

Long-term *in vivo* CRISPR/Cas9 gene editing in liver triggers host immunity and clonal mutations in DNA damage response genes beyond off-target effects

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Short running title: *In vivo* liver CRISPR elicits immunity and clonal mutations

Dear Editor,

Genome editing techniques, particularly those based on CRISPR and its derived variants, hold great potential for developing long-term, curative therapies for various human diseases. However, beyond the well-known issue of off-target effects, recent *in vitro* studies have indicated that Cas9-based editing can cause large-scale genomic rearrangements (Kosicki, Tomberg, and Bradley 2018), induce durable DNA damage responses (Xu et al. 2020; Haapaniemi et al. 2018), and enrich for p53-inactivating mutations (Enache et al. 2020). Notably, p53-dependent toxicity has been shown to impair Cas9-mediated editing in human pluripotent stem cells (Ilhry et al. 2018). Conversely, *TP53*-null cells acquire a competitive growth advantage under Cas9 expression in competition assays (Enache et al. 2020). Consequently, p53 pathway genes are frequently enriched in CRISPR fitness screens (Haapaniemi et al. 2018). These findings suggest that Cas9 not only induces genomic instability but also imposes cellular stress that selectively enriches cell clones with high tumorigenic potential. Nonetheless, normal target tissues in gene-editing settings are considerably less proliferative than *in vitro* cell lines. Moreover, *in vivo* studies have revealed host immune responses triggered by bacterial-derived Cas9 proteins (Nelson et al. 2019), which are absent from cell line models. Therefore, a more comprehensive evaluation of the long-term safety of *in vivo* genome editing is urgently required. Since the liver exhibits proliferative capacities to maintain tissue homeostasis after injury (He et al. 2021; Chen et al. 2020; Wei et al. 2021), here we attempt to utilize liver as a *in vivo* model to observe and assess the risks associated with long-term editing.

Firstly, we generated *in vivo* mouse models by delivering *Staphylococcus aureus* Cas9 (SaCas9) and gRNAs targeting *Pcsk9* or *Angptl3* via single adeno-associated virus serotype 8 (AAV8). Two gRNAs per gene with >50% editing efficacy, as determined from our screening in mouse Hepa1-6 cells (**Figs. S1A–C**), were selected. Adult mice were retro-orbitally injected with AAV8 vectors harboring an all-in-one SaCas9 expression system (**Fig. 1A**). A control group received AAV8 containing a non-targeting gRNA (NT group). Targeted amplicon deep sequencing revealed substantial *in vivo* editing efficiency for *Pcsk9*-G1 and *Angptl3*-G4 gRNAs. *Pcsk9*-G1 achieved the highest efficiency, with $42.1 \pm 5.9\%$ indels detected at 10 months post-injection (**Fig. 1B**). Correspondingly, a single infusion of the *Pcsk9*-G1 construct resulted in near-complete knockdown of hepatic PCSK9, accompanied by approximately 90% and 40% reductions in serum PCSK9 and LDL-C levels, respectively (**Figs. 1C, 1D**). In contrast, the *Angptl3*-G4 group showed about 20% reduction in serum ANGPTL3 and LDL-C (**Figs. S2A, S2B**). Therefore, these data indicate

the successful establishment of long-term models in which efficient genome editing lead to stable phenotypic reductions in target proteins.

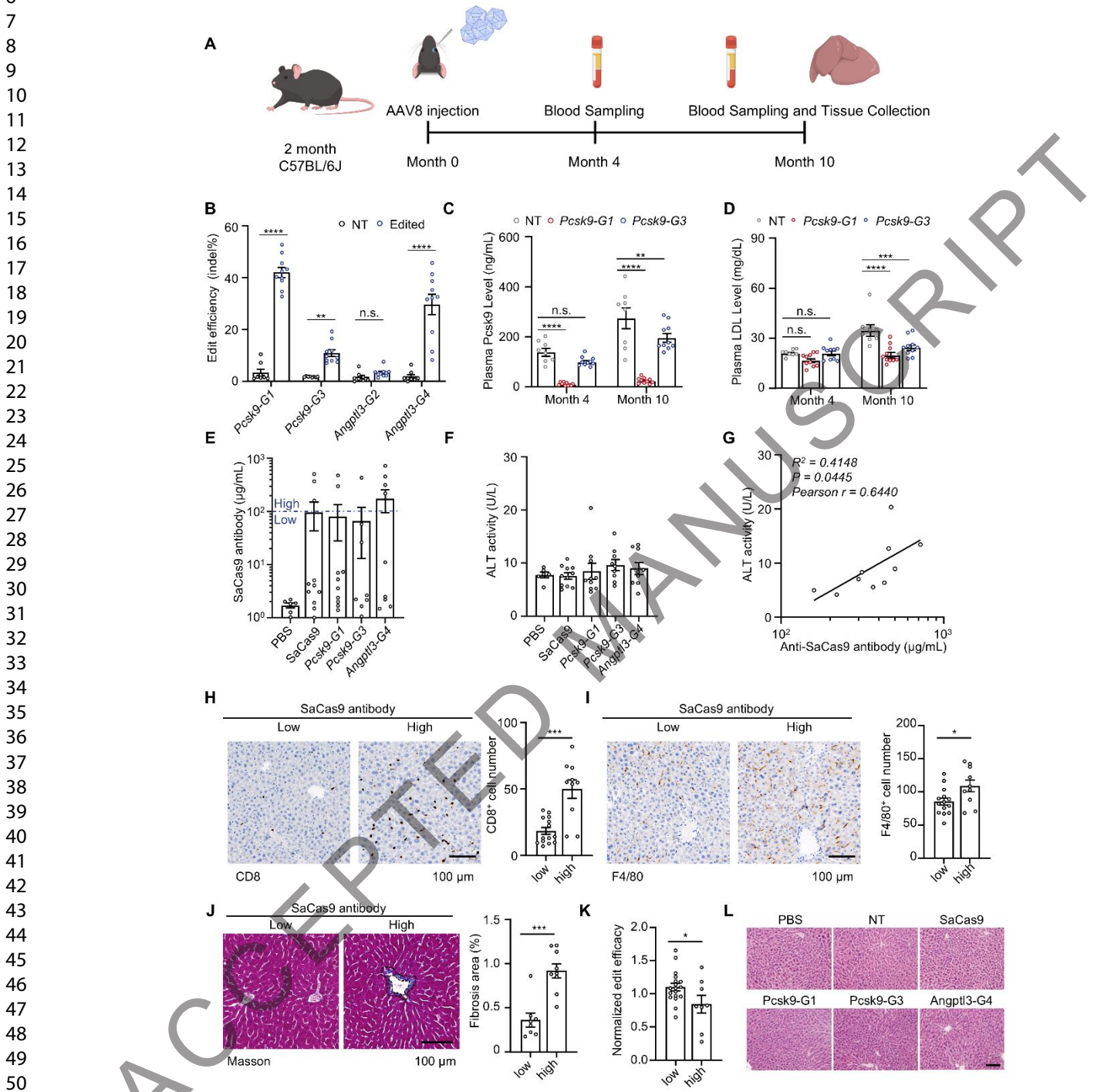


Fig. 1. Long-term *in vivo* gene editing induces chronic liver inflammation and fibrosis. (A) Workflow of blood sampling and tissue collection for AAV8-injected C57BL/6J mice. AAV8 was administered via retro-orbital injection. Created with BioGDP.com. (B) Indel frequency at the targeted liver locus in mice receiving a single AAV8 SaCas9 injection with *Pcsk9-G1*, *Pcsk9-G3*, *Angptl3-G2*, and *Angptl3-G4*, assessed by targeted deep sequencing (NT, $n = 8$; each edited group, $n = 10$). (C–D) The serum Pcsk9 protein (c), and LDL (D) levels in NT, *Pcsk9-G1*, *Pcsk9-G3* groups at month 4 and month 10 (NT, $n = 8$; each edited group, $n = 10$). (E–F) Serum SaCas9 antibody levels (E) and ALT activity (F) of PBS ($n = 6$), SaCas9 alone ($n = 11$), *Pcsk9-G1* ($n = 10$), *Pcsk9-G3* ($n = 10$), *Angptl3-G2* ($n = 10$), and *Angptl3-G4* ($n = 10$)). (G) Scatter plot of ALT activity (U/L) vs. Anti-SaCas9 antibody ($\mu\text{g/mL}$) showing a positive correlation ($R^2 = 0.4148$, $P = 0.0445$, Pearson $r = 0.6440$). (H–I) Immunohistochemistry for CD8 (H) and F4/80 (I) cells. Low and high SaCas9 antibody groups are shown. CD8⁺ cell number and F4/80⁺ cell number are quantified. (J–K) Masson's trichrome staining (J) and fibrosis area (%) (K) for low and high SaCas9 antibody groups. (L) Histology images for PBS, NT, SaCas9, *Pcsk9-G1*, *Pcsk9-G3*, and *Angptl3-G4* groups.

8), and *Angptl3*-G4 ($n = 9$) groups at 10 months post injection. In (E), the dashed line indicates the 100 $\mu\text{g/mL}$ cutoff used to define the low and high antibody groups. (G) Correlation between serum ALT activity and anti-SaCas9 antibody levels. P values and Pearson's r are derived from correlation analysis ($n = 10$). (H–I) Immunohistochemical staining for CD8 (H) and F4/80 (I) in liver sections from mice with low ($<100 \mu\text{g/mL}$, $n = 14$) or high ($\geq 100 \mu\text{g/mL}$, $n = 10$) SaCas9 antibody levels. (J) Masson's trichrome staining showing liver fibrosis in mice with low ($<100 \mu\text{g/mL}$, $n = 8$) or high ($\geq 100 \mu\text{g/mL}$, $n = 9$) SaCas9 antibody levels. Blue staining indicates collagen fibers. (K) Normalized editing efficiency in mice with low ($n = 18$) or high ($n = 8$) SaCas9 antibody levels. Values are normalized to the group mean of *Pcsk9*-G1, *Pcsk9*-G3, and *Angptl3*-G4. (L) Representative H&E staining of liver section from the indicated groups. Data are mean $\pm$ SEM. Statistical significance was determined by one-way ANOVA with Bonferroni's post-hoc test (B), one-way ANOVA with Dunnett's post-hoc test (C–F), and by unpaired two-tailed Student's t-test in (H–K); n.s., not significant; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

A principal safety concern for in vivo gene editing is the potential immunogenicity of non-self bacterial Cas proteins. We therefore characterized the adaptive immune response to SaCas9 and its functional consequences. Anti-SaCas9 antibody levels exceeding 100 $\mu\text{g/mL}$ were detected in 25.6% (10/39) of Cas9-expressing mice (Fig. 1E, Table S1). Serum alanine aminotransferase (ALT) levels were not markedly elevated in any targeting group compared to the PBS control (Fig. 1F). We additionally examined serum anti-AAV8 antibody levels. Although 35 of 36 tested individuals exhibited anti-AAV8 antibody titers above baseline ($>0.008 \text{ mg/mL}$, defined by PBS-treated individuals; Table S1), these titers showed virtually no correlation with ALT levels (Fig. S3A). In contrast, in mice with high anti-SaCas9 antibody titers, a positive correlation was observed between ALT activity and serum antibody levels (Fig. 1G). Notably, the inflammatory cytokines IL-1 β , IL-6, and TNF- α also showed modest increases in the high-antibody group (Figs. S3B–D), suggesting a mild liver inflammation associated with the humoral immune response to SaCas9. We then performed histological analysis to further characterize the immune response and potential liver damage. Notably, the livers from high-antibody group exhibited significant infiltration of T cells and macrophages (Figs. 1H, 1I), and significantly larger fibrosis area (Fig. 1J). Moreover, we found significant positive correlation between anti-SaCas9 antibody levels and fibrosis area (Fig. S3E). Editing efficacy was lower in the high-antibody group (Fig. 1K), and was negatively correlated with anti-SaCas9 antibody levels (Fig. S3F). Collectively, these data indicate that a humoral immune response against SaCas9 can promote immune cell infiltration and liver fibrosis. Nonetheless, overall liver tissue morphology remained comparable across groups (Fig. 1L), with no significant body weight changes (Fig. S3G) or malignant transformation observed at the experimental endpoint. Overall, this pro-fibrotic, inflammatory microenvironment is a recognized risk factor for tumorigenesis (Ringelhan et al. 2018) and may also reduce editing efficacy by negatively impacting the survival of SaCas9-expressing hepatocytes.

Having established that adaptive immune response to SaCas9 can affect the editing efficacy, we next characterized the molecular adaptations in liver tissues that successfully maintained editing. Principal component analysis (PCA) of RNA sequencing (RNA-seq) on mouse liver tissues showed that individuals transfected with SaCas9 formed distinct clusters separate from PBS-, AAV-GFP-, and AAV-gRNA-only control groups (Fig. 2A). Using a nominal P -value < 0.05 and $|\log_2 \text{fold change}| \geq 0.585$ as thresholds, we identified differentially expressed genes (DEGs) in the *Pcsk9*-G1 and *Angptl3*-G4 groups compared to the NT group (Supplementary file 1; Figs. 2B, 2C); as the adjusted P -value < 0.05 threshold yielded too few DEGs for meaningful downstream analysis (Figs. S4A, S4B). To distinguish transcriptomic changes resulting specifically from CRISPR editing from those caused by target gene silencing, we defined “key DEGs” through a two-step filtering strategy: (1) we intersected DEGs from the *Pcsk9*-G1 and *Angptl3* G4 groups to retain genes commonly affected by Cas9 editing, and (2) we removed genes that overlapped

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with DEGs reported in previous *Pcsk9* knockdown studies using non-DSB methods (Gemberling et al. 2021; Thakore et al. 2018), thereby excluding transcriptional changes attributable to *Pcsk9* silencing (**Fig. 2D, Table S2**). Notably, gene set enrichment analysis (GSEA) indicated a positive enrichment of inflammatory pathways in the NT group compared to the AAV-GFP group (**Fig. S4C**). Additionally, TIMER immune inflation analysis (Li et al. 2020) showed that individuals from the high antibody group showed increased CD8⁺ T cell and macrophage cell score (**Fig. S4D**), supporting a pro-inflammatory effect of the Cas9 protein. GSEA also revealed a significant negative enrichment of the p53 signaling pathway in both the *Pcsk9-G1* and *Angptl3 G4* groups compared to the NT group (**Figs. 2E, 2F**). Functional analysis using the DAVID tool showed that upregulated key DEGs were enriched in pathways related to positive regulation of cell population proliferation, and cholesterol metabolism (**Figs. 2G, Table S3**), reflecting both intended on-target effects and potential Cas9-induced stress. Notably, *Angptl8*, which has been implicated in promoting liver fibrosis (Zhang et al. 2023), was the most significantly upregulated gene among the key DEGs. Downregulated key DEGs were associated with apoptosis and transcriptional mis-regulation in cancer. Accordingly, *Gadd45g*, a p53 target gene that functions as a tumor suppressor by inhibiting cell growth and inducing apoptosis (Zhang et al. 2014), was significantly downregulated in both editing groups (**Figs. 2B, 2C**). Interestingly, stable transfection of *Pcsk9-G1* to normal mouse liver cell line (AML12) also showed downregulation of *Gadd45g*, as well as *Trp53* (**Figs. S4E, S4F**). These transcriptional profiles indicate that efficient long-term editing *in vivo* may involve a selection process that favors cell survival, similar to what has been observed *in vitro* (Enache et al. 2020).

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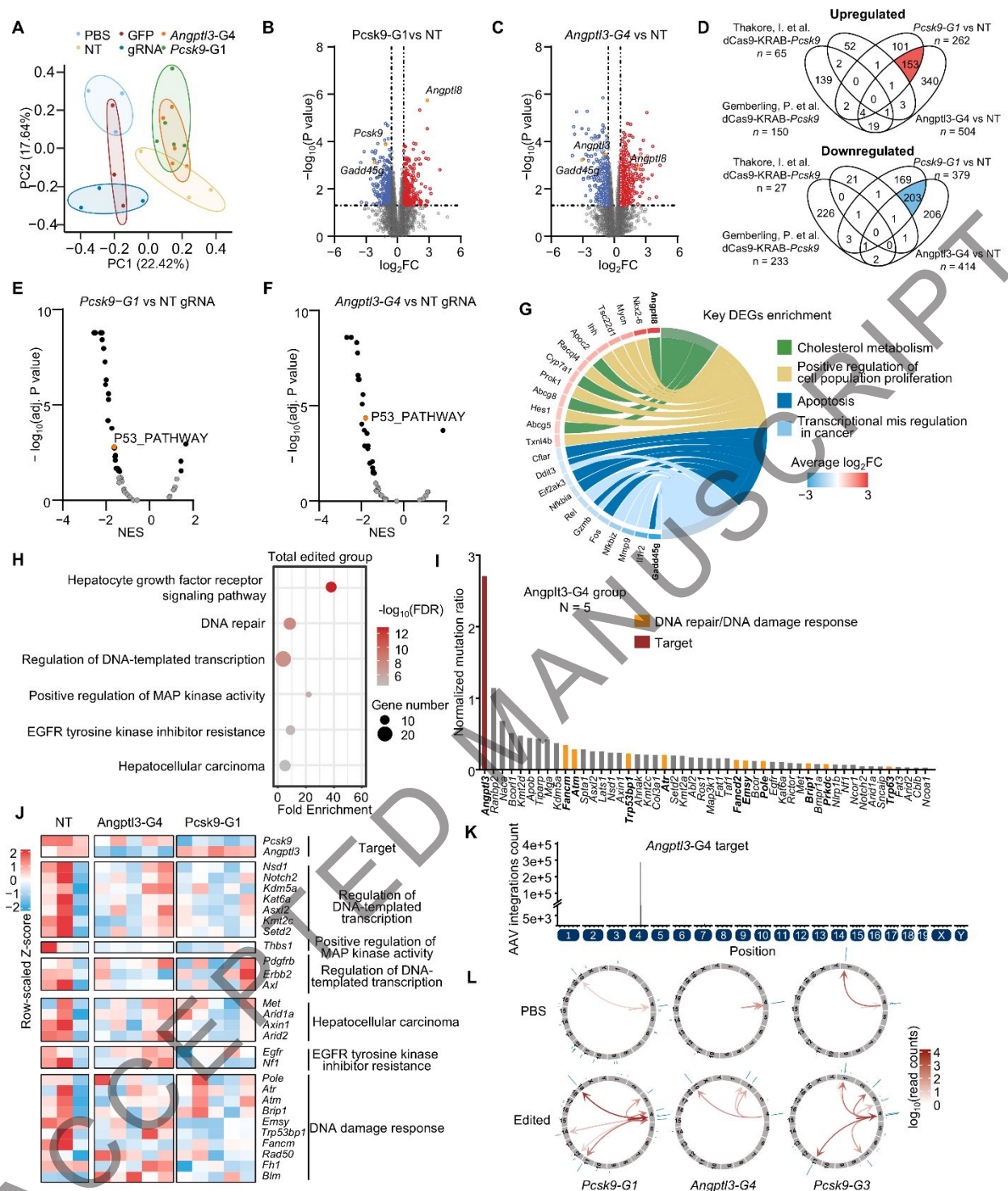


Fig. 2. Cas9-mediated gene editing leads to enrichment of p53-inactivating clones and genome instability

(A) Principal-component analysis (PCA) of RNA-seq samples showing the first two principal components (PC1 and PC2). **(B–C)** Volcano plots of differentially expressed genes (DEGs) in *Pcsk9-G1* **(B)** or *Angptl3-G4* **(C)** liver tissues compared to non-treated (NT) controls. DEGs were defined using $|\log_2FC| \geq 0.585$ and raw $P < 0.05$; upregulated DEGs are highlighted in red and downregulated DEGs in blue. Genes of interest are highlighted in orange. **(D)** Venn diagram illustrating the definition of key DEGs. The overlap between *Pcsk9-G1* and *Angptl3-G4* groups is first identified. Genes found to overlap with DEGs from two published *Pcsk9* knockdown datasets (non-DSB methods)

are then excluded; the remaining shared genes are defined as key DEGs. Red and blue indicate upregulated and downregulated genes, respectively. *n*, number of genes in each set. **(E–F)** GSEA enrichment of MSigDB Hallmark pathways in *Pcsk9-G1* **(E)**, or *Angptl3-G4* **(F)** groups vs NT group. Significantly enriched pathways (adjusted *P* < 0.05) are shown in black; the p53 pathway is highlighted in orange. **(G)** DAVID enrichment analysis of significant pathways associated with key DEGs. Significant pathways (right) and the average fold change of key DEGs (left) are displayed. Strings connecting the panels indicate gene membership within specific pathways. **(H)** Dot plot of DAVID enrichment results. Dot size represents gene count; color indicates false discovery rate (FDR). **(I)** Mutated genes ranked by normalized non-silent mutation ratio in the *Angptl3-G4* group. Genes related to DNA repair and the DNA damage response are highlighted in orange; the targeted *Angptl3* gene is highlighted in red. **(J)** Heatmap of transcript levels (row Z-scores) for frequently mutated genes across groups. Genes are clustered by DAVID enrichment terms. **(K)** Detection of AAV integrations at gRNA-targeted sites. Blue bars represent mouse chromosomes. **(L)** Circos plots depicting translocation events from targeted sites to other genomic regions in PBS-treated (*n* = 3) and edited (*n* = 3) mice. Red arrows indicate translocation events occurring more than twice. Arrow colors and the outer bar scale represent read counts.

To directly test the hypothesis that edited hepatocytes evade p53-mediated apoptosis and undergo clonal selection, we performed high-depth whole-exome sequencing to quantify non-synonymous single-nucleotide variants (SNVs) and indels. After filtering out background mutations present in PBS-injected controls, we found non-silent mutations on 181 genes in total from the edited groups (**Tables S4, S5**). DAVID analysis revealed that the mutated genes were significantly enriched in pathways related to hepatocyte growth, cancer and DNA repair (**Fig. 2H, Table S6**), suggesting a selective expansion of clones harboring mutations that may confer a survival advantage during long-term gene editing. Notably, the *Angptl3-G4* group exhibited a high mutation frequency in *Atm* and *Atr* (**Figs. 2I, S5**). ATM loss would disrupt DNA damage response (DDR) signaling and p53 activation (Lee et al. 2025), potentially enabling edited hepatocytes to bypass apoptosis and gain a selective survival advantage. However, with the exception of the targeted genes (*Pcsk9* and *Angptl3*), none of the other frequently mutated genes showed significant differential expression in the RNA-seq data (**Fig. 2J**). The dissociation between genomic mutations and bulk transcriptional profiles suggests early clonal expansion, where mutated subpopulations are not yet abundant enough to shift the transcriptome of the entire tissue.

Given our observations, we next asked whether the combined effects of chronic inflammation, a pro-survival transcriptome, and the emerging mutational landscape would lead to a measurable increase in genomic instability. First, deep sequencing of the top ten computationally predicted off-target sites revealed no significant increase in indel frequencies (**Fig. S6A–C, Table S7**), indicating the absence of detectable gRNA-dependent off-target double-strand breaks (DSBs). To further characterize Cas9-associated structural variations, we assessed chromosomal translocations between the targeted sites and other genomic loci using a transposon-based library preparation method (Nelson et al. 2019). This approach employs a biotin-labeled forward primer and a transposon-integrated DNA tag-specific reverse primer to enrich for large structural variants in an unbiased manner (**Fig. S7A**). As a proof of concept, our method detected clear AAV integration at the targeted genomic locus compared with the NT group (**Figs. 2K, S7B**), consistent with recent reports (Nelson et al. 2019; Buddle et al. 2026). We then analyzed translocation events between the gRNA-targeted sites (“bait” regions) and other genomic regions (“prey” regions) in the highest-edited individuals from each group. All edited groups exhibited translocation frequencies above baseline (**Fig. 2L**). Importantly, the prey regions displayed low sequence similarity to the gRNA spacer sequence (**Fig. S7C**), suggesting that these translocation junctions are unlikely to arise from gRNA-dependent off-target cutting. Collectively, these results demonstrate that long-term in vivo genome editing induces chromosomal

translocations between on-target DSBs and endogenous genomic breaks through gRNA-independent mechanisms.

Collectively, our long-term *in vivo* assessment reveals that persistent CRISPR-Cas9 genome editing initiates a concerning cascade. While achieving intended therapeutic modifications, the sustained expression of bacterial-derived SaCas9 elicits a potent adaptive immune response, establishing a chronic inflammatory microenvironment in the liver. Given the well-established role of chronic inflammation in promoting hepatocellular carcinoma (HCC) (Fernandes et al. 2024), these findings may indicate the risk of malignant transformation.

Although hepatocytes *in vivo* proliferate far more slowly than established cell lines (Chen et al. 2020), evident clonal expansion occurs after injury (Chen et al. 2020; He et al. 2021; Wei et al. 2021). Moreover, chronic pressures including gene editing, inflammation, and fibrosis produce somatic mutations that increase the heterogeneity in liver tissue (Brunner et al. 2019). Therefore, together with the additional proliferative advantage conferred by p53-related mutations under Cas9-induced stress (Enache et al. 2020), our results indicate that early clonal expansion of cells harboring p53-related mutations can occur *in vivo* after long-term Cas9-mediated gene editing, albeit less robustly than in cultured cell lines.

Nonetheless, limited by the likely small population of mutant clones, we cannot directly correlate low-frequency gene mutations with global transcriptome perturbations using bulk sequencing. In addition to the averaging effect that obscures signals from heterogeneous clones, bulk analysis also loses spatial and temporal resolution. Consistent with this, recent single-cell sequencing studies have revealed differential proliferative potential across the zonation of the hepatic lobule (He et al. 2021; Wei et al. 2021), whereas barcode and fluorescent lineage tracing have elucidated the clonal competition underlying tumorigenesis (Lu et al. 2024) and liver regeneration (He et al. 2021; Wei et al. 2021). Future studies should integrate spatial transcriptomics with lineage tracing to connect hepatocyte clonal expansion during regeneration with the selective pressures imposed by gene editing, thereby clarifying the oncogenic risks associated with long-term *in vivo* gene editing.

Together, these findings indicate that the safety evaluation of *in vivo* therapeutic gene editing must be broadened to encompass immunogenicity, clonal dynamics, and genome-wide structural variations. To address these concerns, future strategies could aim to evade immune recognition against the editor, refine nuclease performance, and improve long-term mutation surveillance. Importantly, because CRISPR-Cas proteins are often derived from bacteria that commonly infect humans, pre-existing immune responses to the effector proteins are frequently detected in the general population (Ewaisha and Anderson 2023; Tang et al. 2022; Ferdosi et al. 2019). One plausible strategy is to explore immune-orthogonal orthologues of both viral capsids and Cas9 (Moreno et al. 2019). In addition, transient delivery of Cas9 mRNA via lipid nanoparticles (LNPs) has been shown to improve safety (Tsuchida et al. 2024), ~~albeit with reduced editing efficiency~~, albeit at the cost of reduced editing efficiency. Notably, beyond enhancing Cas9 fidelity, fusing Cas9 with an exonuclease may suppress translocations by inhibiting end joining (Yin et al. 2022). Finally, unintended mutations with tumorigenic potential must be rigorously defined and monitored, which will require more comprehensive functional assessment studies (Lee et al. 2025).

Footnotes

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3 J.H. and T.C. conceptualized the study. T.C. performed the major experiments, analyzed the data, and drafted the
4 manuscript. A.Z., D.Z., and S.D. analyzed the RNA-seq, translocation, and WES data, and interpreted the results.
5 W.W. prepared the animal samples for sequencing. J.W. produced the AAV8 vector. M.M. performed the histology
6 experiments. S.L. analyzed the animal serum. Y.C., H.L., S.D., and X.H. provided critical advice on study design.
7 All authors reviewed and approved the final version of the manuscript.
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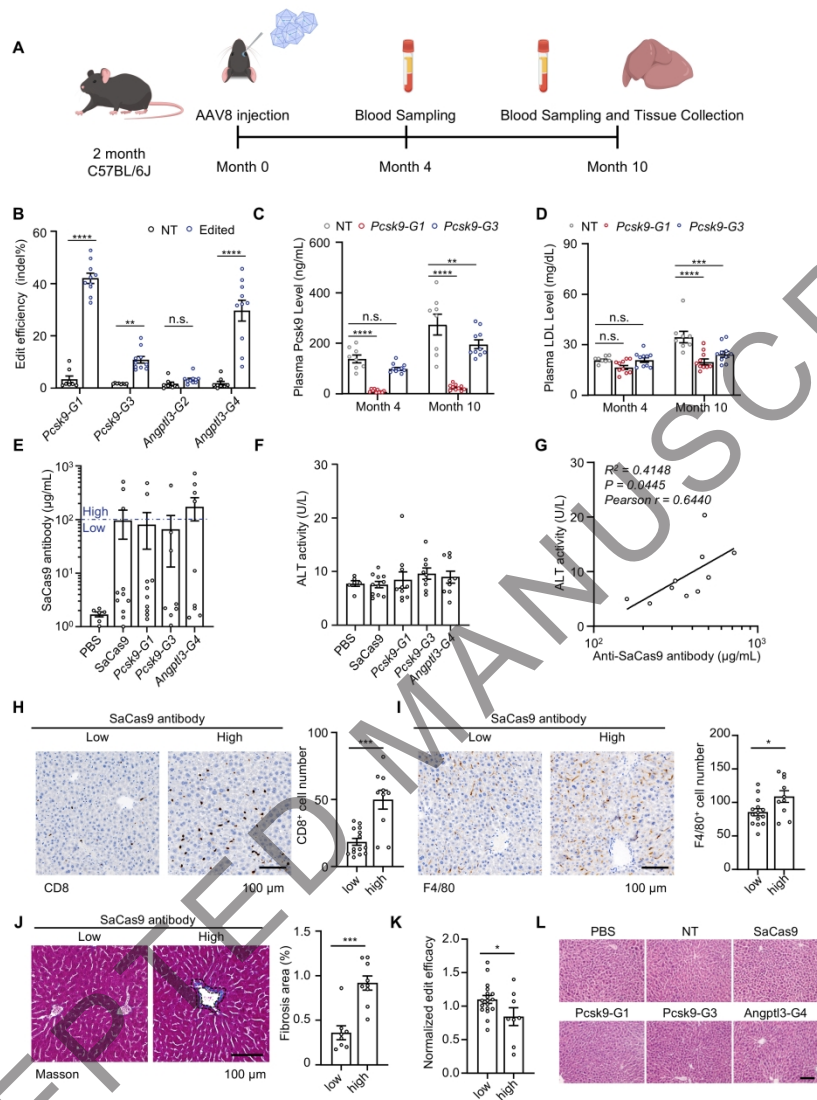


Fig. 1. Long-term in vivo gene editing induces chronic liver inflammation and fibrosis. (A) Workflow of blood sampling and tissue collection for AAV8-injected C57BL/6J mice. AAV8 was administered via retro-orbital injection. Created with BioGDP.com. (B) Indel frequency at the targeted liver locus in mice receiving a single AAV8 SaCas9 injection with Pcsk9-G1, Pcsk9-G3, Angptl3-G2, and Angptl3-G4, assessed by targeted deep sequencing (NT, n = 8; each edited group, n = 10). (C-D) The serum Pcsk9 protein (c), and LDL (D) levels in NT, Pcsk9-G1, Pcsk9-G3 groups at month 4 and month 10 (NT, n = 8; each edited group, n = 10). (E-F) Serum SaCas9 antibody levels (E) and ALT activity (F) of PBS (n = 6), SaCas9 alone (n = 11), Pcsk9-G1 (n = 10), Pcsk9-G3 (n = 8), and Angptl3-G4 (n = 9) groups at 10 months post injection. In (E), the dashed line indicates the 100 µg/mL cutoff used to define the low and high antibody groups. (G) Correlation between serum ALT activity and anti-SaCas9 antibody levels. P values and Pearson's r are derived from correlation analysis (n = 10). (H-I) Immunohistochemical staining for CD8 (H) and F4/80 (I) in liver sections from mice with low (<100 µg/mL, n = 14) or high (≥100 µg/mL, n = 10) SaCas9 antibody levels. (J) Masson's trichrome staining showing liver fibrosis in mice with low (<100 µg/mL, n = 8) or high (≥100 µg/mL, n = 9) SaCas9 antibody levels. Blue staining indicates collagen fibers. (K) Normalized editing efficiency in mice with low (n = 18) or high (n = 8) SaCas9 antibody levels. Values are

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3 normalized to the group mean of Pcsk9-G1, Pcsk9-G3, and Angptl3-G4. (L) Representative H&E staining of
4 liver section from the indicated groups. Data are mean $\pm$ SEM. Statistical significance was determined by
5 one-way ANOVA with Bonferroni's post-hoc test (B), one-way ANOVA with Dunnett's post-hoc test (C-F),
6 and by unpaired two-tailed Student's t-test in (H-K); n.s., not significant; *P < 0.05, **P < 0.01, ***P
7 < 0.001, ****P < 0.0001.

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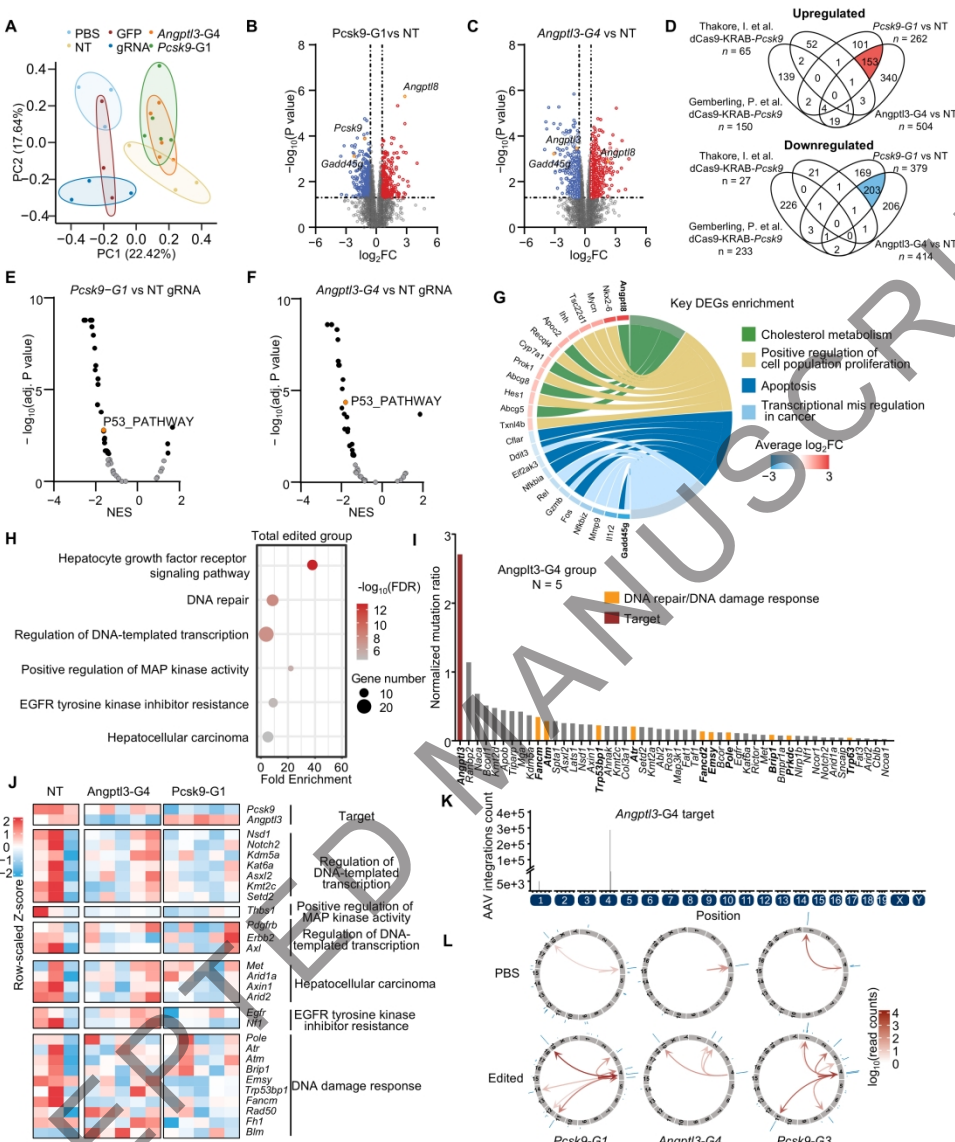


Fig. 2. Cas9-mediated gene editing leads to enrichment of p53-inactivating clones and genome instability (A) Principal-component analysis (PCA) of RNA-seq samples showing the first two principal components (PC1 and PC2). (B–C) Volcano plots of differentially expressed genes (DEGs) in Pcsk9-G1 (B) or Angptl3-G4 (C) liver tissues compared to non-treated (NT) controls. DEGs were defined using $|\log_2FC| \geq 0.585$ and raw $P < 0.05$; upregulated DEGs are highlighted in red and downregulated DEGs in blue. Genes of interest are highlighted in orange. (D) Venn diagram illustrating the definition of key DEGs. The overlap between Pcsk9-G1 and Angptl3-G4 groups is first identified. Genes found to overlap with DEGs from two published Pcsk9 knockdown datasets (non DSB methods) are then excluded; the remaining shared genes are defined as key DEGs. Red and blue indicate upregulated and downregulated genes, respectively. n, number of genes in each set. (E–F) GSEA enrichment of MSigDB Hallmark pathways in Pcsk9-G1 (E), or Angptl3-G4 (F) groups vs NT group. Significantly enriched pathways (adjusted $P < 0.05$) are shown in black; the p53 pathway is highlighted in orange. (G) DAVID enrichment analysis of significant pathways associated with key DEGs. Significant pathways (right) and the average fold change of key DEGs (left) are displayed. Strings connecting the panels indicate gene membership within specific pathways. (H) Dot plot of DAVID enrichment results. Dot size represents gene count; color indicates false discovery rate (FDR). (I) Mutated genes ranked

by normalized non-silent mutation ratio in the Angptl3-G4 group. Genes related to DNA repair and the DNA damage response are highlighted in orange; the targeted Angptl3 gene is highlighted in red. (J) Heatmap of transcript levels (row Z-scores) for frequently mutated genes across groups. Genes are clustered by DAVID enrichment terms. (K) Detection of AAV integrations at gRNA-targeted sites. Blue bars represent mouse chromosomes. (L) Circos plots depicting translocation events from targeted sites to other genomic regions in PBS-treated (n = 3) and edited (n = 3) mice. Red arrows indicate translocation events occurring more than twice. Arrow colors and the outer bar scale represent read counts.

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