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Supplemental information

LPA disruption with AAV-CRISPR potently lowers plasma apo(a) in transgenic mouse model: A proof-of-concept study

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Supplemental Information

Supplemental Figures and Legends

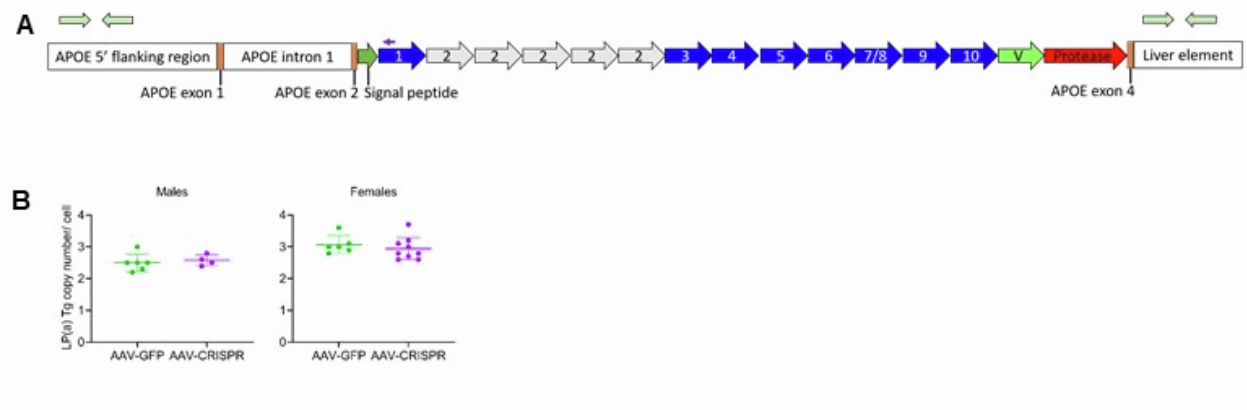

Figure S1. *LPA* transgene construct copy number analysis in *LPA*$^{+/0}$ *Ldlr*$^{-/-}$ (See also Figure 1). A. Schematic of the *LPA* transgene construct and primers used for copy number analysis (indicated by green arrows, primer binding sites are not drawn to scale but indicate which elements on the construct they bind). B. ddPCR results showing an average of 3 *LPA* transgene copies/diploid genome (c/dg) in male and female *LPA*$^{+/0}$ Ldlr$^{-/-}$ mice treated with AAV-GFP or AAV-CRISPR. Statistical analysis was done using Welch's t-test with *p<0.05. Values are mean $\pm$ SD

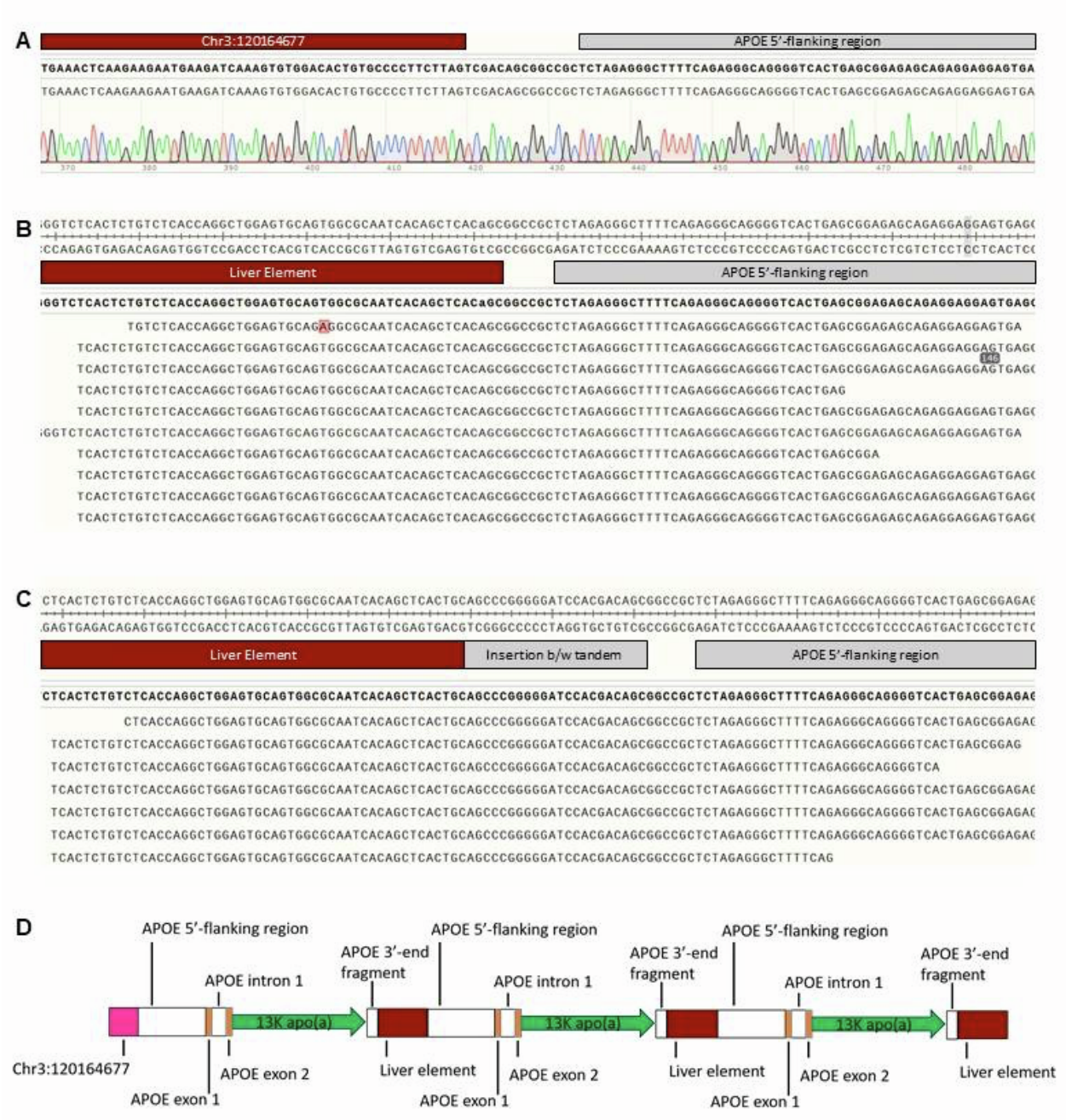

Figure S2. Sequencing results from adaptor ligation-mediated PCR method to identify *LPA* transgene knock-in location in *LPA*$^{+/0}$ *Ldlr*$^{-/-}$ (see also Figure 1). A. Sanger sequencing results showing the junction of chr3:120164677 and the 5' end of the *LPA* construct. NGS results show the B. type 1 and C. type 2 tandem junction of the 3' and 5' end of the *LPA*

construct. D. Schematics of the integrated *LPA* transgene. *LPA*$^{+/0}$ Ldlr$^{-/-}$ mice have three tandem repeats of the *LPA* construct in direct orientations integrated at chr3:120164677.

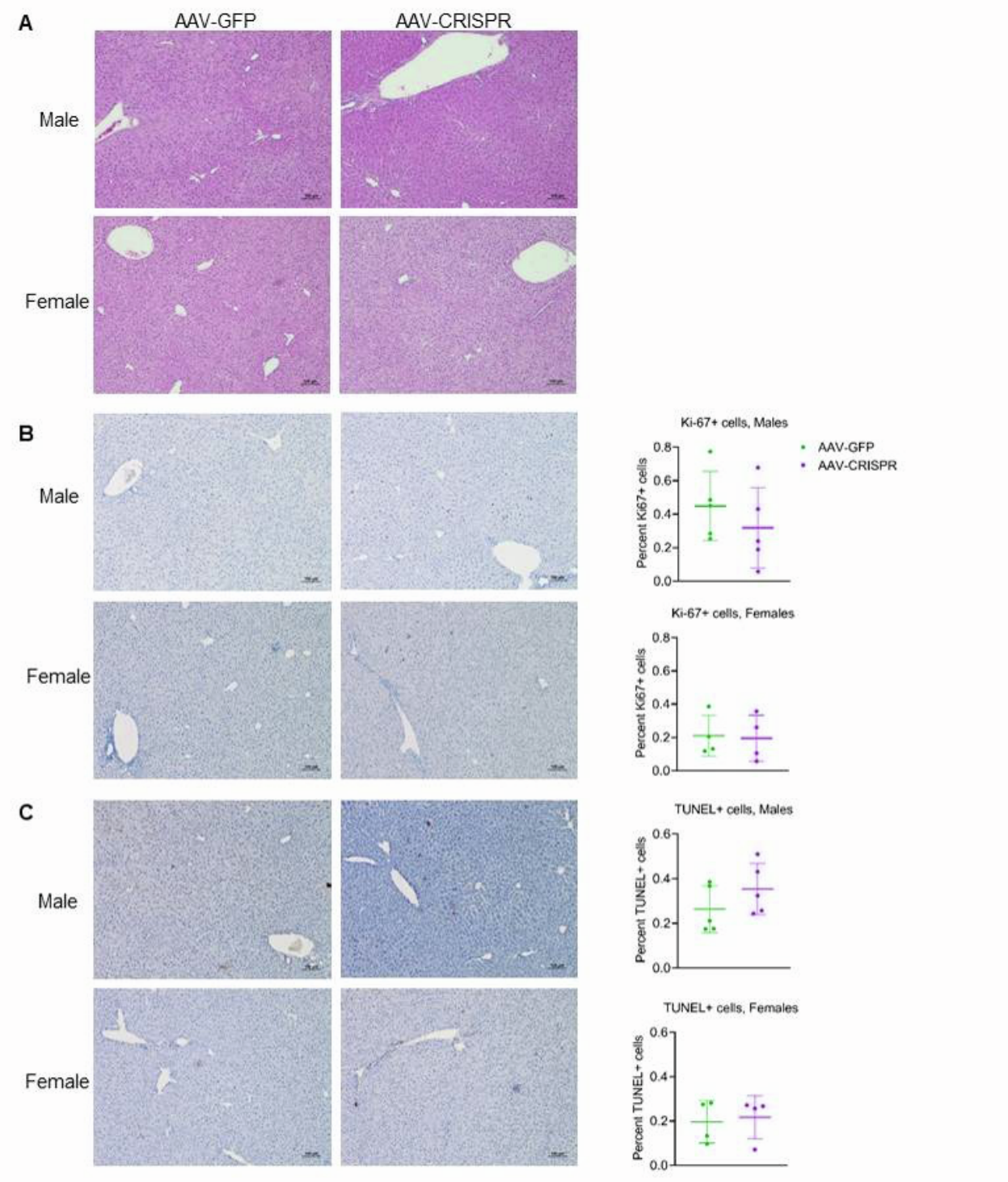

Figure S3. Histology analysis on AAV-GFP and AAV-CRISPR treated mice (See also Figure 2). A. Representative images for hematoxylin and eosin stained male and female mouse livers injected with either AAV-GFP or AAV-CRISPR. B. Representative images and

quantification of Ki67 stained male and female mouse livers. C. Representative images and quantification of TUNEL stained male and female mouse livers. Statistical analysis was done using Mann-Whitney test on non-normally distributed data with *p<0.05 (TUNEL+cells females). Statistical analysis was done using a Welch's *t*-test on normally distributed data with *p<0.05. (KI67+ cells males and females, and TUNEL+ cells males). Values are mean $\pm$ SD.

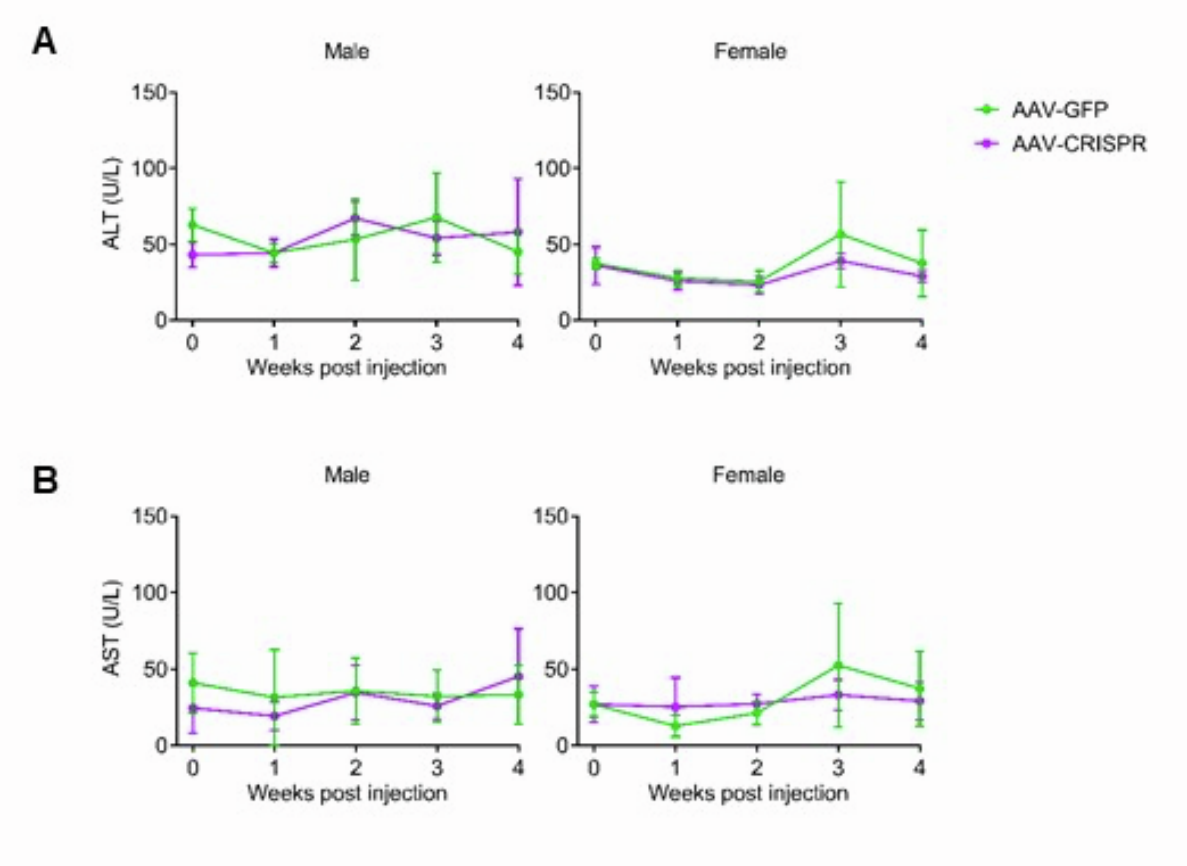

Figure S4. ALT and AST analyses (See also Figure 2). A. ALT analysis on plasma from mice treated with AAV-GFP or AAV-CRISPR. B. AST analysis on plasma from mice treated with AAV-GFP or AAV-CRISPR. Statistical analysis was done using a Welch's *t*-test at each time point with *p<0.05. Values are mean $\pm$ SD.

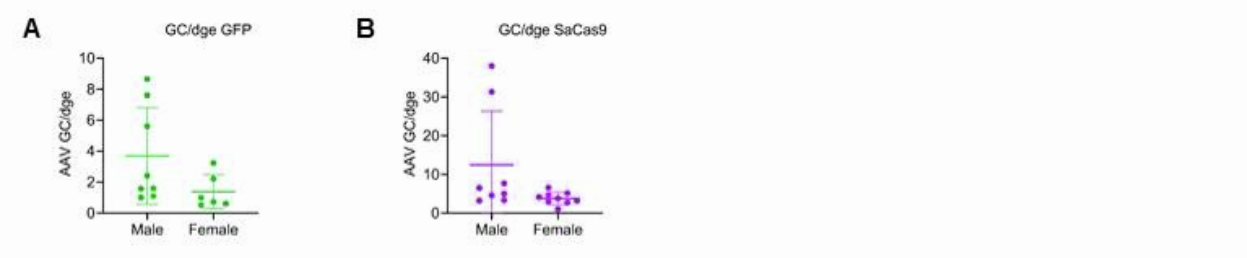

Figure S5. AAV vector genome copies per diploid genome analysis (See also Figure 2). qPCR for vector genomes in mice that received either A. GFP copy number in AAV-GFP treated mice or B. SaCas9 copy number in AAV-CRISPR treated mice. Statistical anlysis was done using Mann-Whitney test with *p<0.05. Values are mean $\pm$ SD.

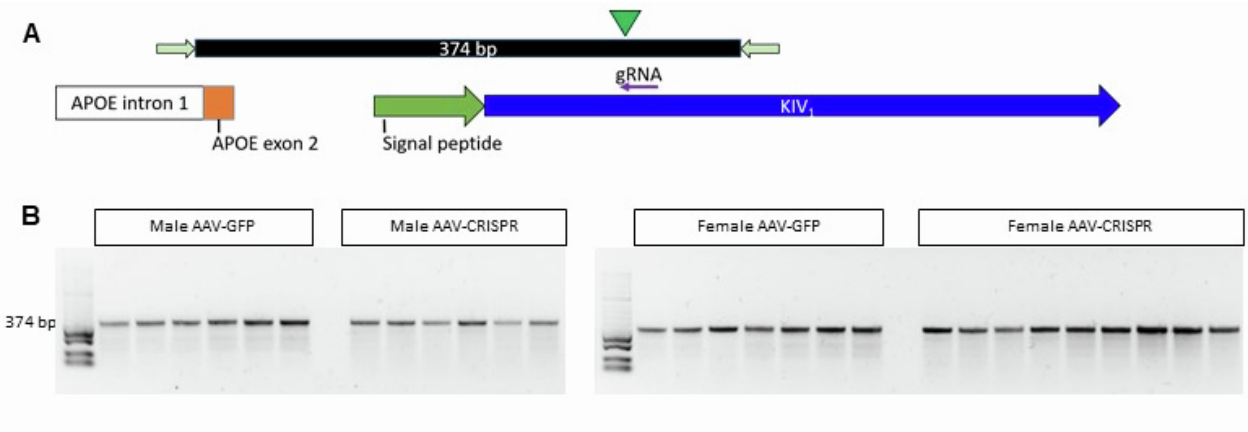

Figure S6. On-target insertion and deletion analysis of *LPA*$^{+/0}$ *Ldlr*$^{-/-}$ mouse livers injected with AAV-CRISPR (See also Figure 3). A. Schematics of KIV_1 specific primer design used for T7E1 and ICE analysis (indicated by green arrows, ICE analysis not shown). The on-target site is indicated by the green triangle. The purple arrow shows the 5' to 3' of gRNA specific for the KIV_1. B. No measurable cleavage activity was detected by T7E1 analysis.

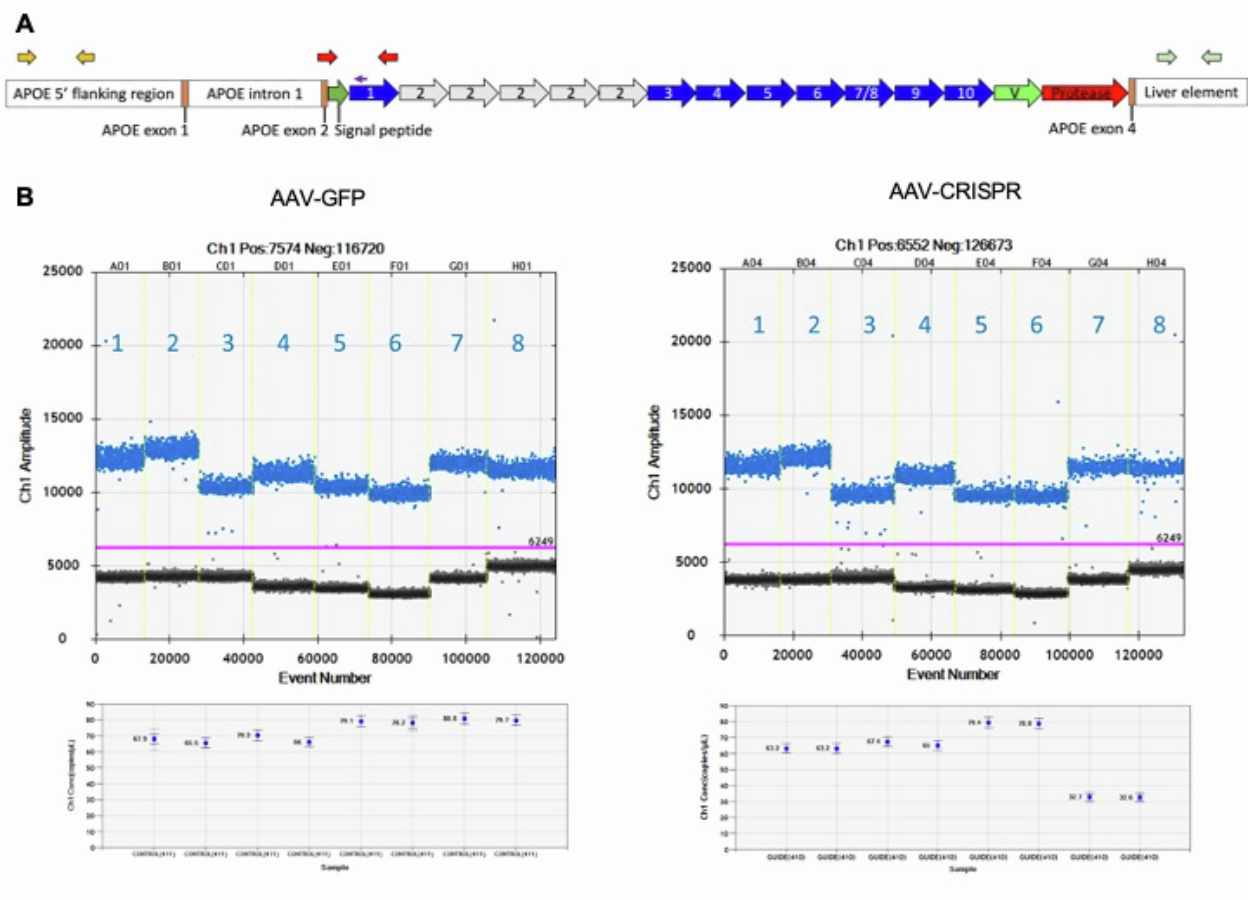

Figure S7. Representative Evagreen-based ddPCR copy number assay amplitude droplet separation and copy number quantification (See also Figure 3). A. Schematics of a KIV_1 specific primer pair used to quantify the frequency of un-rearranged on-target site on KIV_1 by ddPCR (indicated by red arrows). Yellow and green arrows indicate primer pairs used for copy number analysis at the APOE 5' flanking region or the liver element in the apo(a) transgene, respectively. (Primer binding sites are not drawn to scale but indicate which elements on the construct they bind). The purple arrow shows the 5' to 3' of gRNA specific for the KIV_1. The *TFRC* and *RPP30* genes were used as genomic reference sites for the ddPCR but primer binding sites are not shown. B. Representative ddPCR results for AAV- GFP and AAV-CRISPR treated mice. Lane 1: *TFRC* primer set 1, 2: *TFRC* primer set 2, 3: *RPP30* primer set 1, 4:

RPP30 primer set 2, 5: APOE 5’ end of *LPA* construct, 6: Liver element 3’ end of *LPA* construct, 7: *LPA* on-target in KIV_1 primer set 1 and 8: *LPA* on-target in KIV_1 primer set 2.

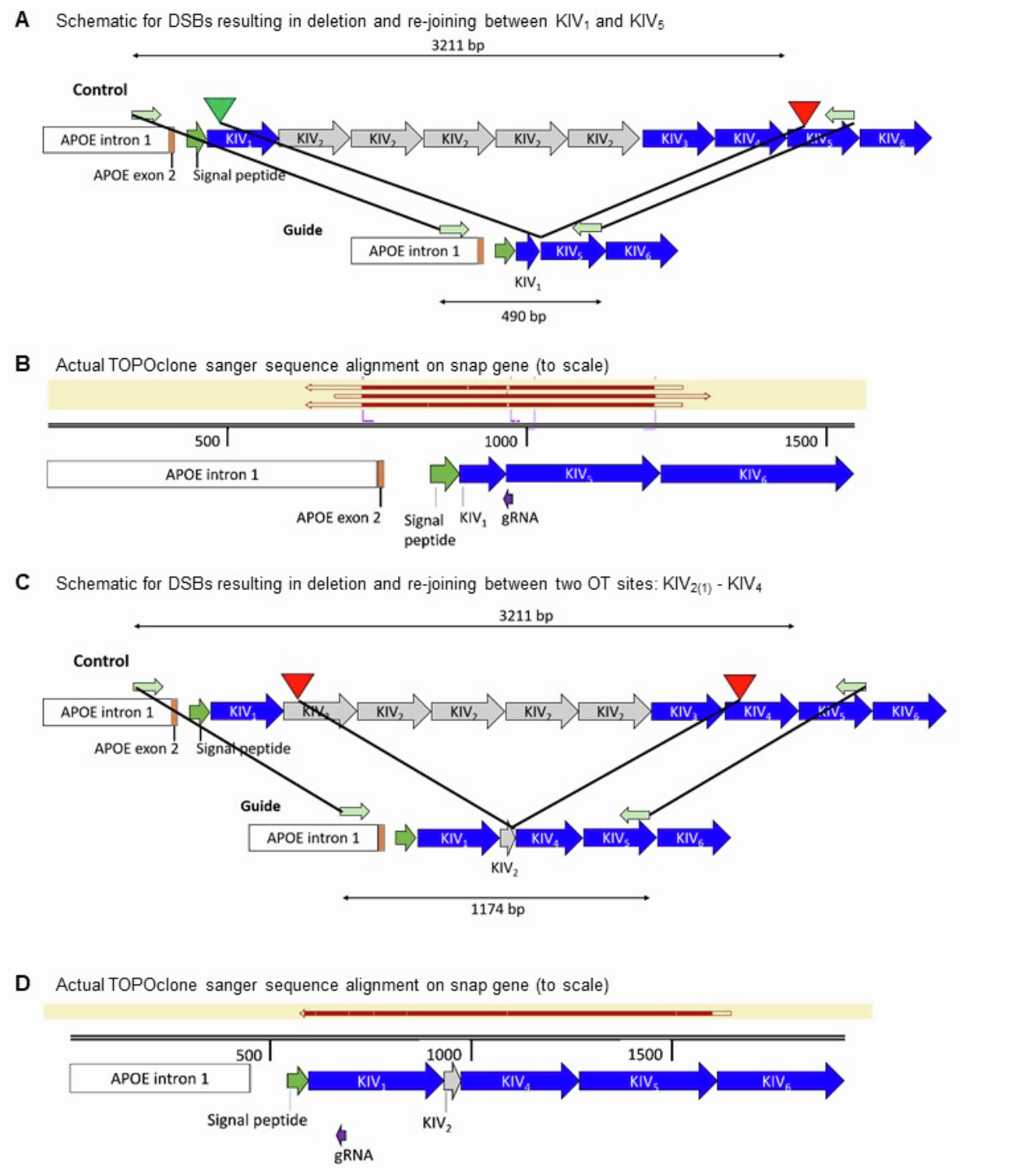

Figure S8. Schematics and aligned TOPO clone sequences for validation of CAST-seq identified large deletion between KIV_1 and KIV_5 (See also Figure 3). A. Schematic for TOPO clone sequencing of truncated amplicons from AAV-CRISPR treated mice showing the product

of 2.7kb large deletion created by DSBs re-joining or chromosomal rearrangements between KIV_1 and KIV_5 DSBs. B. TOPO clone sanger sequence alignment on SnapGene. C. Schematic for topo clone sequencing of truncated amplicons from AAV-CRISPR treated mice showing the product of 2kb large deletion created by DSBs re-joining or chromosomal rearrangements between two OT DSBs: $KIV_{2(1)}$ and KIV_4. D. TOPO clone sanger sequence alignment on SnapGene. On-target cut sites are indicated by green inverted triangles and OT cut sites are indicated by red inverted triangles. Specific primer pair design is indicated by light green arrows.

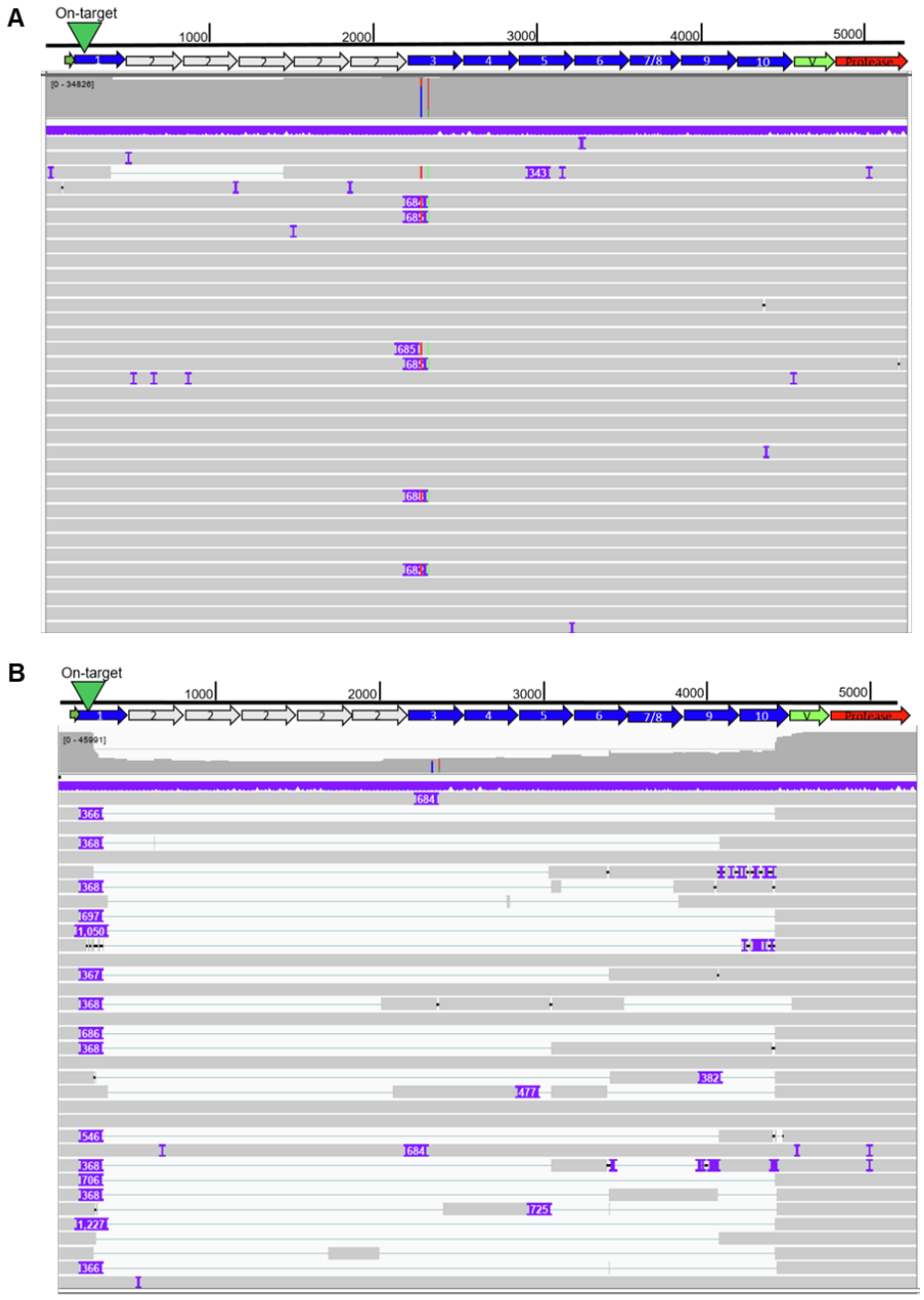
A
On-target
1000
2000
3000
4000
5000
Protease
B
On-target
1000
2000
3000
4000
5000
Protease
C
AAV-GFP
AAV-CRISPR
Read frequency%
80
60
40
20
0
-5000
-4800
-4600
-4400
-4200
-4000
-3800
-3600
-3400
-3200
-3000
-2800
-2600
-2400
-2200
-2000
-1800
-1600
-1400
-1200
-1000
-800
-600
-400
-200
0
200
400
600
800
1000
Large insertion or deletion sizes (bp)

Figure S9. PacBio SMRT sequencing analysis (See also Figure 3). PacBio SMRT sequencing generated HiFi reads alignment as viewed on IGV with either A. AAV-GFP or B.AAV-CRISPR treated samples. A. In AAV-GFP sample, 70% of reads were the expected full-length 13K cDNA, while 17% of reads had an unexpected longer 15K sequence with a 684 bp insertion at KIV_3 (**See Figure S10**). The remaining 12% of reads in the control sample were diverse homologous deletions of up to eight KIV domains most likely due to PCR artifacts. B. In AAV-CRISPR sample, 22% of reads had expected 13K cDNA sequences, while 6% of reads had 15K cDNA, and 72% of reads had deletions ranging from 1710 bp to 4079 bp. In contrast to the homologous deletions observed in AAV-GFP, deletions in the AAV-CRISPR sample are the products of CRISPR DSBs at ON and OT sites. We also observed alleles containing multiple deletions at ON-OT and between OTs. C. Histogram showing the frequency of reads corresponding to 13K, 15K, and deletions in AAV-GFP or AAV-CRISPR treated samples. Note that the read frequency does not represent the allele frequency due to template length bias during PCR amplification, but nonetheless provides information on a relative abundance and full-length sequences of LPA transgene alleles.

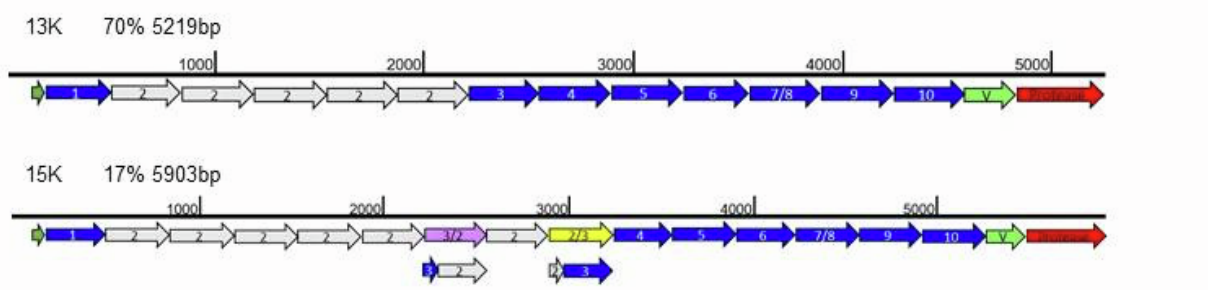

Figure S10. Schematic for transgene construct sequences for 13K or 15K (See also Figure 3). The longer 15K reads in the control sample contained a $KIV_{3/2}$ hybrid, $KIV_{2(6)}$, and $KIV_{2/3}$ hybrid contiguous sequence which could have resulted from homologous recombination in the germline of founder mice.[29] Another explanation for these unusual reads is a PCR artifact since amplification of highly similar sequences can produce undesired products such as recombination or chimera formation.[30] This remains to be validated using amplification-free long-read sequencing of the integrated LPA transgene.[1]

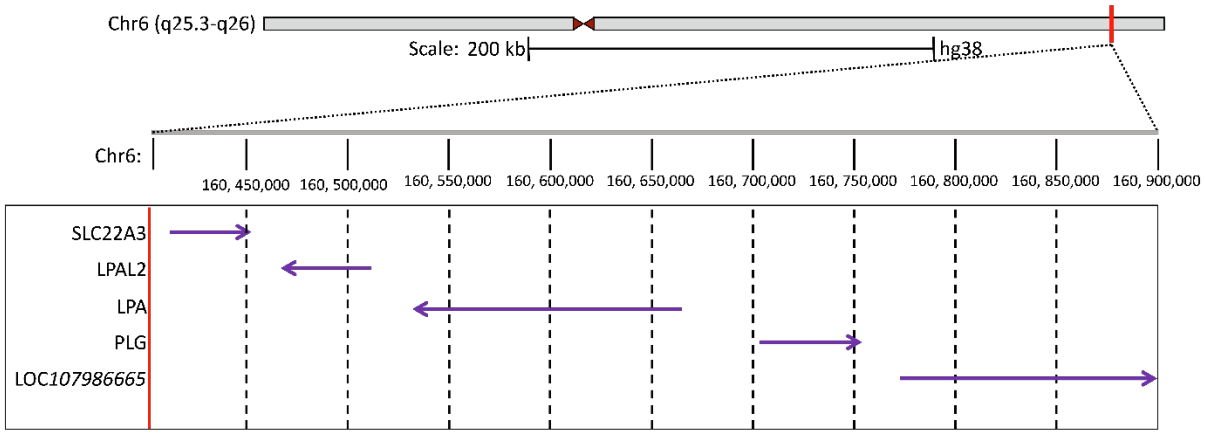

Figure S11. Schematic for the location of pseudogenes nearby *LPA* (See also Figure 4). Two of the GUIDE-seq identified off-target sites are located in *LPAL2* and *LOC107986665* pseudogenes, 173 kbp and 256 kbp away from *LPA* on-target, respectively. *PLG* is located between *LPA* and *LOC107986665.*

Supplemental Tables and Legends

Table S1. Off-target (OT) sites identified by GUIDE-seq in plasmid treated HEK293T (See also Figure 4). GUIDE-seq identified 25 off-target sites which were labeled according to the descending order of read abundance (OT1-OT25). For each off-target site, aligned sequences, location of edit site on Hg38, gene, GUIDE-seq identified read counts and number of mismatches (MMs) to the on-target site are shown. For the 11 off-target sites are at LPA, LPA exon number and corresponding to KIV domain are shown. Due to highly homologous KIV domains, some of the OT sites (ex. OT4 and OT5; OT7 and OT8; OT9 and OT10; OT12, OT13 and OT18) have the same target and PAM sequences but occur at different genomic locations.

Target	Aligned Sequence	Edit Site	Gene	LPA exon (KIV domain)	Distance from On (bp)	Reads	#MMs
On	.....TG.AG.	chr6:+:160650419	LPA	exon2 (KIV_1)	0	630	0
OT1	AT.T.....TG.AGA	chr6:-:160905840	LOC107986665		255421	401	4
OT2	...A..T.....TG.AG.	chr6:+:160599581	LPA	exon20 (KIV_5)	50838	377	2
OT3	A.....TG.AGG	chr6:+:160577217	LPA	exon28 (KIV_9)	73202	363	1
OT4	A..T.....TA.AGA	chr6:+:160594039	LPA	exon22 (KIV_6)	56380	275	4
OT5	A..T.....TG.AGA	chr6:+:160477210	LPAL2		173209	224	3
OT6	A..T.....TG.AGA	chr6:+:160556106	LPA	exon30 (KIV_{10})	94313	185	3
OT7	.....T.....TGAAG.	chr6:+:160500862	LPAL2		149557	108	2
OT8	.....T.....TGAAG.	chr6:+:160482741	LPAL2		167678	81	2
OT9	.....T.....A.....TG.AG.	chr6:+:160611643	LPA	exon16 (KIV_3)	38776	72	2
OT10	.....T.....A.....TG.AG.	chr6:+:160633834	LPA	exon8 (KIV_2)	16585	47	2
OT11	A..T.....A.....TG.AGA	chr6:+:160589634	LPA	exon24 (KIV_7)	60785	37	4
OT12	.....T.....TG.AG.	chr6:+:160617198	LPA	exon14 (KIV_2)	33221	15	1
OT13	.....T.....TG.AG.	chr6:+:160639381	LPA	exon6 (KIV_2)	11039	8	1
OT14	.....T.....TGAAA.	chr6:+:160491442	LPAL2		158977	4	2
OT15	.....T.....AATGAAG.	chr6:+:160605127	LPA	exon18 (KIV_4)	45292	4	4
OT16	...A.....CA..G.....TA.AA.	chr4:+:131259328	RNU6-224P			4	4
OT17	.G.A....C....ACA.....GG.AGC	chr8:+:62003251	C1GALT1P3			3	7
OT18	.....T.....TG.AG.	chr6:+:160644928	LPA	exon4 (KIV_2)	5491	3	1
OT19	....T.....A....A.....TT.AG.	chr5:+:45893210	HCN1			3	3
OT20	....C...C....AG.....T.GA.AAA	chr5:+:151448194	SLC36A1			2	6
OT21	T.....G..A.....AATG.GAA	chr16:+:25134130	LCMT1			2	6
OT22	CTGCT.....C.....GT.AAC	chr10:+:18249228	CACNB2			1	7
OT23	...A..GT.....TG..G.....CA.AAA	chr6:+:20125534	MBOAT1			1	7
OT24	.....G.A.....G..G.AGGAGAG	chr17:-:45243782	FMNL1			1	6
OT25	...T.C.....A....G...CA.GA.AGA	chrX:+:110905812	PAK3			1	7

Table S2. Primers used for AAV titer quantification.

Primer Name	Assay	Primer Sequence Sequence
WRL_0066	AAV titer SaCas9 Fw	GTACTACGAGGAAACCGGGAAC
WRL_0068	AAV titer SaCas9 Rv	GTTGTTGTAGAAGGAGGCGATAAAC
EYFP For2	AAV titer Cre/GFP Fw	GCATCGACTTCAAGGAGGAC
EGFP_Rev2	AAV titer Cre/GFP Rv	TGCACGCTGCCGTCCTCGATG

Table S3. List of primers used as well as corresponding figures and assays.

Mouse			
Primer name	**Figure**	**Assay**	**Primer Sequence**
KI_R1	FigS2	LPA transgene KI location	CACCAACAACTTCACGCTGG
KI_R2_nested			TCACTCCTCCTCTGCTCTCC
KI_F1			ACACTCAGAGCTTTGCAGCT
KI_F2_nested			CACTCTGTCTCACCAGGCTG
KIV1_F	Fig3A, FigS6, FigS7	ddPCR	TCACTGGCGGTTGATTGACA
KIV1_R			GCATTTGGGTAGTTTTCTGTGGT
KIV1_F2	Fig3F, FigS8	PCR and Sanger sequencing	TCACTGGCGGTTGATTGACA
KIV5_del_R			CCTCTGTGCTTGGATCTGGG
APOE_copy_F3	FigS1, FigS7	LPA transgene copy number ddPCR	GGAGAGCAGAGGAGGAGTGA
APOE_copy_R3			CCGACAGCTTCCAGACCTTT
Liver_copy_F4			CAACCCCTCAGTTCCCATCC
Liver_copy_R4			CCGAAATTCCAAGGGGTCGA
TFRC_new_F1			CTTCCCACCATGCTTGTTGC
TFRC_new_R1			CACTTCCGCTGCTGTACGAA
TFRC_new_F2			GCAGTGAGTTCTTCCCACCA
TFRC_new_R2			CGCTGCTGTACGAACCATTT
mRPP30_F1			CTACTGGCTTGGCTCTGTCC
mRPP30_R1			TAGGGACGTCAGGCAGATCA
mRPP30_F2			TCTGCAAGACGACCCAAGAC
mRPP30_F2			GGACAGAGCCAAGCCAGTAG
EYFP For2	Fig S5	AAV GC	GCATCGACTTCAAGGAGGAC
EGFP_Rev2			TGCACGCTGCCGTCCTCGATG
WRL_0066			GTACTACGAGGAAACCGGGAAC
WRL_0068			GTTGTTGTAGAAGGAGGCGATAAAC
Human			
Primer name	**Figure**	**Assay**	**Primer Sequence**
LPA_F1	Data not shown	T7E1, ICE	CTCAGGACCCAGGGTGTTTC
LPA_R1			CAGCTTGCAGTGCCAAATGT
LPA_long_F1	Fig4A,C	on-target unrearranged ddPCR	CACGTCTGTCTGCCTGCTAA
LPA_long_R1			AGTTAGCTTGACGCACACCT
LPA_long_F2			TTTTTGCACGTCTGTCTGCC
LPA_long_R2			GCTTGACGCACACCTTTTCT
CACNA1C_F			GAGAGTGCCTGGTCTTTACTGCAGG
CACNA1C_R			CTCTCCTATAGCTGCATGCACACCC
LPA_CAST_bait_F1	Fig5A,C	CAST-seq	TAGCAGCACCTGAGCAAAGC
LPA_CAST_bait_nest_F			CACCTGAGCAAAGCCATGTG
LPA_decoy_R			ACGCATTTGGGTAGTTTTCTGTG

LPA_decoy_F			ACAGTAAGCAAAACAAGGTCCA
ON_F2	Fig5E	ON/OT deletion confirmation PCR	TTTTTGCACGTCTGTCTGCC
OT2_R1			AGCCAAGTTGAGTCCTGAGC
OT3_R2			TCAAAAGCAAAGGTCCTGAGAC
OT4_R			TTCTAAGCACGTGGCCATGT
OT6_R1			AGCTCCATGTAGCCTTCTGC
OT12_13_18_26_R2			CGCCATCTGCATCTGTCACA
OT1_F1			AGTTAGCTTGACGCACACCT
OT1_R1			AAGCCTGAGACATTCTGCCC
exon5_probe_MGB_FAM	Fig5F	KIV_2 CNV ddPCR	CTTGGCAGGTTCTTCC
KIV2_copy_F			GACAGAGTTATCGAGGCACATAC
KIV2_copy_R			CTATGCGAGTGTGGTGTCATAG
hRPP30_F			GATTTGGACCTGCGAGCG
hRPP30_R			GCGGCTGTCTCCACAAGT
hRPP30_probe_VIC			CTGACCTGAAGGCTCT

Supplemental References

1. Höijer I, Johansson J, Gudmundsson S, Chin CS, Bunikis I, Häggqvist S, Emmanouilidou A, Wilbe M, den Hoed M, Bondeson ML, et al. Amplification-free long-read sequencing reveals unforeseen CRISPR-Cas9 off-target activity. *Genome Biology*. 2020;21(1):1–19.