.docx

DATA VISUALIZATION

The ChIP-seq dataset can be directly visualized using the UCSC genome browser: https://genome.ucsc.edu/s/kellyg/Cas9_ontarget

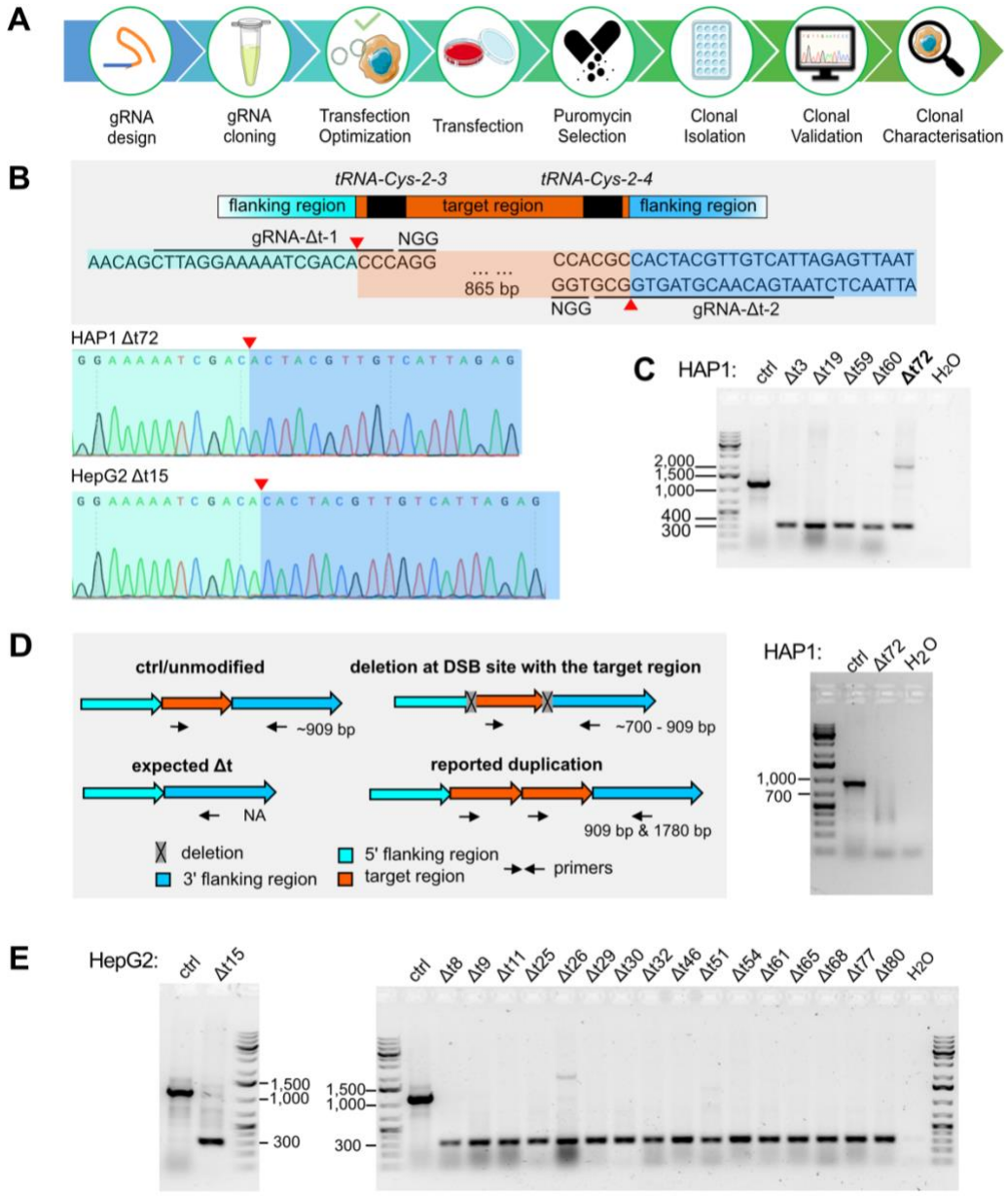

Fig S1. Validation of the tRNA gene locus deletion in HAP1 and HepG2.
(**A**) Schematic illustration of the workflow to obtain single cell-derived deletion clones. (**B**) The schematic representation (top) of our gene deletion design illustrates the 5' and 3' flanking regions (light and dark blue) and the target region (orange) containing the two $tRNA^{Cys}$ genes (black). Dual gRNA sequences with the PAM sequences (NGG) and cut sites (red triangle) are highlighted. Sanger sequencing chromatograms (bottom) show the successful deletion of the targeted genomic region in deletion clones HAP1 Δt72 and HepG2 Δt15. The red triangles indicate the predicted cut sites. The sequences and chromatograms are shadowed according to the colors used to highlight the flanking and target regions in the schematic representation. (**C**) Agarose gel electrophoresis confirms the size of the obtained PCR product to validate a deletion event (320 bp) using primers annealing to the flanking regions of the target sites (Fig. 1B) in the HAP1 clones. The HAP1 clone with duplicated target regions is bolded. Marker bands specify DNA size in bp. (**D**) Schematic illustration (grey box, left) of the PCR primer design strategy and expected amplicon lengths to assess the presence of different on-target outcomes in the HAP1 control (ctrl) clone and deletion clone Δt72. Agarose gel electrophoresis (right) confirms the size of the obtained PCR products. (**E**) As in Fig. S1C but for HepG2 deletion clones.

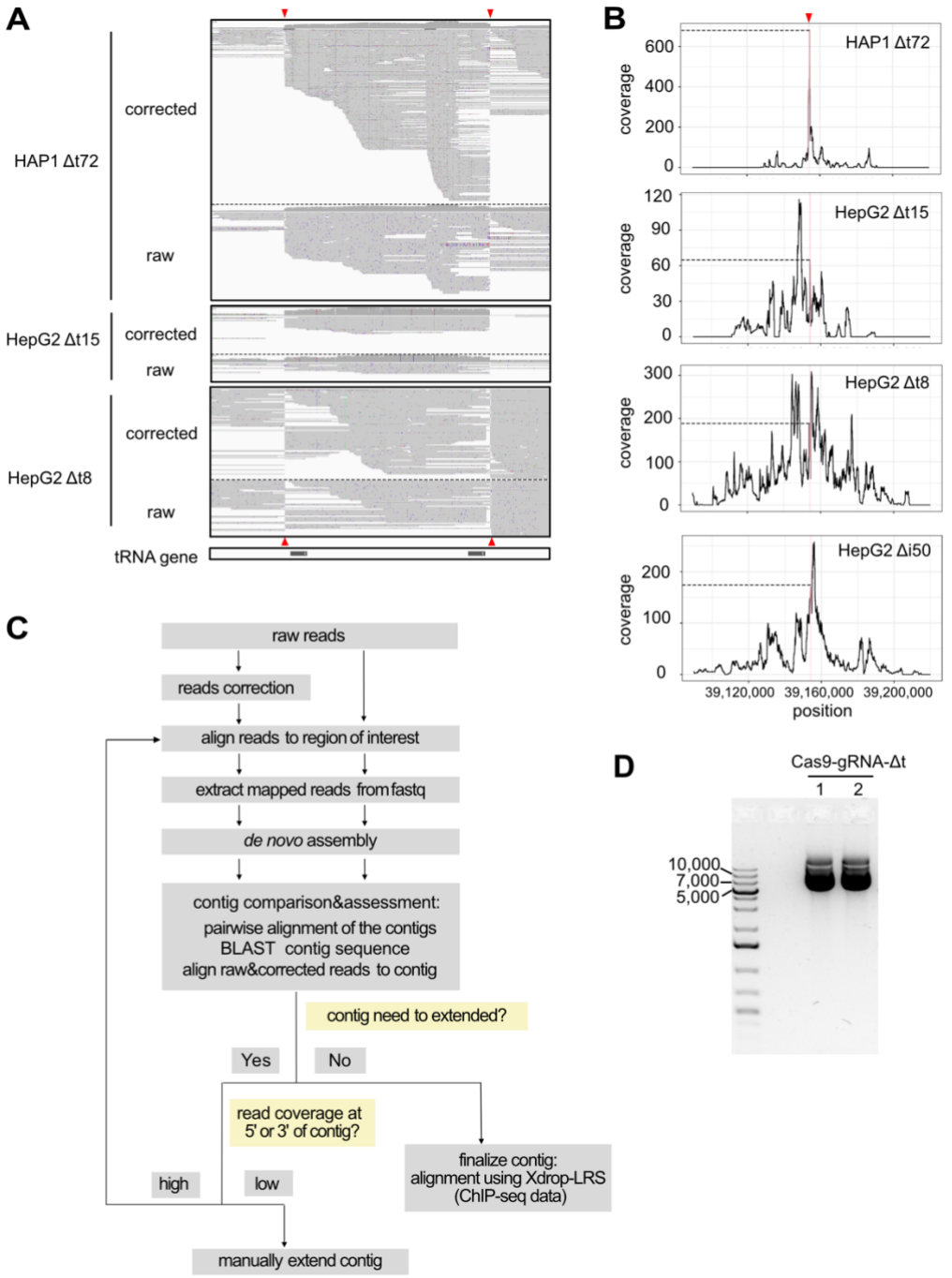

Fig S2. Complex on-target genomic alterations are revealed by Xdrop-LRS.
(**A**) Alignment tracks display Xdrop-LRS corrected and raw reads at the target loci in deletion clones HAP1 Δt72 (top), HepG2 Δt15 (middle) and HepG2 Δt8 (bottom) when aligned to the human reference genome (hg38). DSB sites (red triangles) and tRNA genes (black) are shown. (**B**) Line graphs show coverage when aligning raw reads obtained for the deletion clones HAP1 Δt72 (top), HepG2 Δt15 (upper middle), HepG2 Δt8 (lower middle) and HepG2 Δi50 (bottom) to the human reference genome (hg38). The position was defined by the coordinates from the human reference genome (hg38). Red lines and triangles highlight the location and dashed lines indicate the number of reads covering the target regions. (**C**) *De novo* assembly-based analysis workflow. (**D**) Agarose gel electrophoresis confirms the size of the CRISPR-Cas9-gRNA-Δt-1 and -2 vectors (around 9,170 bp) after plasmid purification. Marker bands specify DNA size in bp.

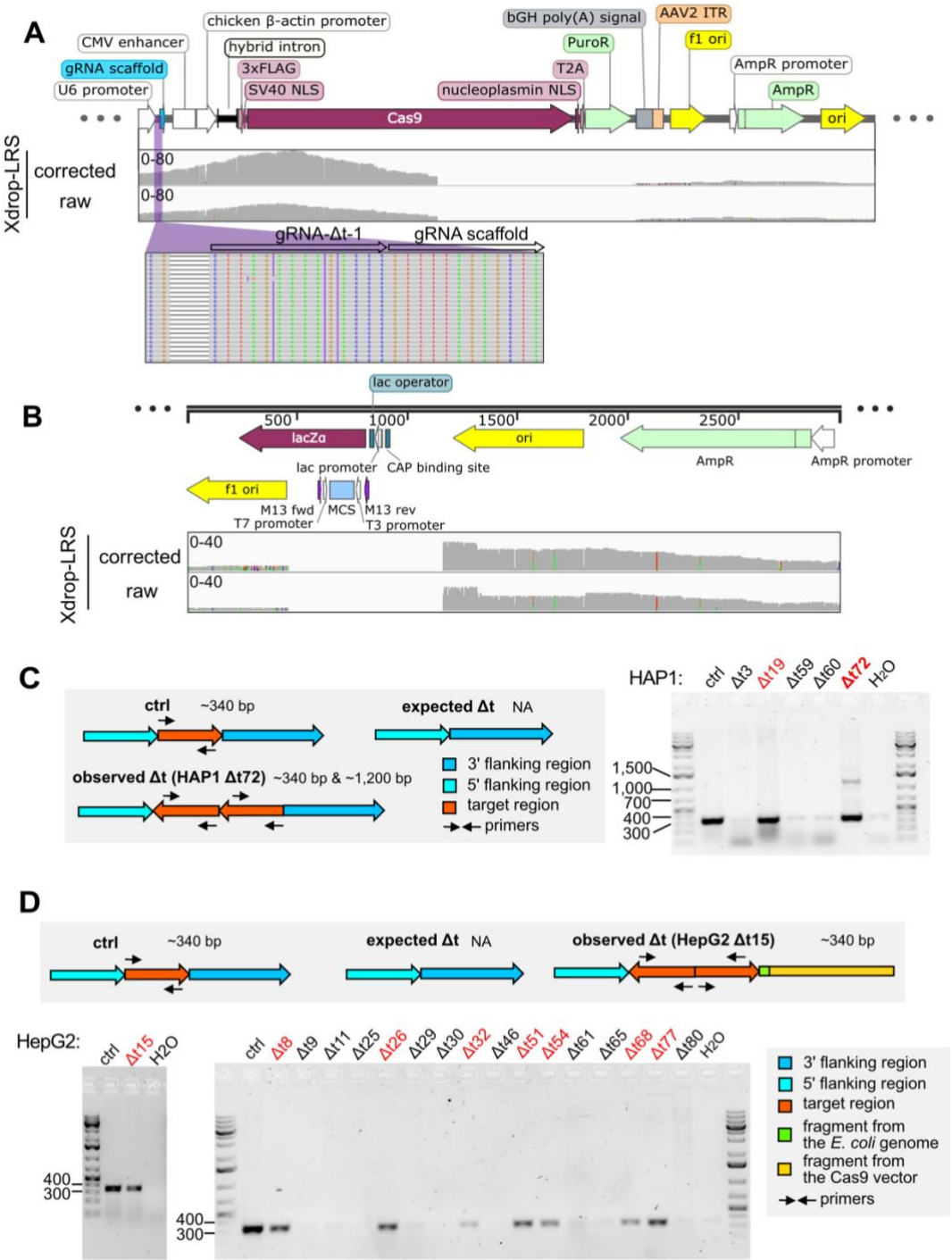

Fig S3. Validation of genomic on-target aberrations in HAP1 and HepG2 deletion clones. (**A-B**) Coverage tracks display deletion clone HepG2 Δt15 Xdrop-LRS corrected and raw reads aligning to the (A) CRISPR-Cas9 vector or (B) pBlueScript vector. Sequence annotations and directions of gene transcription are labelled according to the respective vector maps. The gRNA-Δt-1 cloning site and reads mapping to this region are highlighted (purple horizontal line) and magnified (Fig. S3A, bottom). (**C-D**) Schematic illustrations of additional primer pairs designed to distinguish unmodified control (ctrl), expected and observed deletion events in (C) HAP1 and (D) HepG2. Deletion clones confirmed in C and D with a reinserted target region (single target region 340 bp or if duplicated 340 bp and 1,200 bp) are indicated in red. Marker bands specify DNA size in bp.

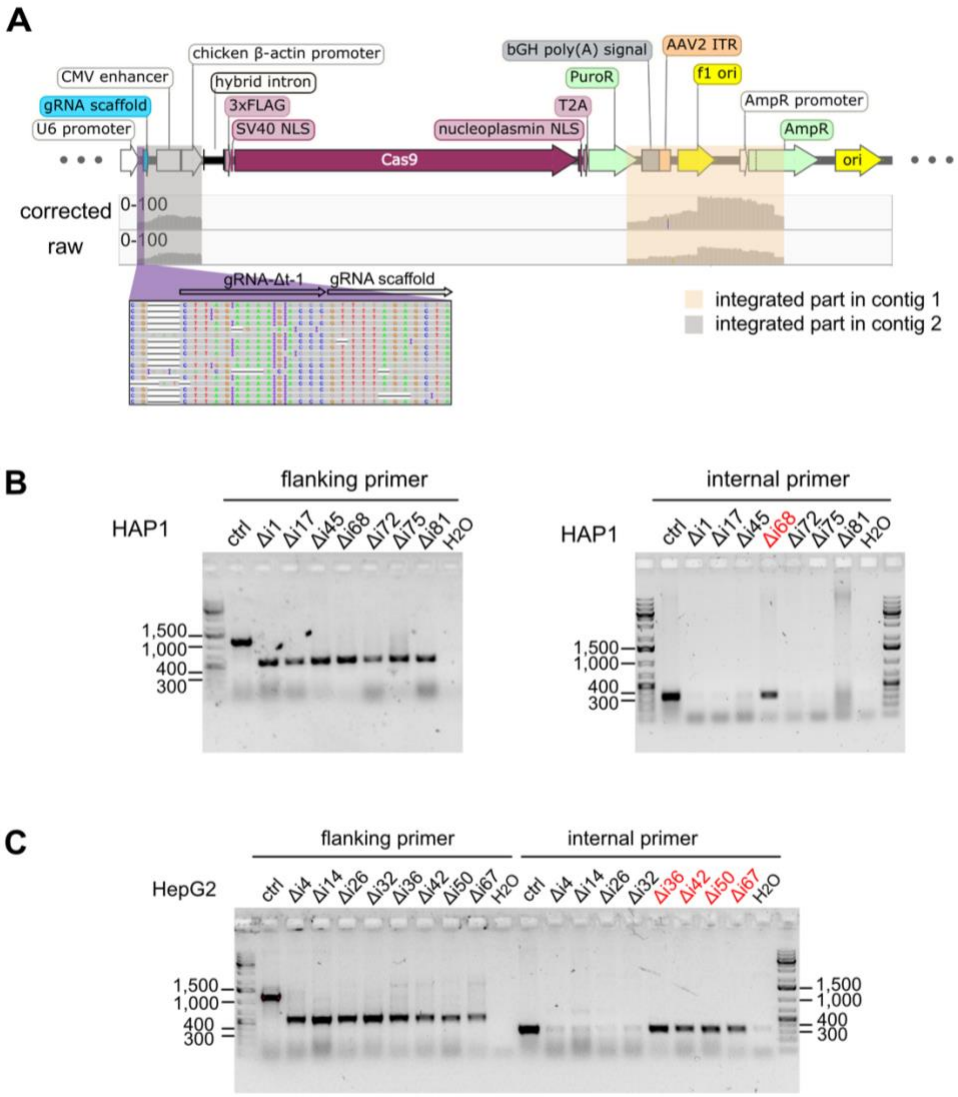

Fig S4. Validation of the intergenic region deletion in HAP1 and HepG2.
(**A**) Coverage tracks show deletion clone HepG2 Δt8 Xdrop-LRS corrected and raw reads aligning to the CRISPR-Cas9 vector (top). The vector fragments that were detectable in contig 1 (amber) and contig 2 (grey) (Fig. 4A-B) are highlighted. The gRNA-Δt-1 cloning site and reads mapping to this region are highlighted (purple horizontal line) and magnified (bottom). (**B-C**) Agarose gel electrophoreses display the size of the obtained PCR products using flanking primer (left) to validate deletion events and the internal primer (right) to examine genomic alterations (Fig. 4D) in (B) HAP1 and (C) HepG2 clones. Clones with genomic alterations are indicated in red.

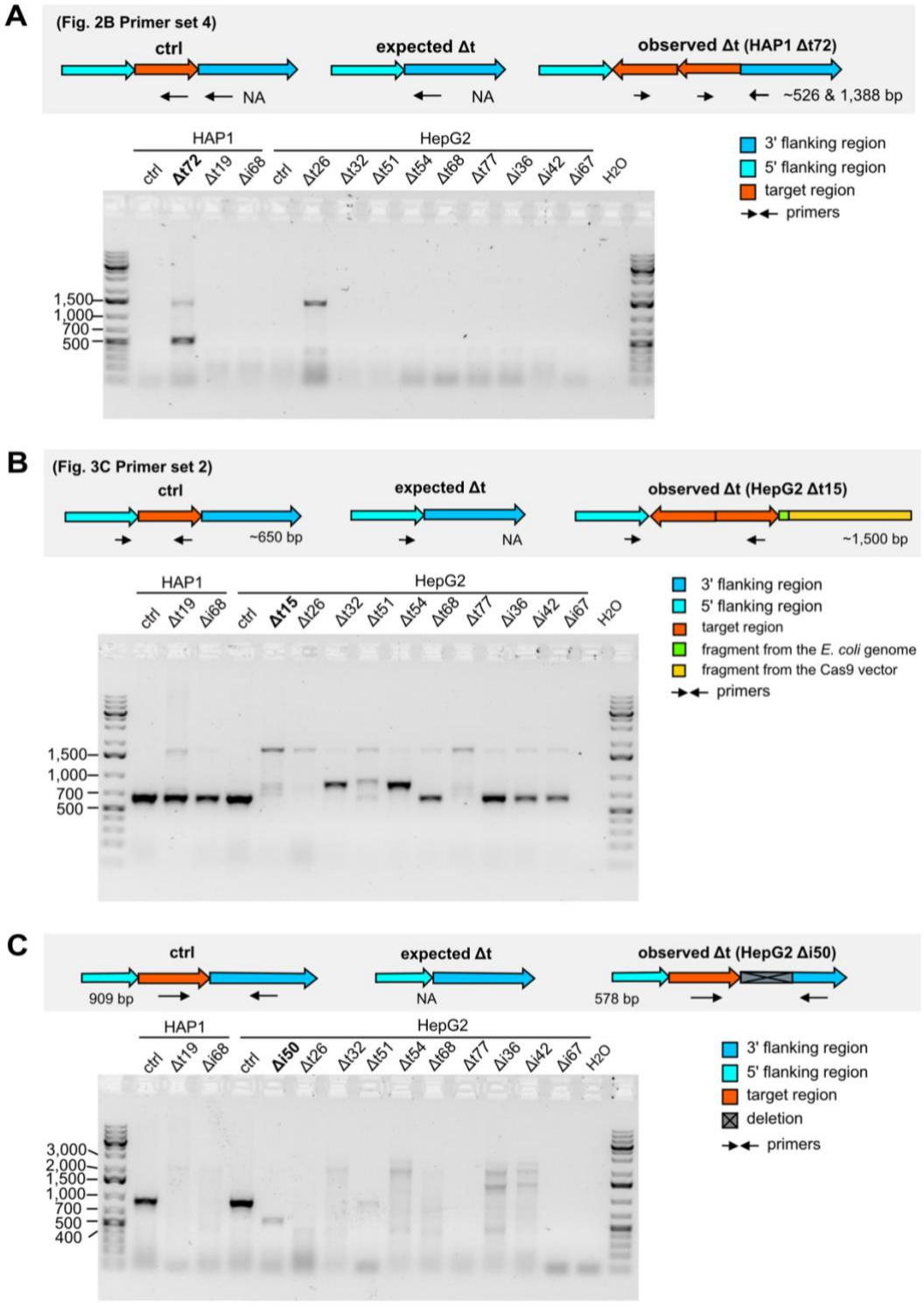

Fig S5. Inversions and deletions are found in deletion clones with a detectable target region.

(**A-C**) Schematic illustration of the additional primer sets designed to identify inversions of the (A) target region, as well as potential genomic alterations at the (B) 5' and (C) 3' DSB sites. Agarose gel electrophoreses separate the size of the obtained PCR products. Deletion clones (highlighted in bold) carry an on-target aberrations found by Xdrop-LRS and are used as positive controls: (A) HAP1 Δt72 (inversion), (B) HepG2 Δt15 (inverted target-derived fragment inserted at 5' DSB) and (C) HepG2 Δi50 (large deletion at 3' DSB).

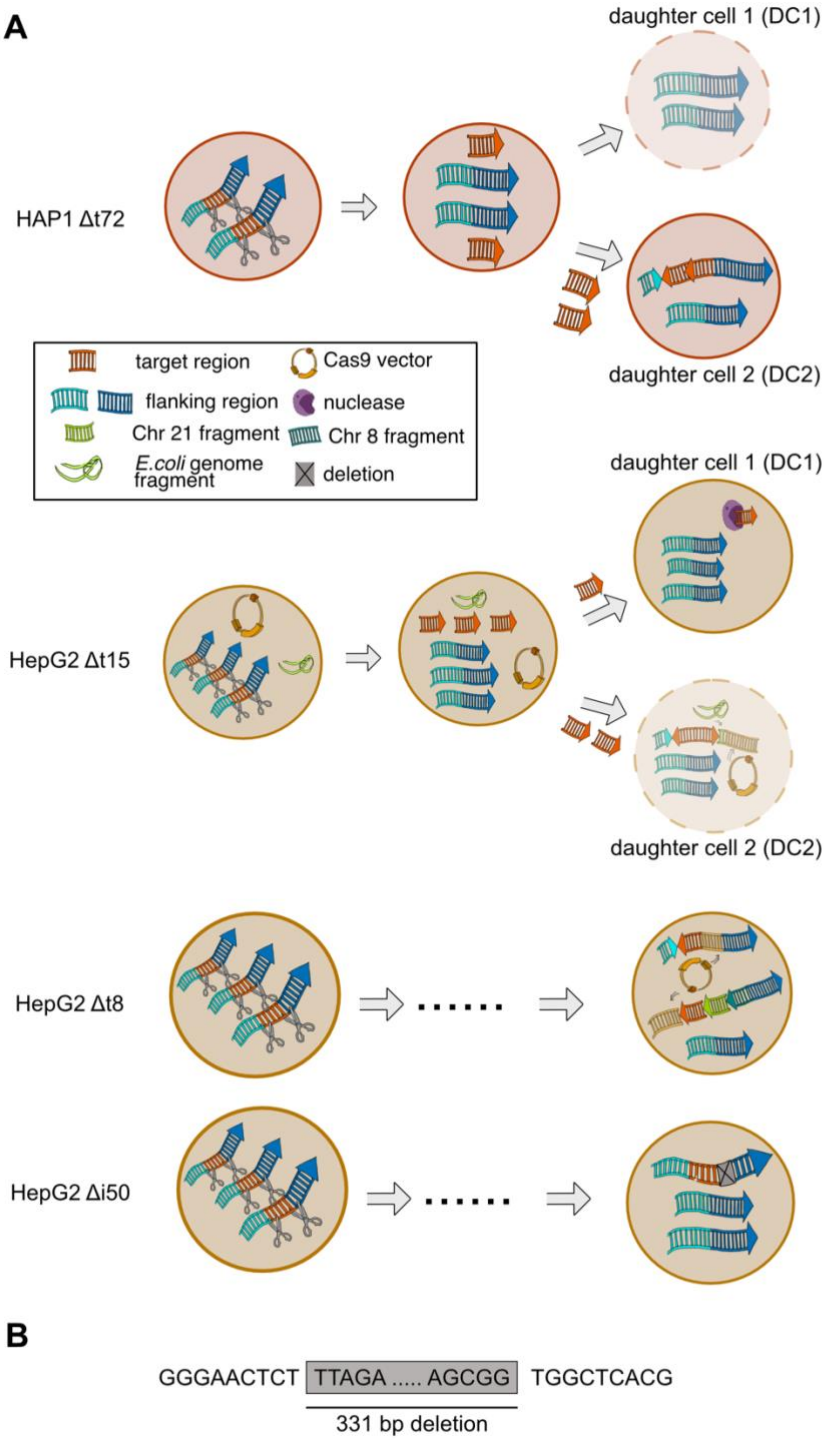

Fig S6. Hypothetical model of on-target genomic alterations in HAP1 and HepG2.
(**A**) CRISPR-Cas9-mediated DSBs cleave the target region from the genome in the mother cell. Deletion clones HAP1 Δt72 and HepG2 Δt15 are heterogenous. Cells deriving from daughter cell 2 (DC2) contain alleles with and without on-target genomic alterations while cells originating from daughter cell 1 (DC1) only carry alleles with the expected deletion. The on-target aberrations result in a cell growth advantage (HAP1 Δt72) or disadvantage (HepG2 Δt15). Over time, cells with a cell growth advantage became dominant. Cells with growth disadvantage are drawn with dashed outer lines and lighter colors. Cells deriving from deletion clones HepG2 Δt8 and Δi50 were heterozygous. (**B**) The sequence of the large deletion breakpoint junctions in the deletion clone HepG2 Δi50 is shown. The grey box indicates the deletion.