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Supplemental information

Targeting the *Apoa1* locus

for liver-directed gene therapy

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List and sequence of plasmids used.

1. 1507-pAAV-U6-*Apoa1*-gRNA2-SA-HLP-SaCas9-HA-OLLAS-spA 6968 bp
2. 1729-pAAV-*Apoa1*-Target-2A-mKate-pA 4924 bp
3. 1730-pAAV-*Apoa1*-Target-2A-*APOE*-pA 5158 bp
4. 1731-pAAV-*Apoa1*-Target-2A-*FIX*-pA 5590 bp
5. 1771-pAAV-*Apoa1*-Target-2A-*FAH*-pA 5470 bp
6. 1161-pAAV-CB-EGFP 5478 bp

FASTA sequences:

>1507-pAAV-U6-*Apoa1*-gRNA2-SA-HLP-SaCas9-HA-OLLAS-spA 6968 bp

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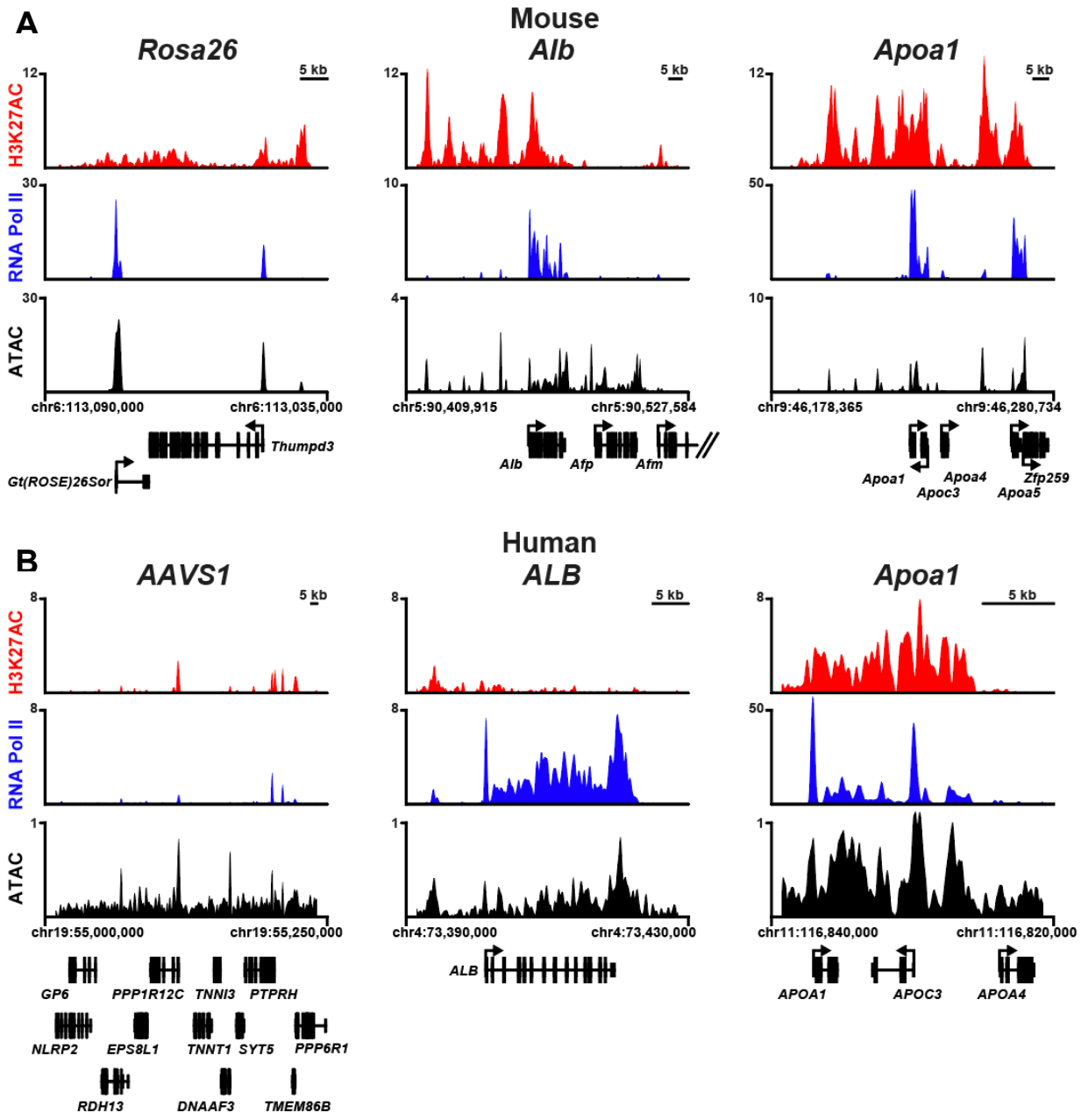

Figure S1: High chromatin accessibility and active transcription at the *Apoa1* locus in mouse and human liver. (A) ChIP-seq profile of histone H3K27 acetylation, RNA Polymerase II binding, and ATAC-seq chromatin accessibility for a safe harbor locus (*Rosa26*), and select loci for genes that are highly expressed in murine liver (*Alb* and *Apoa1)*. (B) ChIP-seq profiling and ATAC-sequencing for a safe harbor locus, adeno-associated virus integration site 1 (*AAVS1*) as well as the same highly expressed loci (*ALB, APOA1*) in human hepatocytes from an adult female liver from the ENCODE database.

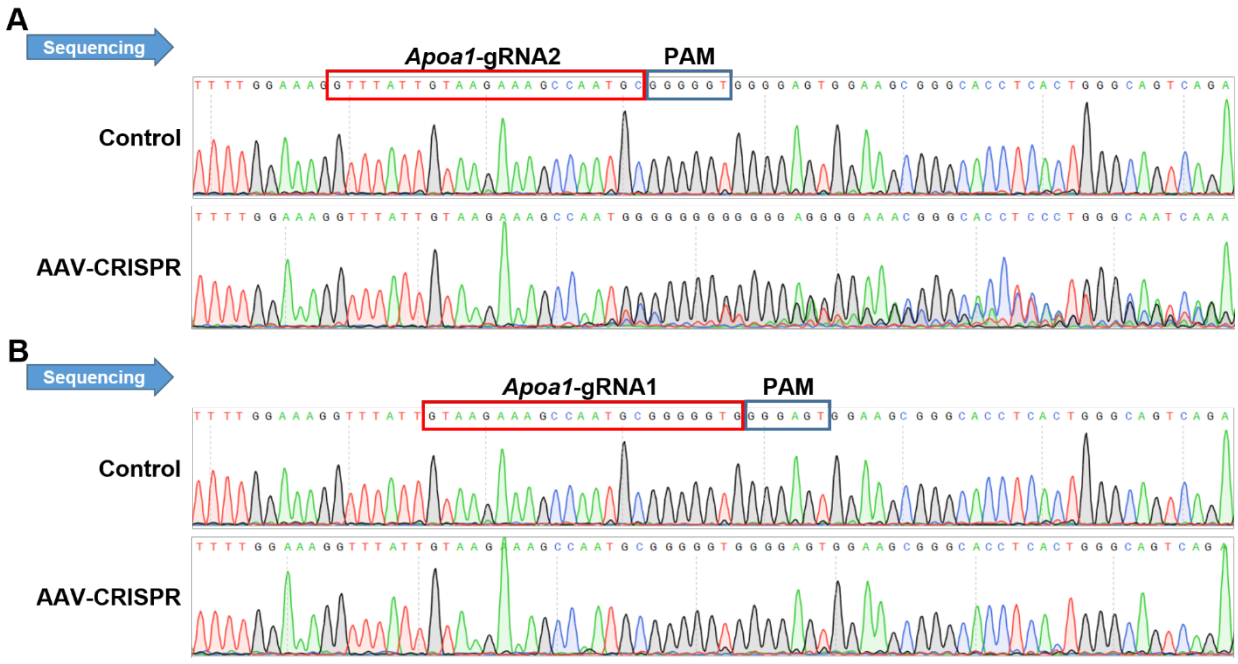

Figure S2: Screening of gRNAs for targeting *Apoa1* 3'UTR. (A) Sanger sequencing chromatograms of *Apoa1* 3'UTR showed multiple sequence traces in liver of AAV-CRISPR-injected mice due to NHEJ-derived indel formation. (B) *Apoa1*-gRNA1 failed in inducing indel formation. Control: wild type *Apoa1* 3'UTR sequence. gRNA and PAM sequences are marked in red and blue, respectively. Sequencing direction is indicated by the arrow.

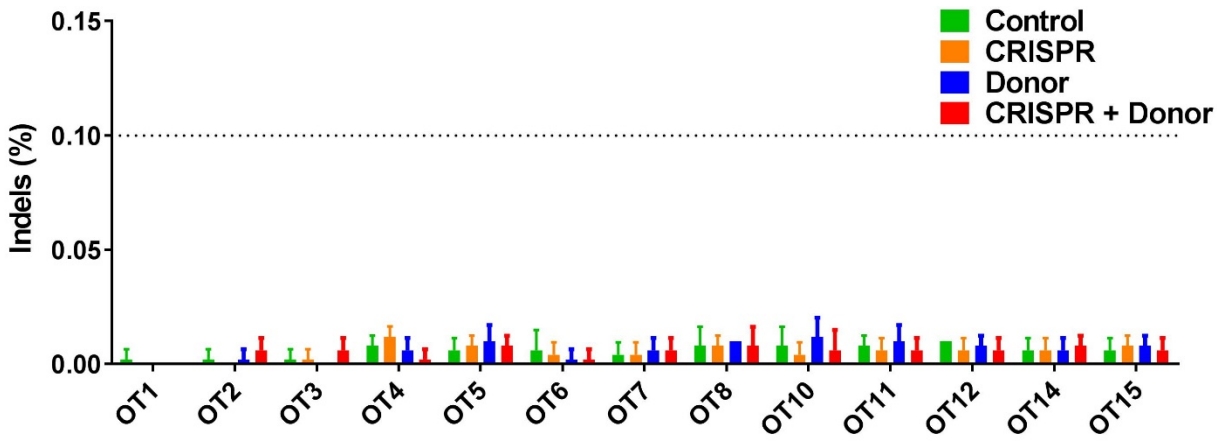

Figure S3: No detectable Off Target (OT) activity of the SaCas9 gRNA targeting the *Apoa1* 3'UTR. Deep sequencing analysis of indel formation rate in off target sites predicted by COSMID. Data are shown as mean ± standard deviation (n=5). A one-way ANOVA followed by Tukey test revealed no significant differences for OTs among groups. All the indel formation rates are below the limit of detection (0.1%).

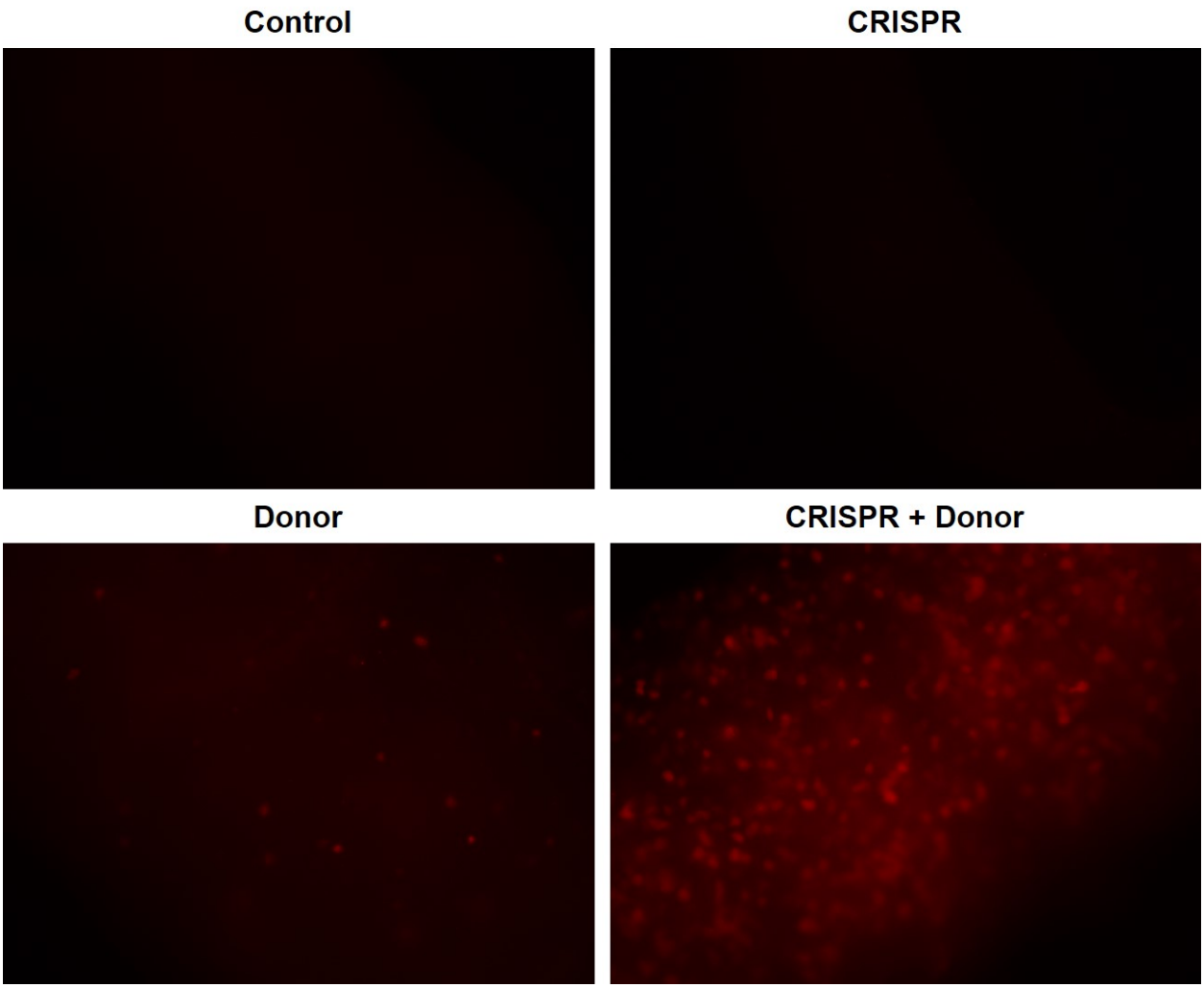

Figure S4: Efficient expression of mKate2 in *Apoa1*-targeted mice. Direct mKate2 fluorescence in fresh liver slices from *Apoa1*-targeted mice. A 10x objective lens and 80 ms exposure time were used.

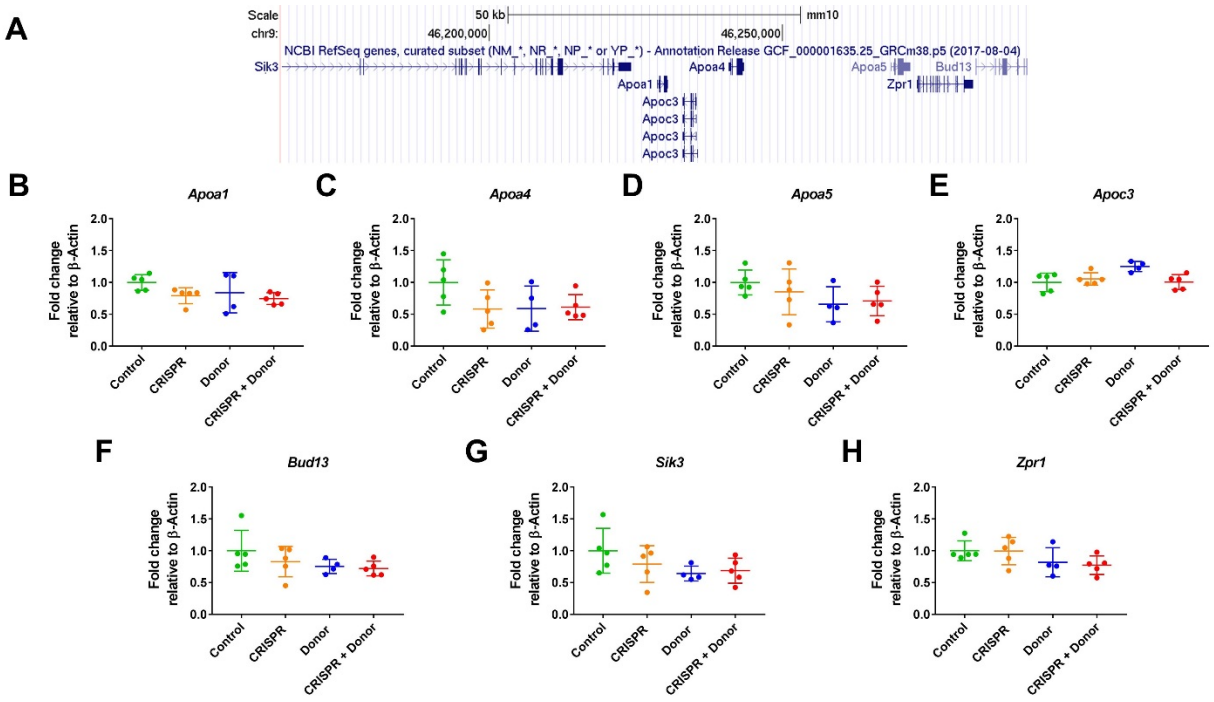

Figure S5: Targeting of *Apoa1* does not adversely affect expression of neighboring genes. (A) Schematic diagram of *Apoa1* and neighboring loci on Chromosome 9 by UCSC genome browser (*Mus Musculus* genome assembly GRCm38/mm10). Expression of *Apoa1* (B) and neighboring genes at the *Apoa1* locus by qPCR: (C) Apolipoprotein a4 (*Apoa4*), (D) Apolipoprotein a5 (*Apoa5*), (E) Apolipoprotein c3 (*Apoc3*), (F) *Bud13*, (G) SIK family kinase 3 (*Sik3*) and (H) Zinc finger protein 1 (*Zpr1*). Data are expressed as fold change relative to *β-Actin* and shown as mean ± standard deviation (n=5 for all groups except for Donor, n=4). A one-way ANOVA followed by Tukey test revealed no significant differences among groups in all the panels.

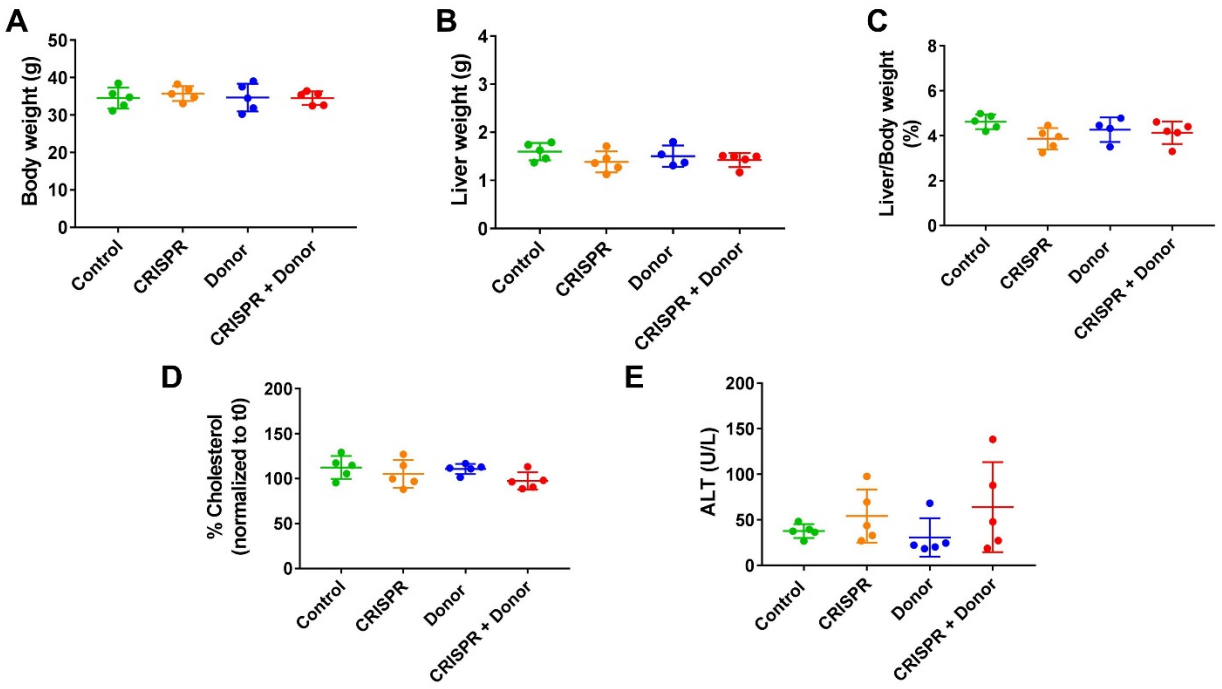

Figure S6: Targeting of *Apoa1* is safe and well-tolerated. (A) Body and (B) liver weights at 12 weeks post-AAV injection. (C) Liver/body weight ratios. (D) Total plasma cholesterol levels normalized to baseline at time 0. (E) ALT levels in plasma at endpoint. Data are shown as mean ± standard deviation (n=5). A one-way ANOVA followed by Tukey test revealed no significant differences among groups in all the panels.

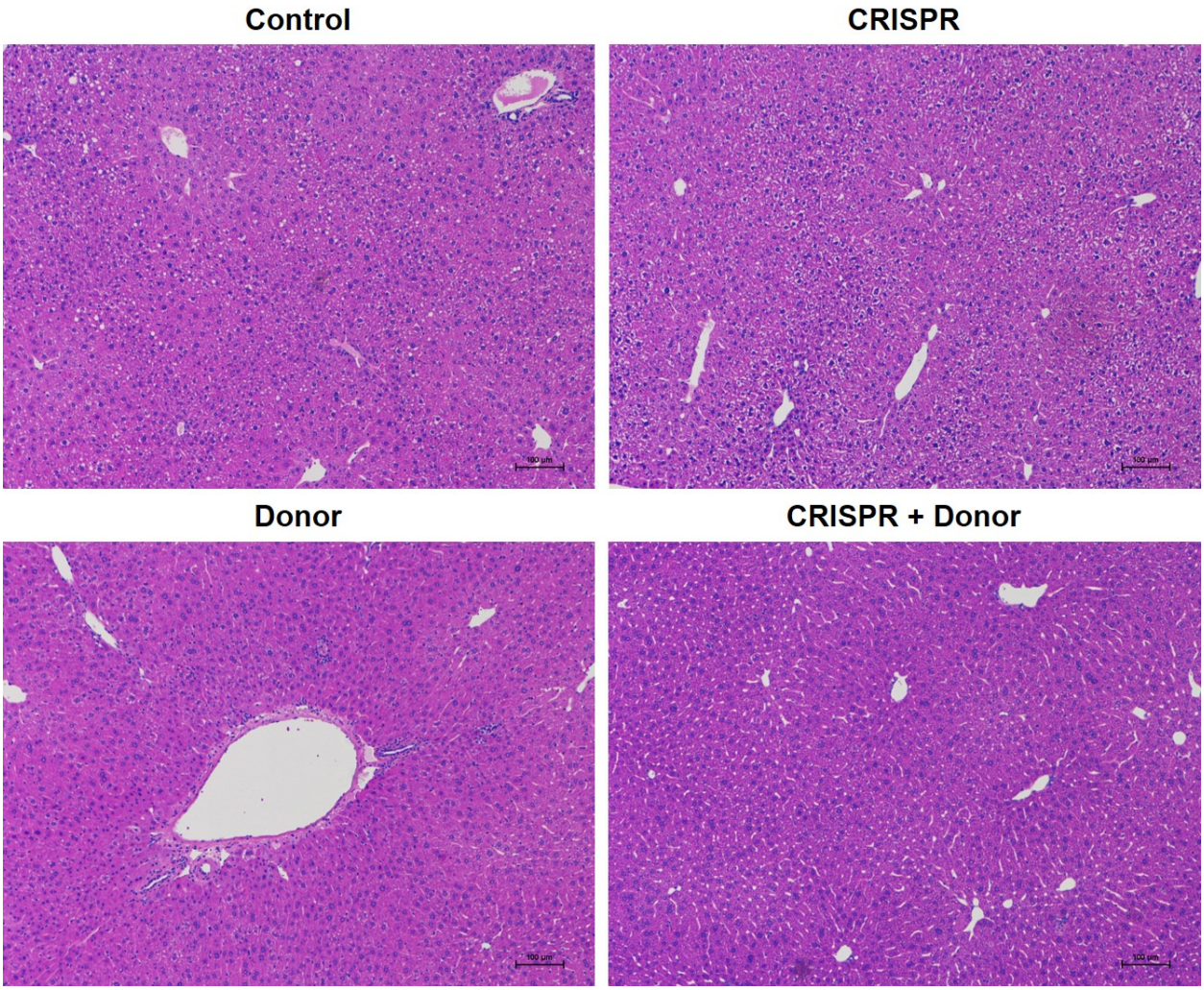

Figure S7: No histopathological abnormalities in liver from *Apoa1*-targeted mice. Representative H&E staining of livers from *Apoa1*-targeted mice. Scale bar is 100 µm.

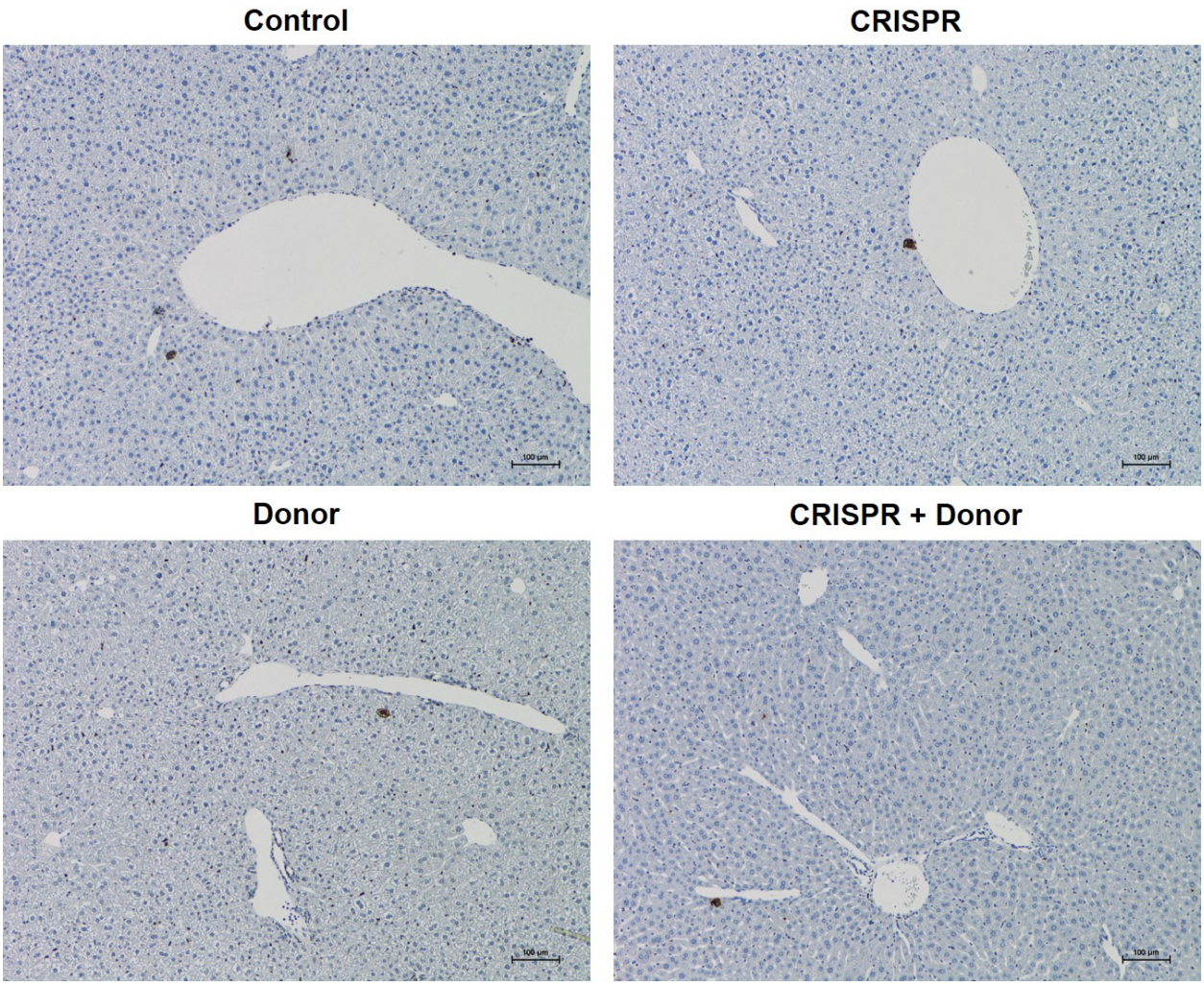

Figure S8: Normal incidence of hepatocyte apoptosis in *Apoa1*-targeted mice. Representative TUNEL staining of livers from *Apoa1*-targeted mice at 12 weeks post-injection. Scale bar is 100 μm.

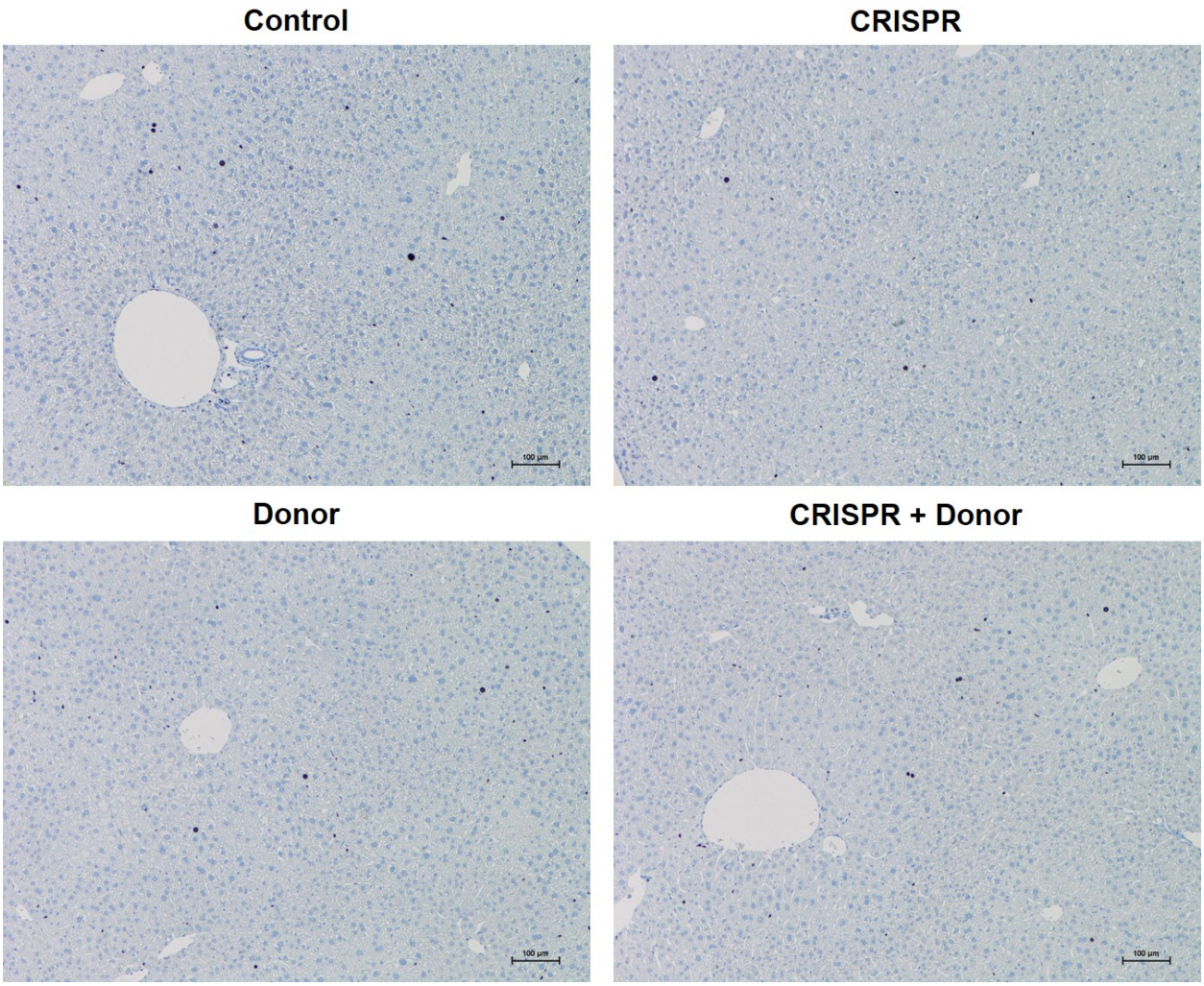

Figure S9: Normal rates of proliferation in livers of *Apoa1*-targeted mice. Representative Ki67 staining of livers from *Apoa1*-targeted mice. Scale bar is 100 µm.

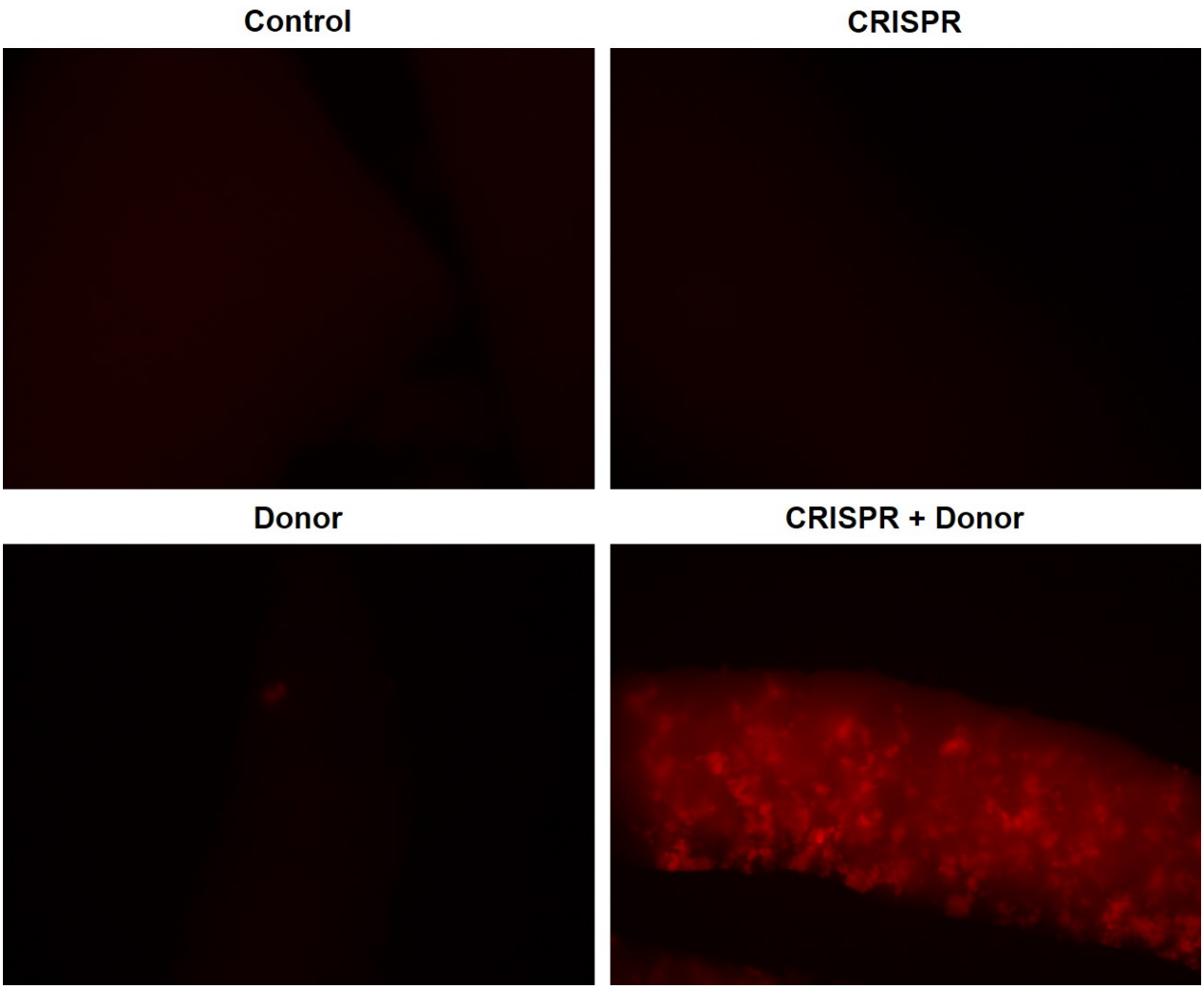

Figure S10: Efficient expression of mKate2 in *Apoa1*-targeted mice injected at P4. Direct mKate2 fluorescence in fresh liver slices from *Apoa1*-targeted mice. Representative images are shown. A 4x objective lens and 45 ms exposure time were used.

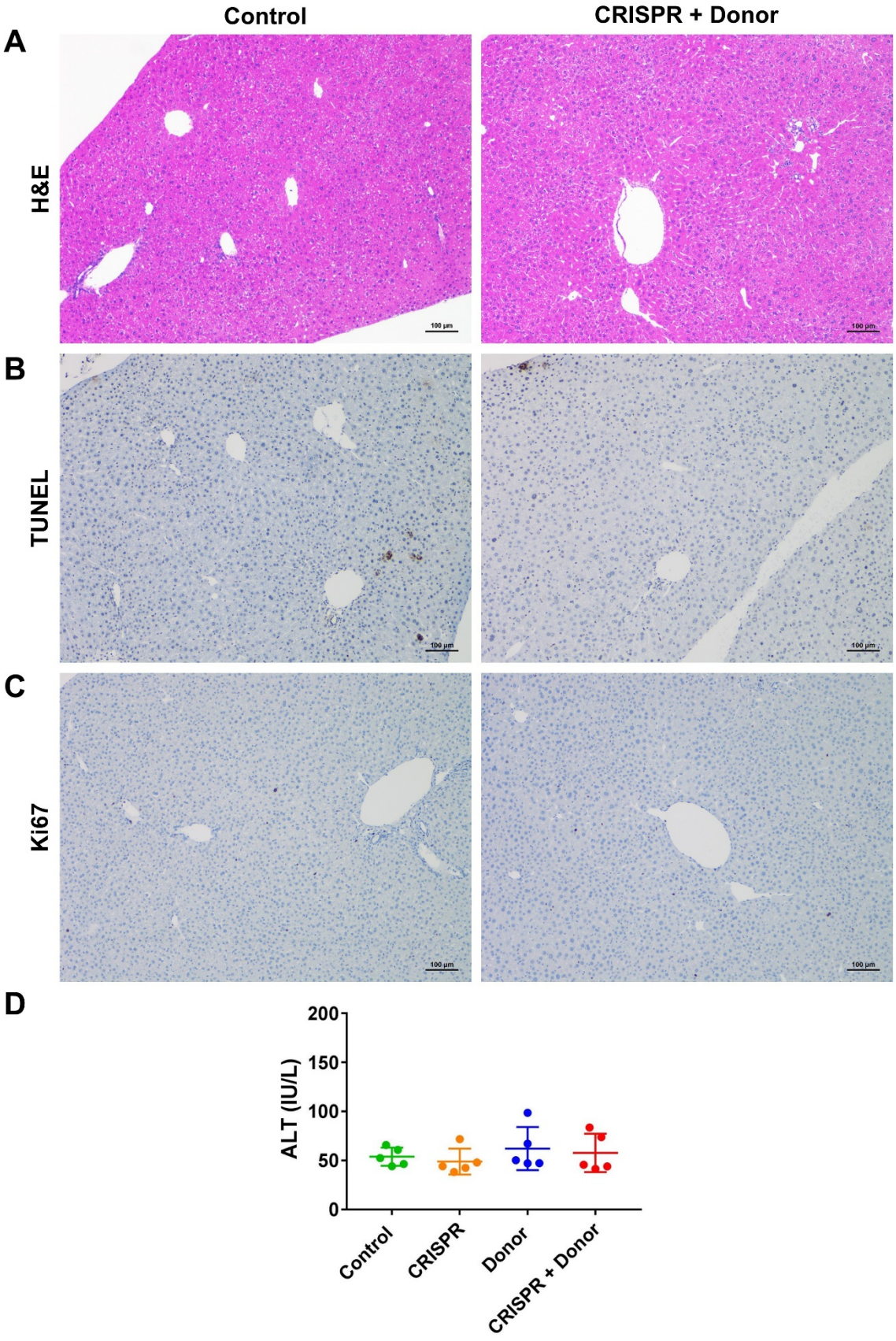
Control
CRISPR + Donor
A
H&E
B
TUNEL
C
Ki67
D
ALT (IU/L)
200
150
100
50
0
Control
CRISPR
Donor
CRISPR + Donor

Figure S11: No histopathological abnormalities or toxicity in liver from P4-injected *Apoa1*-targeted mice. Representative (A) H&E, (B) TUNEL and (C) Ki67 staining of livers from *Apoa1*-targeted mice. Scale bar is 100 μm. (D) ALT levels in plasma at endpoint. Data are shown as mean ± standard deviation (n=5). A one-way ANOVA followed by Tukey test revealed no significant differences in ALT levels among groups.

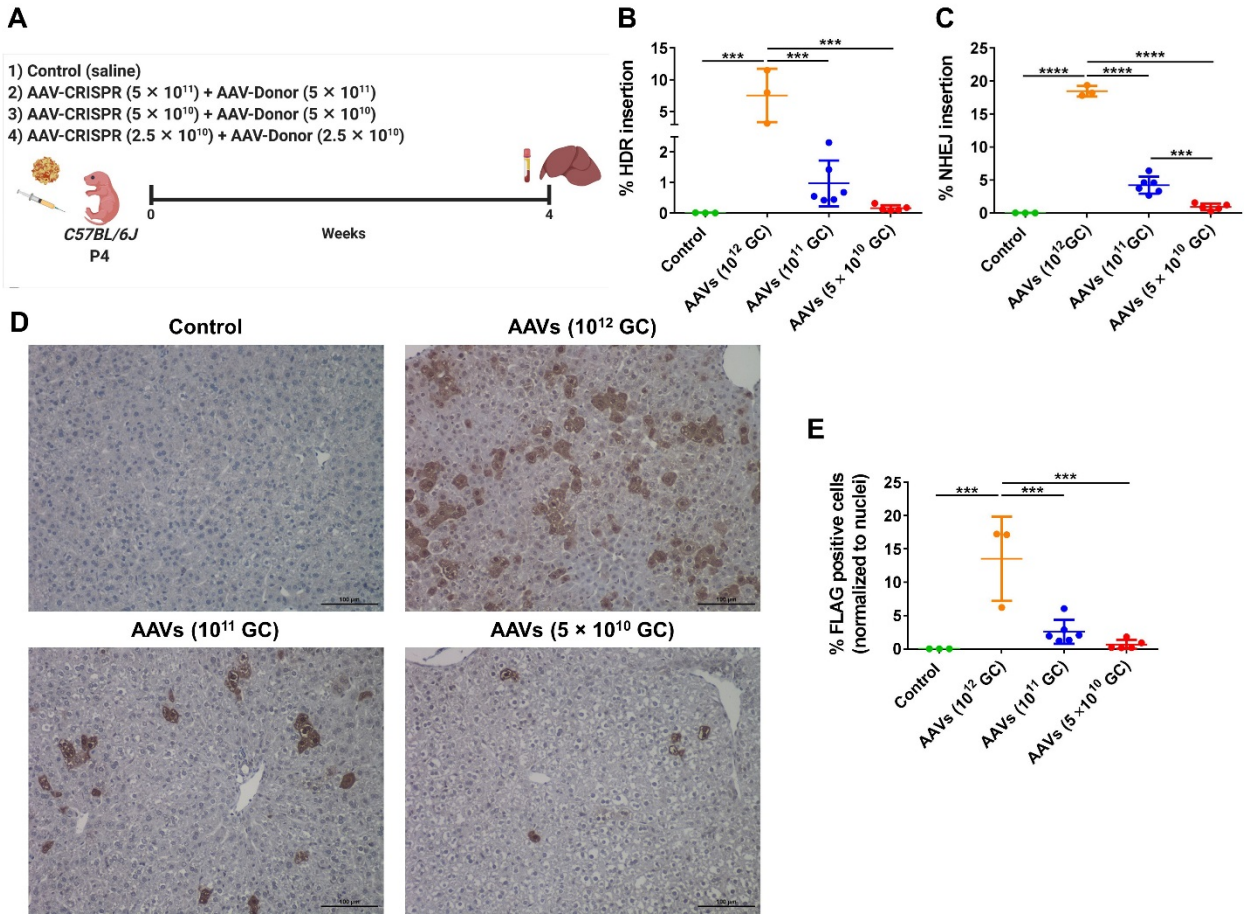

Figure S12: Dose response study of *Apoa1* targeting in pups injected AAV-CRISPR + AAV-Donor. (A) P4 *C57BL/6J* pups were subcutaneosly injected with three doses of AAV-CRISPR and an AAV-Donor encoding mKate2 in a 1:1 molar ratio (total AAVs: 10^{12}, 10^{11} or 5×10^{10} GC) or saline (control). Mice were sacrificed at 4 weeks of age and the liver was evaluated for the targeting at the *Apoa1* locus and the expression of mKate2. (B) Frequency of correct HDR targeting of AAV-Donor by ddPCR. (C) Frequency of *Apoa1* alleles with NHEJ insertions of AAV genomes by ddPCR. (D) Representative immunohistochemistry for mKate2-FLAG (brown cells) in *Apoa1*-targeted mice. Scale bar is 100 µm. (E) Quantification of FLAG positive hepatocytes relative to total nuclei per field. Data are shown as mean ± standard deviation (n=3 control mice, 3 mice injected with 10^{12} GC, 6 mice injected with 10^{11} GC and 5 mice injected with 5×10^{10} GC), with significance determined by One-way ANOVA followed by Tukey test. *** $p<0.001$, **** $p<0.0001$. (A) created with BioRender.

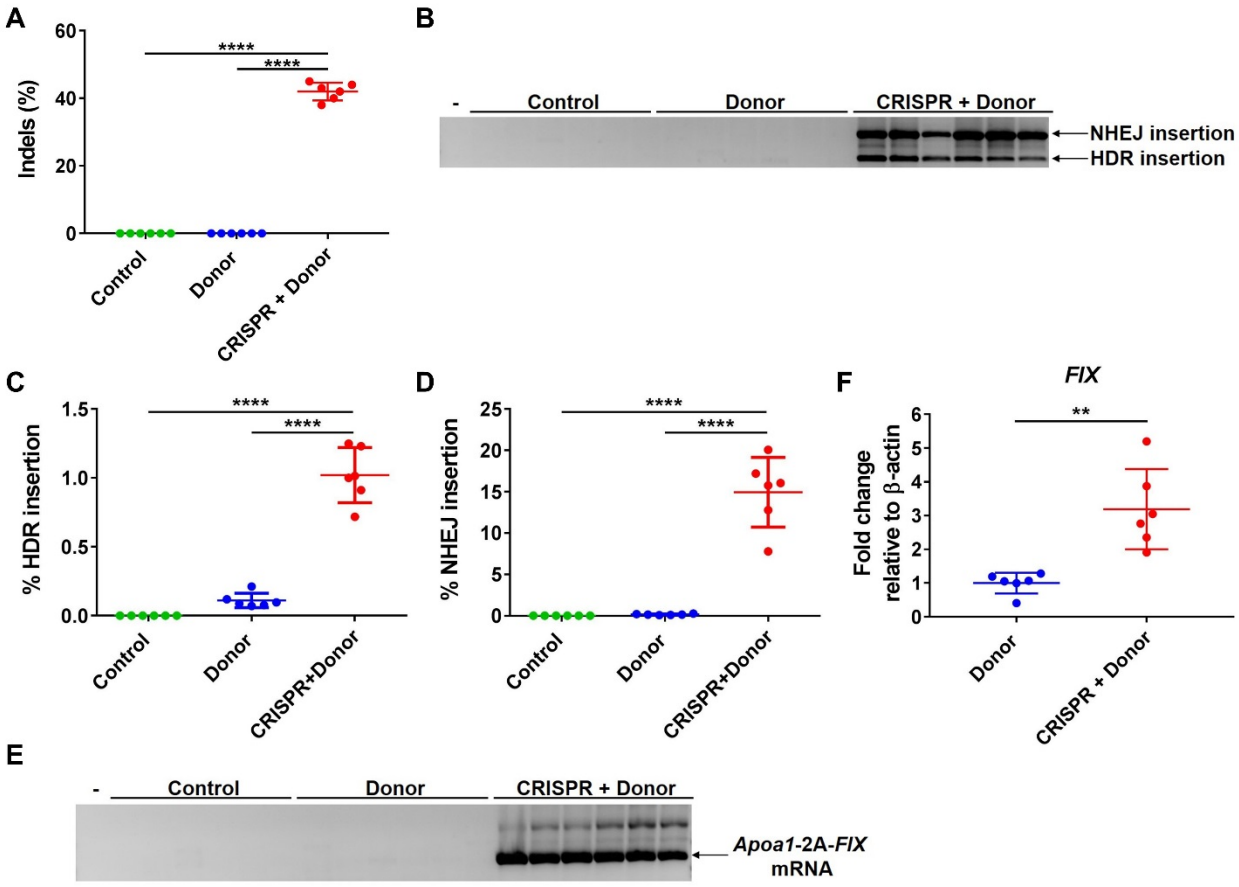

Figure S13: **Specific AAV-Donor integration in the 3'UTR of *Apoa1* in livers from *C57BL/6J* mice.** (A) Rate of indel formation in the 3'UTR of *Apoa1* by ICE analysis. (B) Integration PCR on liver DNA showed two main products corresponding to HDR (941 bp) and NHEJ (1866 bp) insertion of AAV-Donor in the *Apoa1* cut site. Minus (-) indicates a water only PCR control. (C) Frequency of HDR targeting of AAV-Donor by ddPCR. (D) Frequency of *Apoa1* alleles with NHEJ insertions of AAV genomes by ddPCR. (E) Detection of the bicistronic *Apoa1*-2A-*FIX* mRNA (724 bp) by end point PCR on cDNA. Minus (-) indicates a water only PCR control. (F) qPCR analysis of *FIX* expression in livers from Donor and CRISPR + Donor mice. qPCR data are expressed as fold change relative to β-Actin. Data are shown as mean ± standard deviation (n=6). One-way ANOVA followed by Tukey test in panels A, C and D. Two-tailed Student's t-test in panel F. **p<0.01, **** p<0.0001.

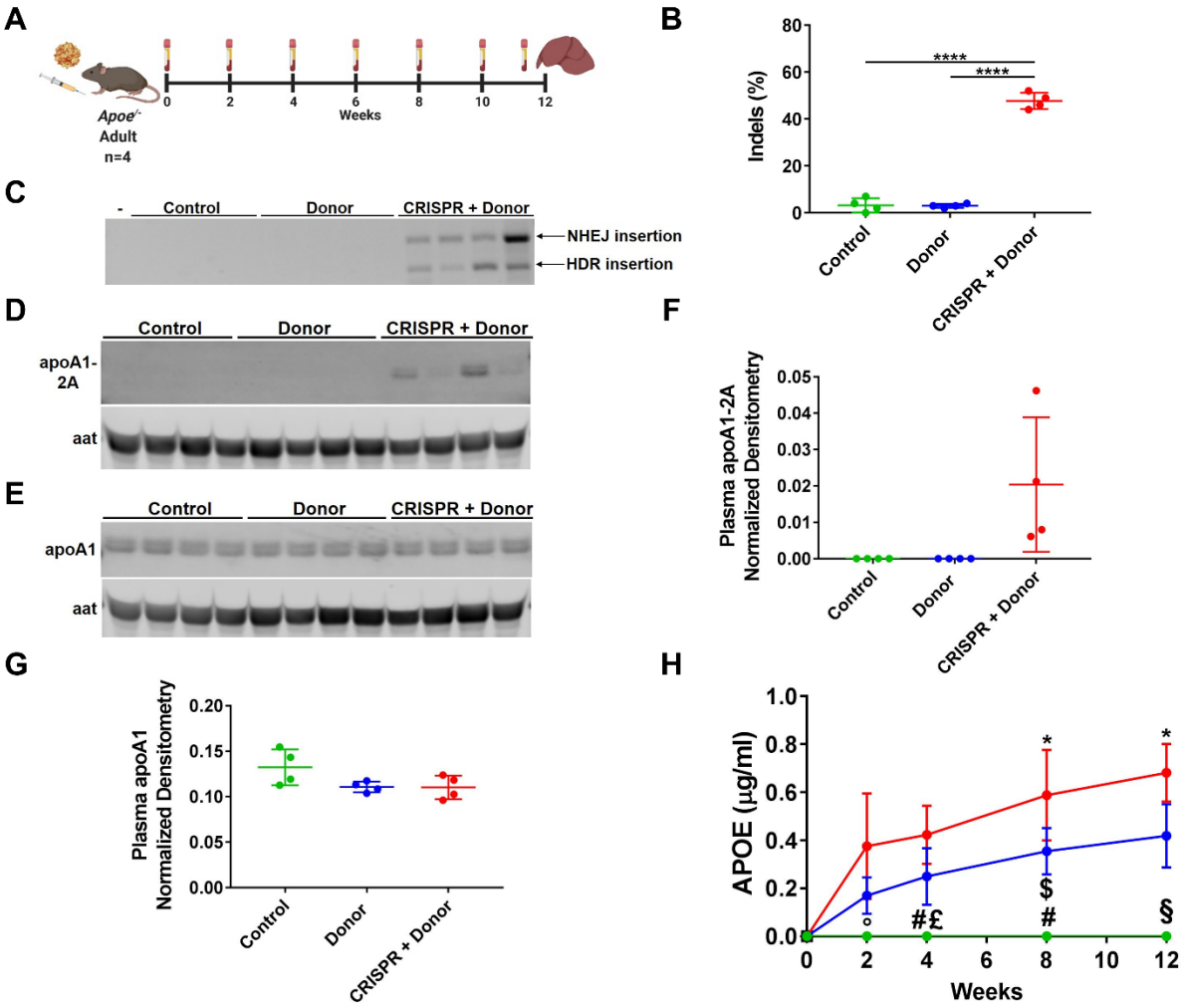

Figure S14: Efficient expression of a secreted therapeutic protein from the *Apoa1* locus. (A) Adult *Apoe*$^{-/-}$ mice were intraperitoneally injected with AAV-CRISPR (5×10^{11} GC) and/or an AAV-Donor (5×10^{11} GC) encoding the human *APOE* gene. Control mice were injected with AAV-GFP (5×10^{11} GC). Plasma was collected every two weeks up to 12 weeks post-injection for analysis of human APOE protein. (B) Rate of indel formation in the 3'UTR of *Apoa1* by ICE analysis. (C) Integration PCR on liver DNA showed two main products corresponding to HDR (1289 bp) and NHEJ (2213 bp) insertion of AAV-Donor in the *Apoa1* cut site. Minus (-) indicates a water only PCR control. Western blot analysis of 2A-tagged apoA1 (D) and total apoA1 (E) in plasma isolated at 12 weeks post-injection, with aat as loading control. Densitometry analysis of apoA1-2A (F) and apoA1 (G) relative to aat loading control. (H) Quantitative measurement of plasma APOE over time by ELISA. Green line: control; Blue line: Donor; Red line: CRISPR + Donor mice. Data are shown as mean ± standard deviation (n=4). A one-way ANOVA followed by Tukey test was used for determining significance in panels B, F and G. A two-way ANOVA followed by Tukey test was used for determining significance in panel H. **** $p<0.0001$, * $p<0.05$ CRISPR + Donor vs Donor, ° $p<0.001$ Control vs CRISPR + Donor, # $p<0.0001$ Control vs CRISPR + Donor, £ $p<0.05$ Control vs Donor, $ $p<0.001$ Control vs Donor, § $p<0.0001$ Control vs Donor and CRISPR + Donor. (A) created with BioRender.

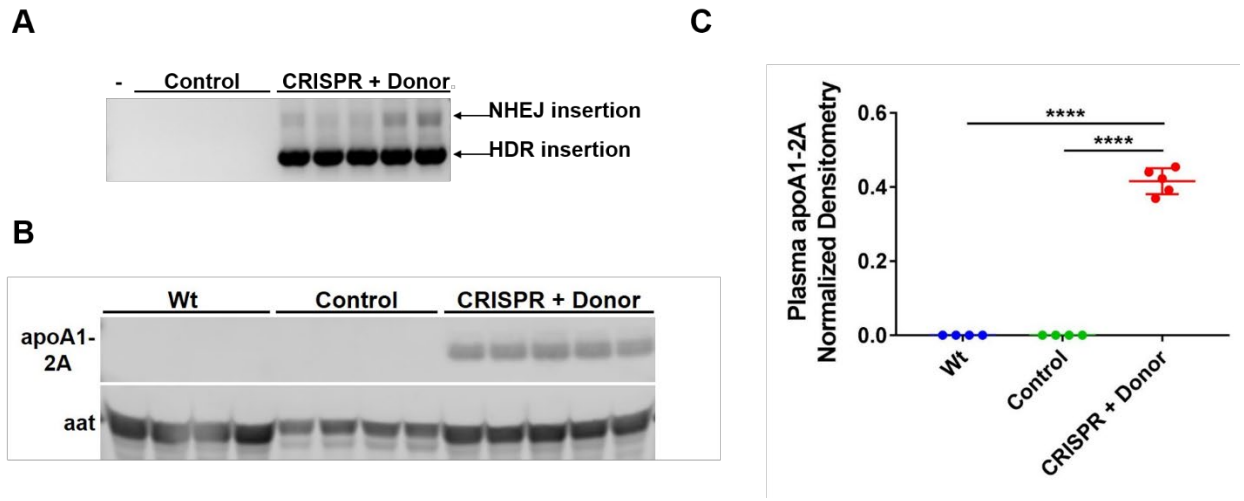

Figure S15: Enrichment of HDR-mediated integrations of AAV-Donor in the *Apoa1* locus in livers from *Fah*$^{-/-}$ mice. (A) Integration PCR on liver DNA showed mostly the HDR (886 bp) insertion rather than the NHEJ (1810 bp) insertion of AAV-Donor in the *Apoa1* cut site. Minus (-) indicates a water only PCR control. (B) Western blot analysis of 2A-tagged apoA1 in plasma isolated at endpoint, with aat as loading control. (C) Densitometry analysis of apoA1-2A relative to aat loading control. Wild type (Wt) indicates plasma samples from *C57BL/6J* mice as a control for physiological levels of aat. Control mice showed lower levels of plasma aat likely due to the severe liver damage. Data are shown as mean ± standard deviation (n=4 Wt and Control, 5 CRISPR + Donor). A one-way ANOVA followed by Tukey test was used for determining significance. **** $p<0.0001$.

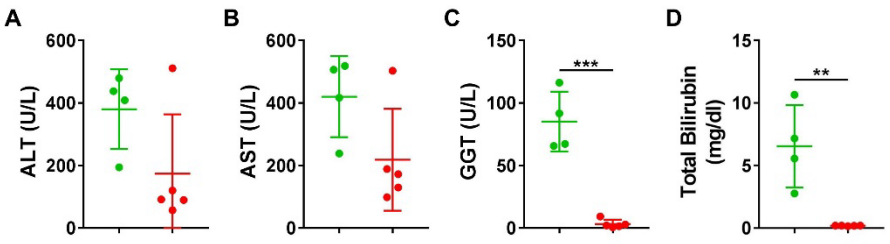

Figure S16: Rescue of liver toxicity in *Apoa1*-targeted *Fah*$^{-/-}$ mice. (A) ALT, (B) AST, (C) GGT and (D) total bilirubin levels in plasma of *Apoa1*-targeted mice. Green dots: control; Red dots: CRISPR + Donor mice. Data are shown as mean ± standard deviation (n=4 control and 5 CRISPR + Donor mice). ** $p<0.01$, *** $p<0.001$ by two-tailed Student's t-test.

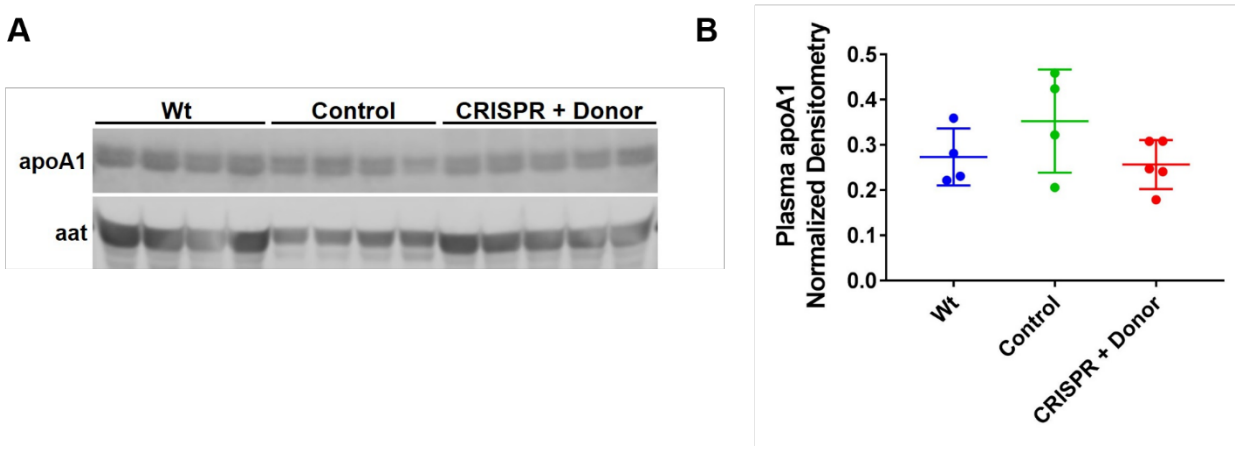

Figure S17: High rates of *Apoa1* targeting does not impact endogenous apoA1 levels. (A) Western blot analysis of total apoA1 in plasma isolated at endpoint, with aat as loading control. (B) Densitometry analysis of apoA1 relative to aat loading control. Wild type (Wt) indicates plasma samples from *C57BL/6J* mice as a control for physiological levels of apoA1 and aat. Control mice showed lower levels of plasma aat likely due to the severe liver damage. Data are shown as mean ± standard deviation (n=4 Wt and Control, 5 CRISPR + Donor). A one-way ANOVA followed by Tukey test did not reveal significant differences in apoA1 levels among groups.

Table S1: On- and off-target (OT) indel formation analysis by deep sequencing.

Available as Excel File.

Table S2: ChIP-seq and ATAC-seq details.

NAME	DESCRIPTION	BIOSAMPLE_ DESCRIPTION	GENOME	ANTIBODY	EXPERIMENT_ ACCESSION	BIOSAMPLE_ ACCESSION	FILE_ ACCESSION	FILE_TYPE	PROCESSING_ PIPELINE
MOUSE_ LIVER_ H3K27AC	H3K27ac ChIP-seq	1 million hepatocytes extracted from 8 week old C57BL/6 mouse liver	mm10	Abcam 4729	NCBI GEO GSE152993	N/A	N/A	FASTQ (SE)	Aquas ChIP-seq
MOUSE_ LIVER_ RNA_ POL2	RNA Pol II ChIP-seq	Mus musculus strain B6NCrl male adult (8 weeks) liver tissue	mm10	Covance MMS-126R	ENCODE ENCSR000 CBR	ENCB S337E NC	ENCFF0 01LNH	FASTQ (SE)	Aquas ChIP-seq
MOUSE_ LIVER_ ATAC	ATAC-seq	Liver from pooled postnatal 0 day mice	mm10	N/A	ENCODE ENCSR609 OHJ	ENCB S559 AAA	ENCFF9 57VLH, ENCFF9 99IJT	FASTQ (PE)	RIESLING
HUMAN_ LIVER_ H3K27AC	H3K27ac ChIP-seq	Homo sapiens female adult (53 years) right lobe of liver tissue	hg38	Active Motif 39133	ENCODE ENCSR981 UJA	ENCB S536T HV	ENCFF9 31EZZ	FASTQ (SE)	Aquas ChIP-seq
HUMAN_ LIVER_ RNA_POL 2	RNA Pol II ChIP-seq	Homo sapiens right lobe of liver female adult (53 years)	hg38	Biolegend 920102	ENCODE ENCSR415 MOW	ENCB S904 HZU	ENCFF1 74GER, ENCFF0 71CTT	FASTQ (PE)	Aquas ChIP-seq
HUMAN_ LIVER_ ATAC	ATAC-seq	Homo sapiens right lobe of liver female adult (53 years)	hg38	N/A	ENCODE ENCSR373 TDL	ENCB S904 HZU	ENCFF0 35UUL, ENCFF2 59ZLS	FASTQ (PE)	RIESLING

Table S3: Primer list.

Name	Sequence (5' to 3')	Purpose
WRL_0066	GTACTACGAGGAAACCGGGAAC	AAV titer SaCas9 Fw
WRL_0068	GTTGTTGTAGAAGGAGGCGATAAAC	AAV titer SaCas9 Rv
WRL_0200	CACATGGGAGAGAGTCACCACATAC	AAV titer mKate2 Fw
WRL_0201	CCACGAGCTTCAGGGCCATG	AAV titer mKate2 Rv
EYFP For2	GCATCGACTTCAAGGAGGAC	AAV titer EGFP Fw
EGFP_Rev2	TGCACGCTGCCGTCCTCGATG	AAV titer EGFP Rv
MDG_0046	GGTCGCTTTTGGGATTACCT	AAV titer *APOE* Fw
MDG_0047	TTCCTCCAGTTCCGATTTGT	AAV titer *APOE* Rv
MDG_0054	ATGCATTCTGTGGAGGCTCT	AAV titer *FIX* Fw
MDG_0056	GCTGATCTCCCTTTGTGGAAG	AAV titer *FIX* Rv
WRL_0397:	CCACTCTGTCAACGGCTGCAAC	AAV titer *FAH* Fw
SynPARevSeq	CACACAAAAAACCAACACACAGATCTAATG	AAV titer *FAH* Rv
MDG_0040	TGAGTGCCAAATCCCTTTTC	Integration PCR *Apoa1* Fw
WRL_0047	GCCCTCGACCGCCTTGATTC	Integration PCR mKate2 Rv
MDG_0047	TTCCTCCAGTTCCGATTTGT	Integration PCR *APOE* Rv
MDG_0168	GGCTCCGCTTCCTCTAGACT	Integration PCR 2A Rv
MDG_0098	TCTACAGTCCGACGATCAACCCACCTGAAGACACTTGG	*Apoa1* deep seq Fw
MDG_0099	GACGTGTGCTCTTCCGATCTGTTCTCACGTCCCTTGATG	*Apoa1* deep seq Rv
MDG_0100	TCTACAGTCCGACGATCACTCCCCCATCATATACACTTCC	OT1 deep seq Fw
MDG_0101	GACGTGTGCTCTTCCGATCGACAGGTACCCAAACAGACCTT	OT1 deep seq Rv
MDG_0102	TCTACAGTCCGACGATCAGGAGTGCATCCGTTATGGAG	OT2 deep seq Fw
MDG_0103	GACGTGTGCTCTTCCGATCTGCACATGAGAGCACTTGAA	OT2 deep seq Rv
MDG_0104	TCTACAGTCCGACGATCACTTGGGAGAGGTGACCAGAC	OT3 deep seq Fw
MDG_0105	GACGTGTGCTCTTCCGATCTCTAACAGGCATCGGACCTT	OT3 deep seq Rv
MDG_0106	TCTACAGTCCGACGATCACTATGGGGTGGCTGCTCTTA	OT4 deep seq Fw
MDG_0107	GACGTGTGCTCTTCCGATCTGAGGTTAATTGGGGTTCCA	OT4 deep seq Rv
MDG_0108	TCTACAGTCCGACGATCAAGGGTAACATGGGTGGTGAA	OT5 deep seq Fw
MDG_0109	GACGTGTGCTCTTCCGATCCCGATCTGCAATCTGCACTA	OT5 deep seq Rv
MDG_0110	TCTACAGTCCGACGATCATGTGCATGCTGAGTGAATGA	OT6 deep seq Fw
MDG_0111	GACGTGTGCTCTTCCGATCGGAGCCAGCTATTCTGCAAC	OT6 deep seq Rv
MDG_0112	TCTACAGTCCGACGATCAACAAGTAAAAATCAGTGAAGACCT	OT7 deep seq Fw
MDG_0113	GACGTGTGCTCTTCCGATCTGCTAGAAACTGTCATGAGAGGAC	OT7 deep seq Rv
MDG_0114	TCTACAGTCCGACGATCACCAGAGAGAAGGAGGGAATCTT	OT8 deep seq Fw
MDG_0115	GACGTGTGCTCTTCCGATCCGATGGAACCCTTTTAGACAAC	OT8 deep seq Rv
MDG_0118	TCTACAGTCCGACGATCATCAACTACACTTTGGTTCCTTCT	OT10 deep seq Fw
MDG_0119	GACGTGTGCTCTTCCGATCAAATGAAAACCATCGAGGAAGA	OT10 deep seq Rv
MDG_0120	TCTACAGTCCGACGATCATTTGCTGAGTCATTTCTCTGTTG	OT11 deep seq Fw
MDG_0121	GACGTGTGCTCTTCCGATCGCATTTGGGAGATCTTTTGC	OT11 deep seq Rv
MDG_0122	TCTACAGTCCGACGATCACACCTGATCAGCAACAAAGTGT	OT12 deep seq Fw
MDG_0123	GACGTGTGCTCTTCCGATCGCTCACTCTGTTTTCCTCCAAC	OT12 deep seq Rv
MDG_0126	TCTACAGTCCGACGATCAACCTTCTGCTAGCCTCCTTTCT	OT14 deep seq Fw
MDG_0127	GACGTGTGCTCTTCCGATCTATCGAGTGGTCCAATCTTGTG	OT14 deep seq Rv
MDG_0128	TCTACAGTCCGACGATCACATTTGCGTGCATACCTGTAGT	OT15 deep seq Fw
MDG_0129	GACGTGTGCTCTTCCGATCTAAGCAAAACAAGCCAGATTCA	OT15 deep seq Rv
P5_1	CTGATCGT	Deep seq P5 barcode Fw
P5_2	ACTCTCGA	Deep seq P5 barcode Fw
P5_3	TGAGCTAG	Deep seq P5 barcode Fw
P5_4	GAGACGAT	Deep seq P5 barcode Fw
P5_5	CTTGTCGA	Deep seq P5 barcode Fw
P7_1	CACTCACG	Deep seq P7 barcode Rv
P7_2	ACACGATC	Deep seq P7 barcode Rv

P7_3	TATCTGAC	Deep seq P7 barcode Rv
P7_4	CACGTCGT	Deep seq P7 barcode Rv
P7_5	TAGCGACG	Deep seq P7 barcode Rv
P7_6	AGCTCTAG	Deep seq P7 barcode Rv
P7_7	ACTAGAGC	Deep seq P7 barcode Rv
P7_8	CGTACGCA	Deep seq P7 barcode Rv
P7_9	CTACACTA	Deep seq P7 barcode Rv
P7_10	TGCTGCTT	Deep seq P7 barcode Rv
P7_11	TCACGCGT	Deep seq P7 barcode Rv
P7_12	GTAGATCG	Deep seq P7 barcode Rv
P7_13	GTGACGCA	Deep seq P7 barcode Rv
P7_14	CATACTAG	Deep seq P7 barcode Rv
P7_15	AGTGTAGA	Deep seq P7 barcode Rv
P7_16	CGAGAGTT	Deep seq P7 barcode Rv
P7_17	GACATAGT	Deep seq P7 barcode Rv
P7_18	ACGCTACT	Deep seq P7 barcode Rv
P7_19	ACTCACTG	Deep seq P7 barcode Rv
P7_20	TGAGTACG	Deep seq P7 barcode Rv
MDG_025a	AAACAGAAGGTGCAGCCCTA	*Apoa1* ICE Fw
MDG_025b	CTCGGCTAGCACAAGAAACC	*Apoa1* ICE Rv
MDG_0150	AGGGAAGGCAGTGAGTTAGA	ddPCR HDR and ddPCR NHEJ Fw
MDG_0202	CGGGATTCTCTTCGACATCC	ddPCR HDR Rv
MDG_0162	AGCGCGCGCCAGAAGCTGCA	ddPCR HDR and ddPCR NHEJ probe
MDG_0151	GATAAGTAGCATGGCGGGTT	ddPCR NHEJ Rv
MDG_0158	GCTGAGCTTATCAGTCTCCC	ddPCR Ref Fw
MDG_0159	GAAGGCACCAAGAACACAGA	ddPCR Ref Rv
MDG_0163	AGCCCAGCCCCTGCCCACACA	ddPCR Ref probe
MDG_0130	TATGTGGATGCGGTCAAAGA	*Apoa1* qPCR Fw
MDG_0131	ACGGTTGAACCCAGAGTGTC	*Apoa1* qPCR Rv
MDG_0132	TACAGGGCTACATGGAACAA	*Apoc3* qPCR Fw
MDG_0133	TCGGACTCCTGCACGCTACTT	*Apoc3* qPCR Rv
MDG_0134	ACCCAGCTAAGCAACAATGC	*Apoa4* qPCR Fw
MDG_0135	TAGCATCCCCAAGTTTGTCC	*Apoa4* qPCR Rv
MDG_0136	GGCTGCAGTCATCACTTGG	*Apoa5* qPCR Fw
MDG_0137	GGCTGAAGTAGTCCCAGAGG	*Apoa5* qPCR Rv
MDG_0138	GACTCTGCCACCACAAGGAT	*Zpr1* qPCR Fw
MDG_0139	ACGGCTGATGAGTCCTTCAA	*Zpr1* qPCR Rv
MDG_0140	GCAAAGGCTCATTTTGAAGC	*Bud13* qPCR Fw
MDG_0141	CTGAATCAGAGGCTTTTTGG	*Bud13* qPCR Rv
MDG_0142	GCTAGCGGAGGGGAGATATT	*Sik3* qPCR Fw
MDG_0143	TTTGAACTTCCGTCGAGCTT	*Sik3* qPCR Rv
mActb RT F1	TTGGGTATGGAATCCTGTGG	*Bact* qPCR Fw
mActb RT R1	CTTCTGCATCCTGTCAGCAA	*Bact* qPCR Rv
MDG_0203	ATTTCGCTAATGTGTATGTG	bicistronic mRNA PCR Fw
MDG_0204	TTCAACAGGGAGAAGTTAGT	bicistronic mRNA PCR Rv
MDG_0230	TCACTCAAAGCACCCAATCA	*FIX* qPCR Fw
MDG_0231	GGGCAGCAGTTACAATCCAT	*FIX* qPCR Rv