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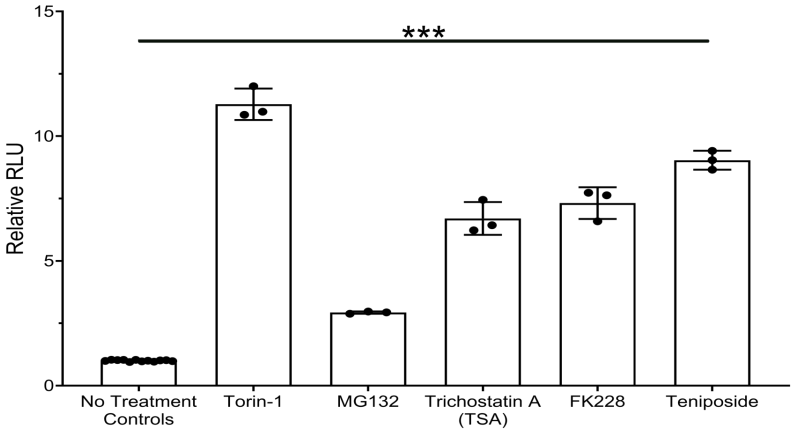
Supplementary information

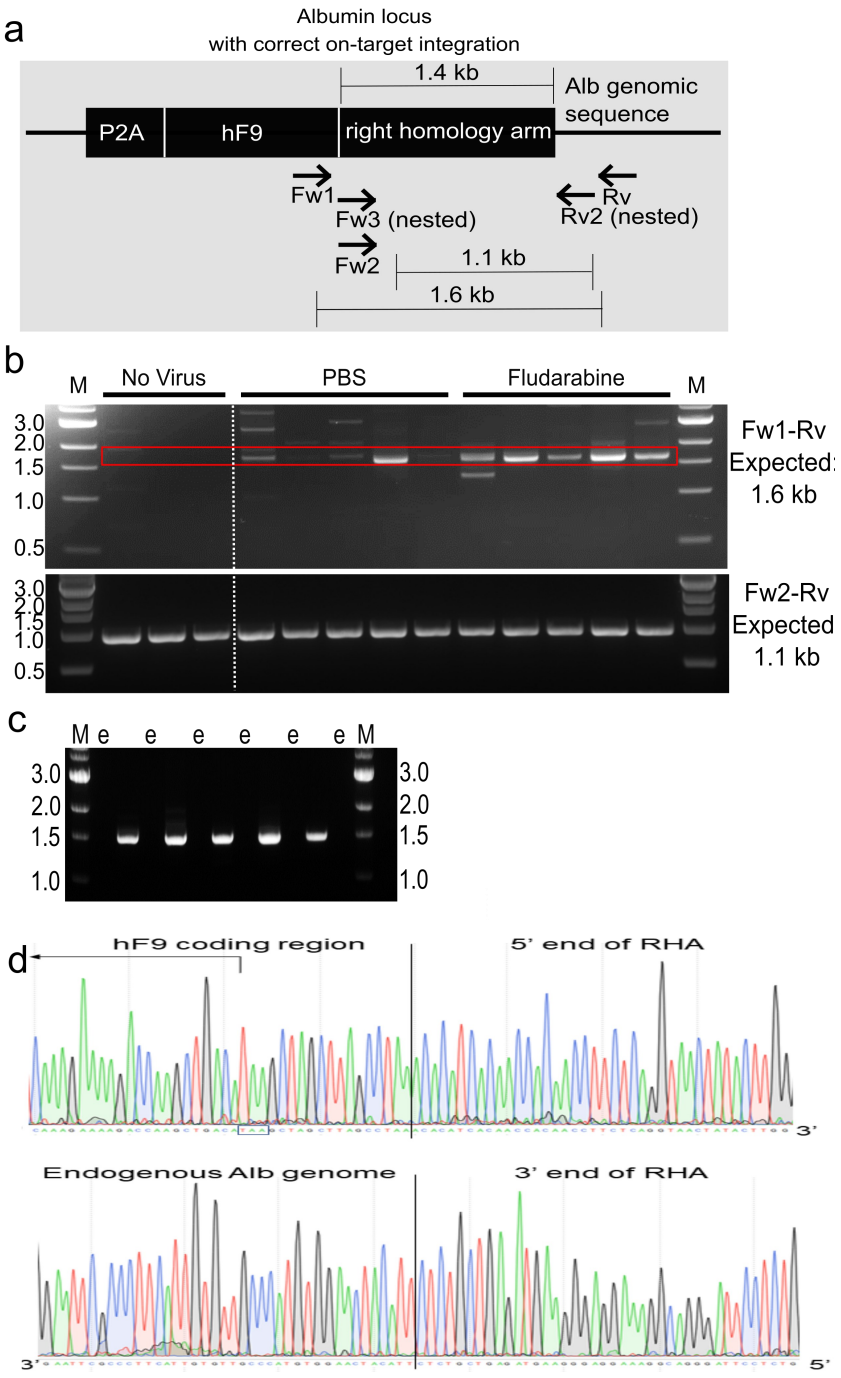
Fludarabine increases nuclease-free AAV- and CRISPR/Cas9-mediated homologous recombination in mice

In the format provided by the authors and unedited



Supplementary Figure 1| Various small molecule compounds enhanced rAAV transduction

The effect of various compounds on standard rAAV transduction was tested in Huh7 cells. Cells were treated with each compound at the indicated concentration and transduced with an AAVDJ vector expressing Fluc from the CAG promoter. Data is compared to relative luciferase activity of control (DMSO-treated) cells. Data is representative of two independent experiments, each with three biological replicates. Data is displayed as the group mean with error bars representing s.d.; n=3 replicate wells. Significance testing was performed by a one-way ANOVA with Dunnett's multiple comparison test. A single asterisk (*) represents a p-value of <0.05, ** is <.01, *** is <.001, and **** is <.0001; ns is not significant.



Supplementary Figure 2| Genomic DNA analysis to validate correct integration at the albumin locus in the liver of mice.

a, Schematic of the gene targeting Alb-P2A-hF9 vector integrated at the albumin genomic region. Also shown are positions of PCR primers (black arrows) used for qualitative analysis on on-target genomic integration. Primers Fw1 (binding in the hF9 transgene) and Rv (binding in the *Alb* genomic locus) are used for initial amplification of integration junctions.

b, Junction capture PCR samples were run on agarose gels to detect genomic integration in the right homology arm region (above). PCR amplification of endogenous albumin locus was used as a control (below) (primers Fw2 and Rv). This data is from one independent experiment.

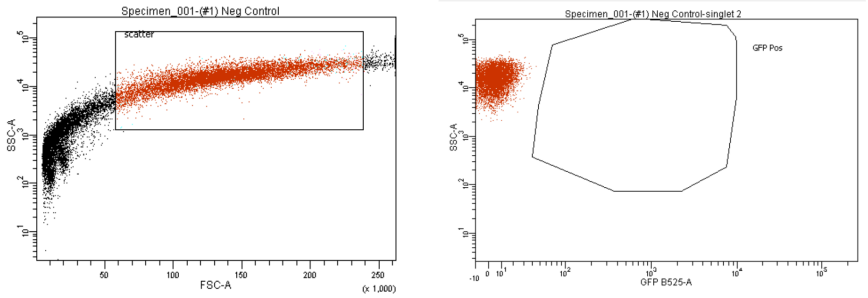
c, Agarose gel image of nested PCR reactions from each fludarabine-treated mouse is shown. Each well represents amplification from an individual mouse, while 'e' represents an empty well. This data is from one independent experiment.

d, Nested PCR amplicons, using primers Fw3 and Rv2 (expected product size of 1.5kb), from fludarabine-treated mice samples were Sanger sequenced. DNA was extracted from the gel and cloned into a plasmid for sequencing analysis using TOPO cloning. Sanger sequencing confirmed on-target genomic integration of the P2A-hF9 cassette without indels or mutations.

a

Tube: (#1) Neg Control

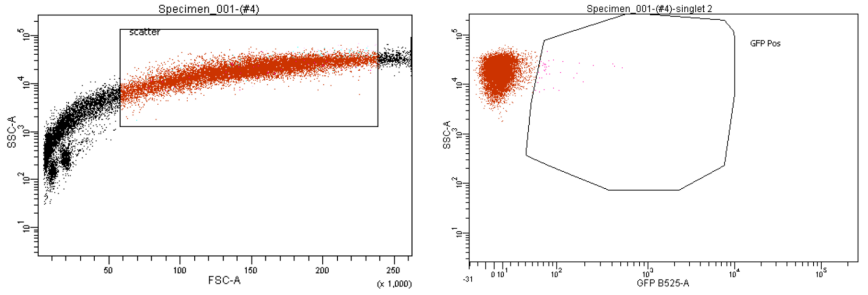
Population	#Events	%Parent	%Total
All Events	20,000	####	100.0
scatter	10,135	50.7	50.7
singlet 1	9,956	98.2	49.8
singlet 2	9,792	98.4	49.0
GFP Pos	0	0.0	0.0



b

Tube: (#4)

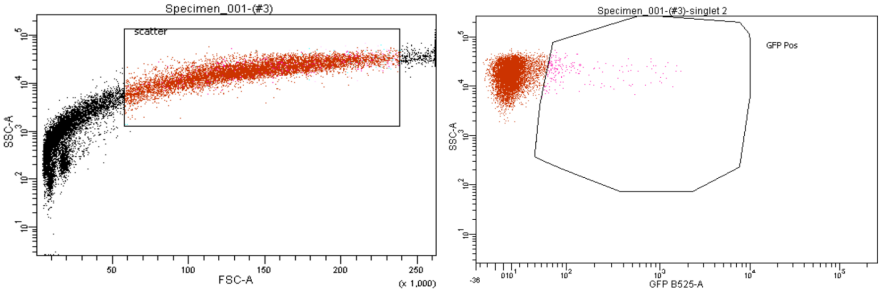
Population	#Events	%Parent	%Total
All Events	20,000	####	100.0
scatter	12,780	63.9	63.9
singlet 1	12,502	97.8	62.5
singlet 2	12,211	97.7	61.1
GFP Pos	37	0.3	0.2



c

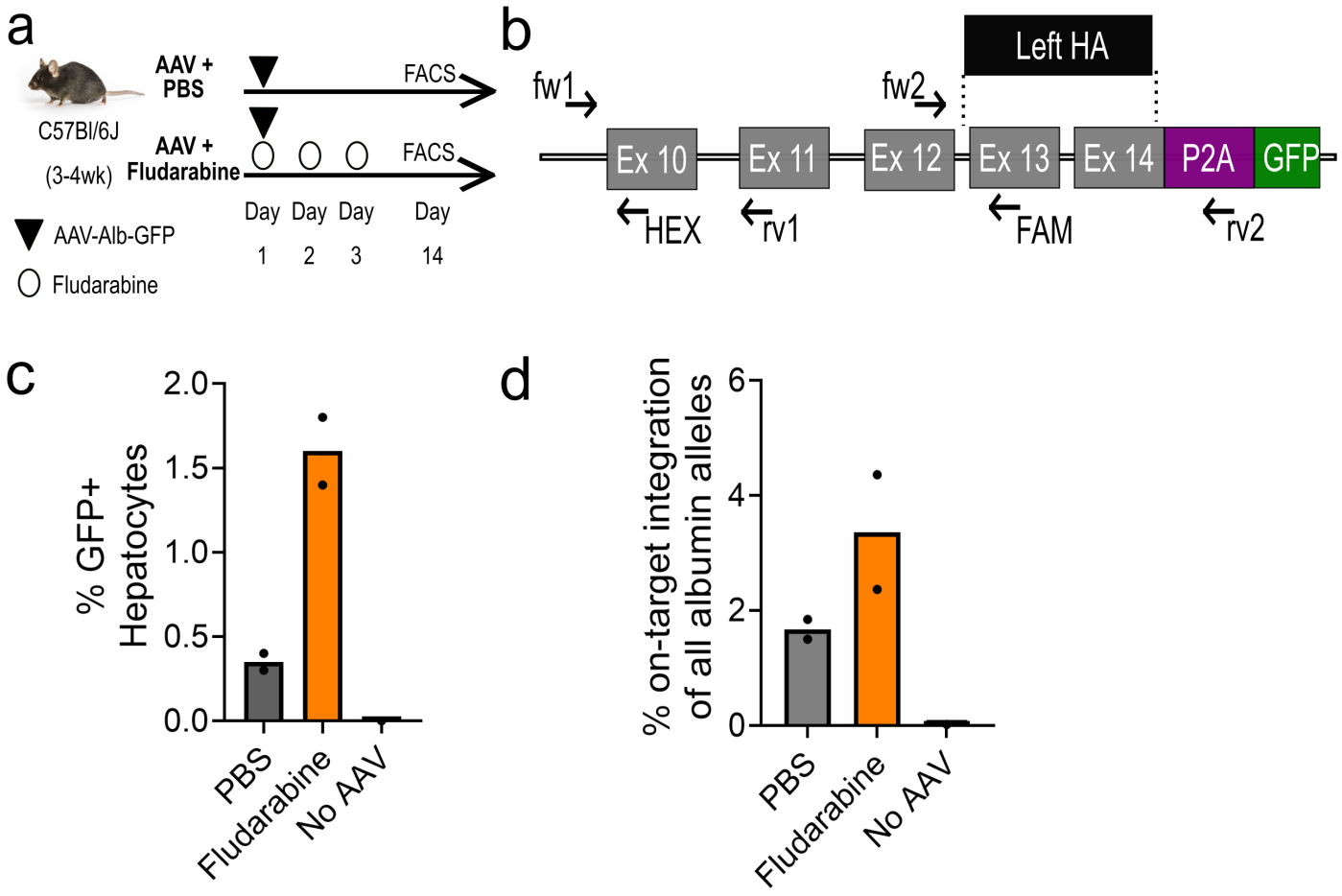
Tube: (#3)

Population	#Events	%Parent	%Total
All Events	20,000	####	100.0
scatter	9,449	47.2	47.2
singlet 1	9,193	97.3	46.0
singlet 2	9,065	98.6	45.3
GFP Pos	194	2.1	1.0



Supplementary Figure 3| FACS sorting of GFP+ hepatocytes

4-week-old male mice were injected with rAAVDJ-Alb-P2A-GFP ($2x10^{12}$ vg/mouse) and PBS or Fludarabine, as described previously (n=2 per group). Two-weeks later, hepatocytes were isolated by liver perfusion and dissociation, then FACS sorted for GFP positivity. Example of gating is shown for **(a)** a negative control non-injected mouse, **(b)** a mouse injected with PBS and rAAVDJ-Alb-P2A-GFP, **(c)** a mouse injected with Fludarabine and rAAVDJ-Alb-P2A-GFP.



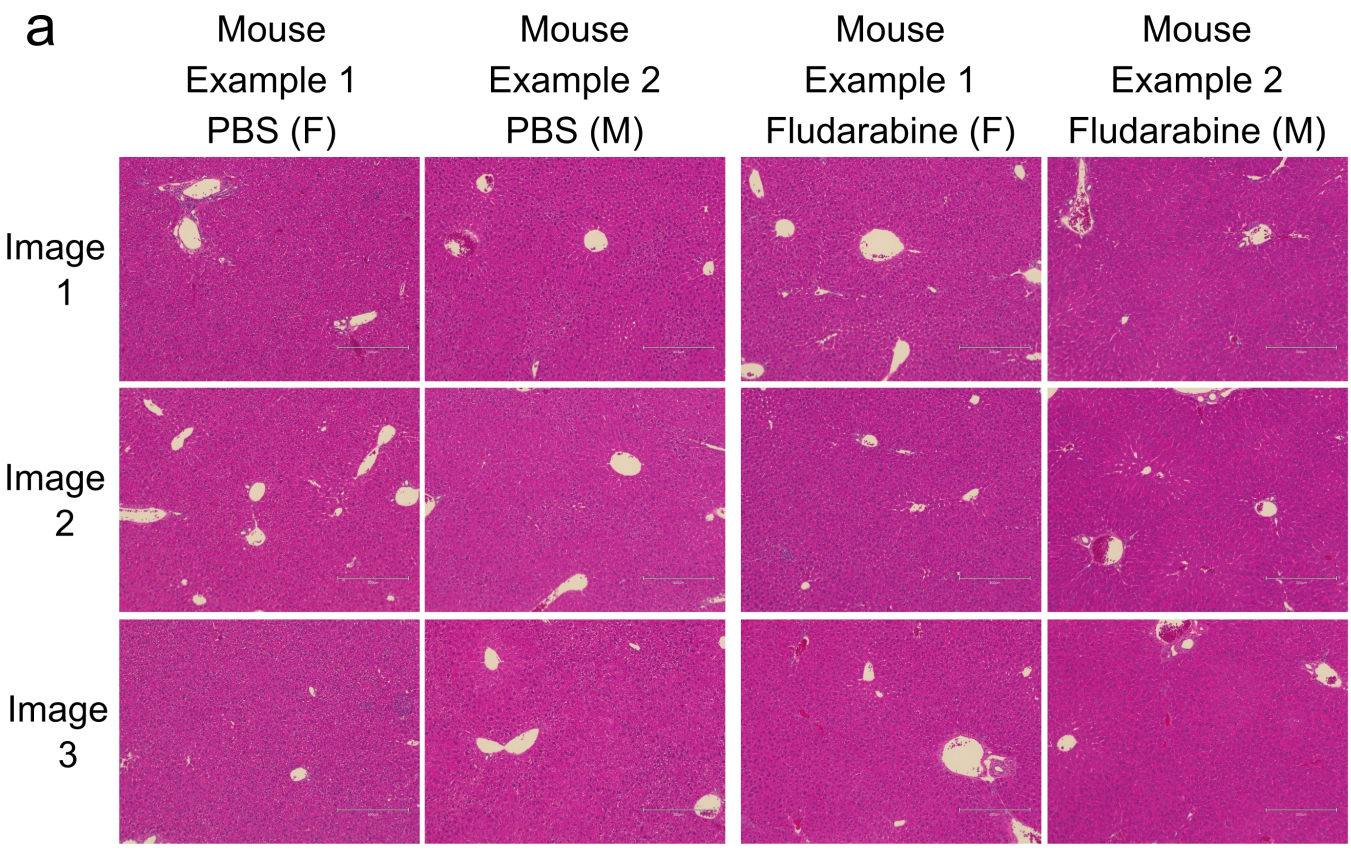
Supplementary Figure 4| ddPCR analysis of on-target homologous recombination in GFP+ FACS sorted hepatocyte gDNA

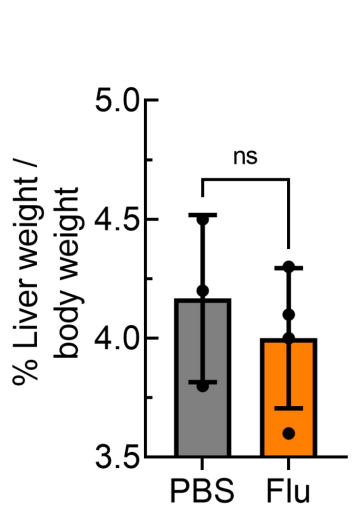
a, 4-week-old male mice were injected with rAAVDJ-Alb-P2A-GFP (2x1012 vg/mouse) and PBS or fludarabine (375 mg/kg/day) for three days (n=2 per group). Two-weeks later, hepatocytes were isolated by liver perfusion and dissociation, then FACS sorted for GFP positivity. FACS sorting was conducted using 'yield' mode, as opposed to 'purity' mode, which could result in a sorted population that is enriched but not purely GFP+.

b, gDNA was extracted from GFP+ hepatocytes and used for ddPCR reactions which amplified an untargeted region of mouse Alb (primers fw1 and rv1) (HEX probe) to quantify total Alb copies. Simultaneously, Alb alleles containing on-target integration of P2A-GFP were quantified using primers fw2 and rv2 (FAM probe).

c, The total number of GFP+ hepatocytes from the gated population during FACS sorting is shown. Bars are group average and each point represents average data from one animal.

d, The percentage of Alb alleles containing on-target integration of P2A-GFP out of total Alb copies is shown. Note the ploidy status of these hepatocytes was not determined and may range from diploid to octoploid or even more, meaning a single hepatocyte can contain many copies of Alb. Bars are group average and each point represents average data from one animal.

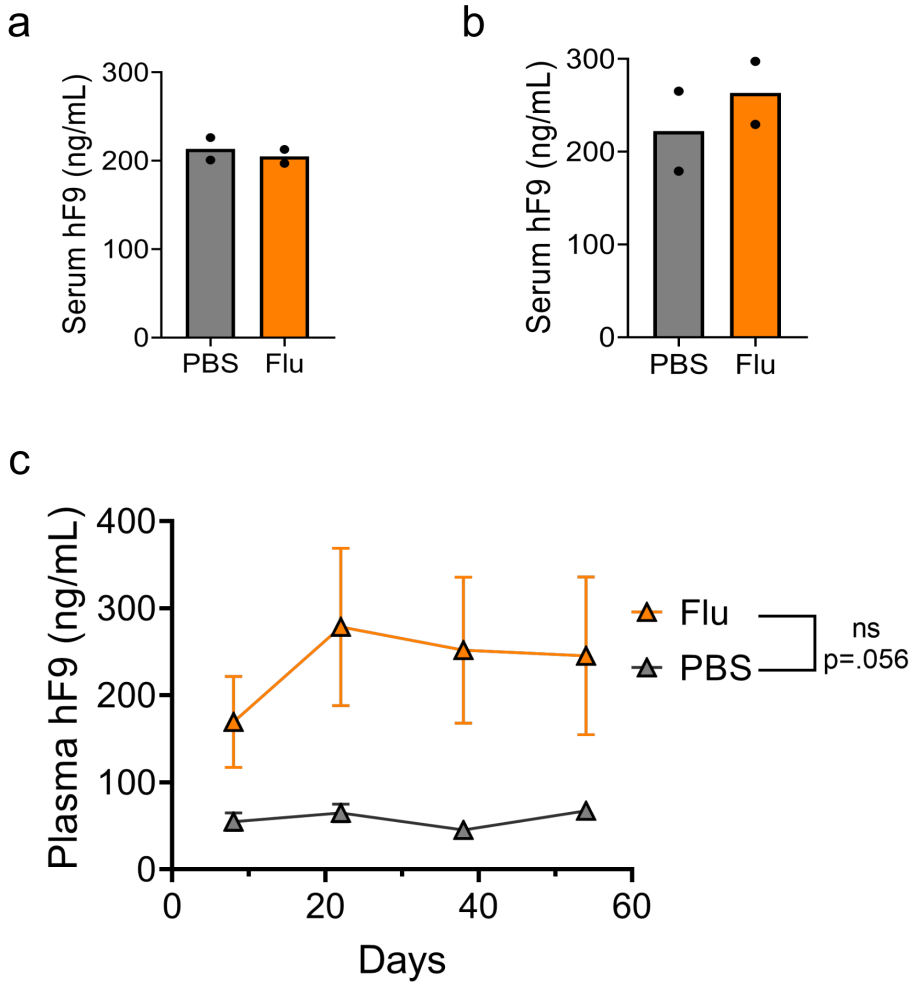




Supplementary Figure 5| Fludarabine treatment in neonatal mice does not lead to tumorigenesis or overt toxicity to the liver.

a, Neonatal mice were injected with i.p. with PBS (n=3) or Fludarabine (375 mg/kg) (n=4) and four hours later with rAAV8-Alb-P2A-hF9 ($2.5x10^{13}$ vg/kg) at one week of age. Fludarabine was administered once more, one day after vector injection. 157 days later, animal livers were collected, fixed in 10% formalin, and sectioned for H&E staining. H&E slides were examined in a blinded manner by a pathologist for analysis of liver condition. Three representative H&E images are provided for two example mice, one female (F) and one male (M), per group. Scale bar is 300 μm.

b, At the time of collection, livers were weighed and are given as the percentage of total body weight. Data is displayed as the group mean with error bars representing s.d. Significance was determined using a two-tailed Student's t-test.

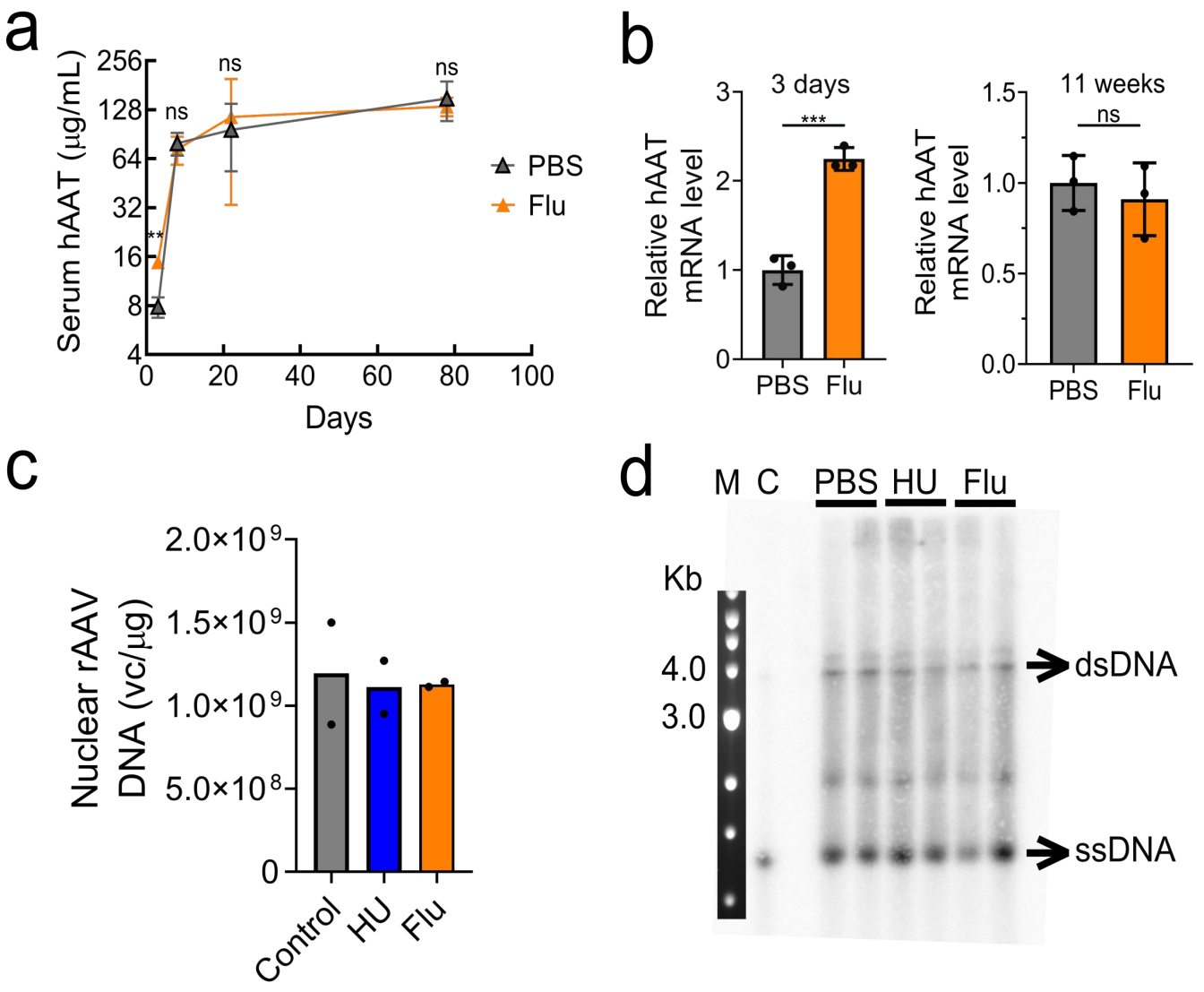


Supplementary Figure 6| Delayed dosing of fludarabine failed to increase the efficiency of gene targeting; administration increased the efficiency of gene targeting at the *ApoE* locus *in vivo*

a, Mice were injected i.v. with rAAV8 Alb-P2A-hF9 targeting vector (1.0E11 vg/mouse). Plasma samples were collected at Day 22 and analyzed for hF9 levels via ELISA. Animals were then grouped for equal expression between a control and treatment group. n=2 per group.

b, Next, PBS (control) or fludarabine (125 mg/kg) was administered i.p. three times per day for three days (days 28-30 post vector-injection). hF9 protein levels were then determined 24 days later

c, 4-week-old mice were treated with Flu (375 mg/kg/day) for three days and injected with 1.0E11 vg of a gene targeting vector, rAAV8-ApoE-P2A-hF9, targeting the murine ApoE locus. Serum was collected at various times across a nearly 60-day time course and hF9 protein levels were determined. Data is displayed as the group mean with error bars representing s.e.m.; n=4 mice per group. Significance was determined using a two-way ANOVA testing.



Supplementary Figure 7| The effect of fludarabine administration on promoter-driven rAAV transduction and the processing of rAAV genomes from ssDNA to dsDNA in cell culture

Flu (125 mg/kg) was administered i.p three times per day for three days in 4-week-old mice. The mice were also injected i.v. with a rAAV8 vector expressing hAAT from the HLP promoter (3.0E10 vg/mouse) on the first day of drug treatment. Blood was collected at indicated time points and liver tissues were extracted at the end of the time course.

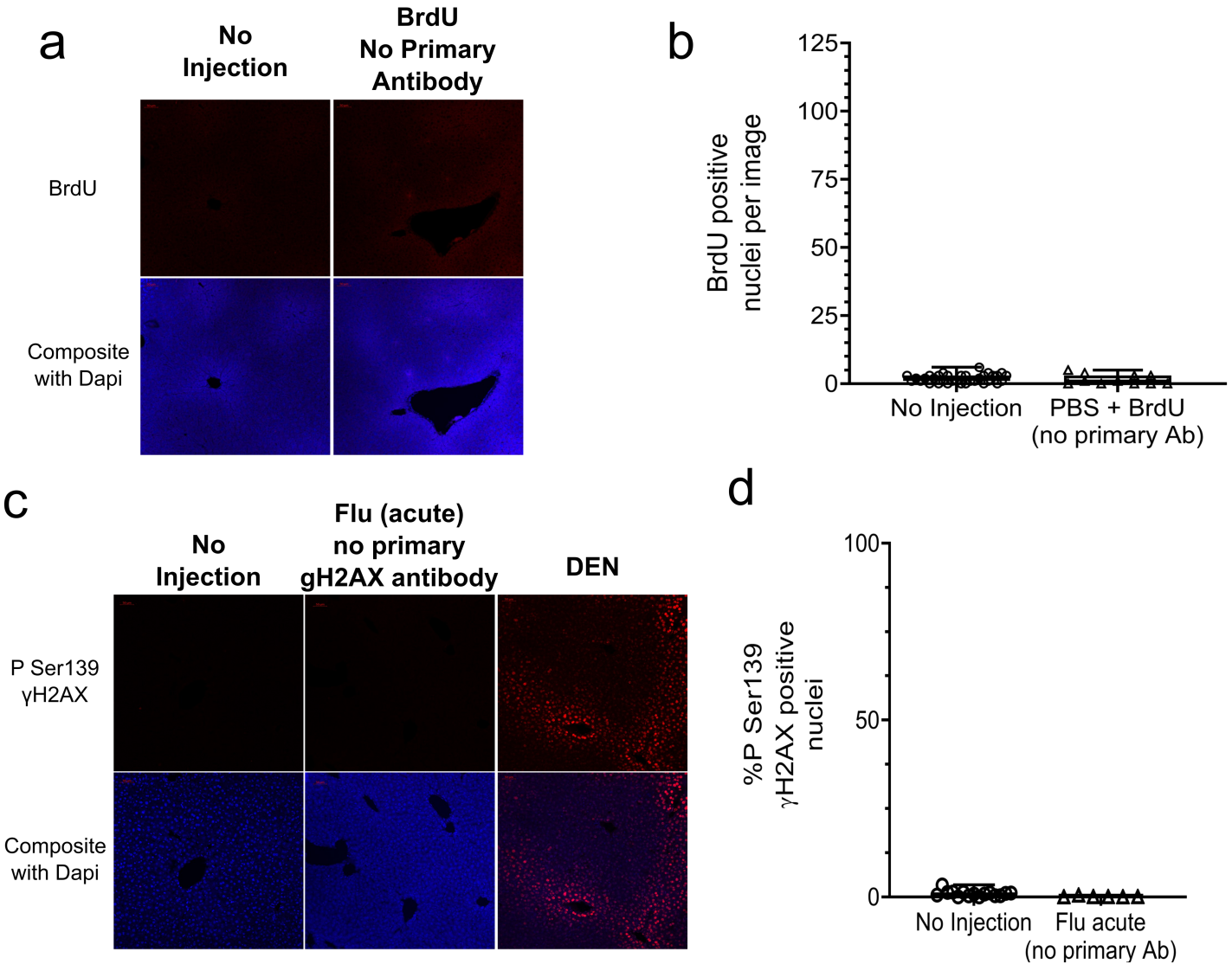
a, Serum hAAT protein levels were determined via ELISA for an 80-day time course. Data is displayed as the group mean with error bars representing s.d.; n=3 mice per group. Significance was determined using one-way ANOVA with Sidak's multiple comparisons test.

b, Total RNA was extracted from liver tissues at three days and 11 weeks, then qPCR was performed to quantify hAAT mRNA expression. Actb mRNA was used for normalization and data shown as relative expression to the PBS-treated group. Data is displayed as the group mean with error bars representing s.d.; n=3 mice per group. Significance was determined using an unpaired two-tailed t test. P-value for the Day 3 mRNA level was .0005 and .568 at Week 11.

For c-d, Huh7 cells were treated with control DMSO or RNR inhibitors, hydroxyurea (HU) (5mM) or fludarabine (Flu) (100μM), for 16 hours and transduced with an AAVDJ CAG-promoter expressing Fluc vector after a media change to washout drugs. 48 hours after transduction, nuclear fractions were isolated and genomic DNA was purified

c, The copy number of rAAV genomes in the nuclear fraction was measured by qPCR. Data shown is with 2 individual replicates along with the average.

d, Southern blot analysis using luciferase probes was performed using the same amount of genomic DNA to distinguish the double- or single-stranded forms of rAAV genomes. Non-denaturing agarose gel (1%) electrophoresis was used to separate single- or double-stranded form of rAAV genomes. DNA ladder (dsDNA) and purified single-stranded AAV genomes from rAAV preps were used as size controls.



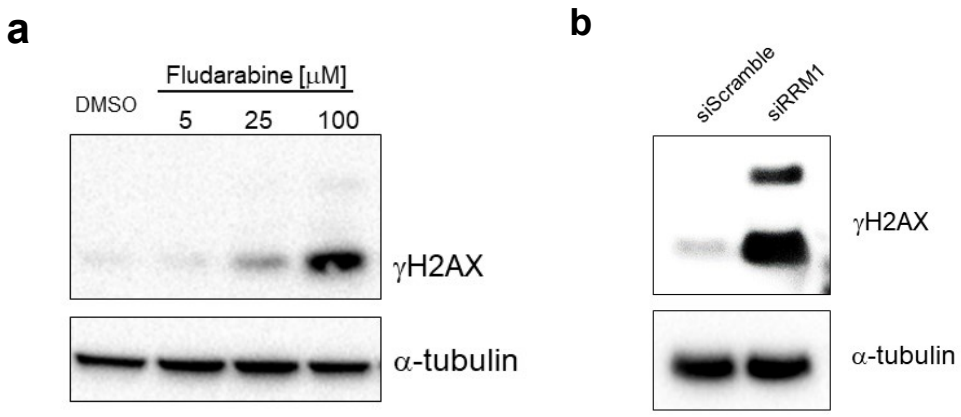
Supplementary Figure 8|- Positive and negative control staining of BrdU or γH2AX in mouse liver (see Figures 3 and 4)

a, Representative 20x confocal images from IHC staining of BrdU in control no injection mouse liver is provided. Also shown is staining of mouse liver from a BrdU and Flu treated animal, stained without the primary anti-BrdU antibody.

b, Images from control stained livers, taken at identical settings to Figure 3b images, were quantified. Each dot represents the data from a single technical replicate image. n=1 animal for no injection and n=2 animals for no primary antibody control stains.

c, Representative 20x confocal images from IHC staining of γH2AX in control no injection or DEN-injected mouse livers are provided. Also shown is staining of mouse liver from a Flu treated acute-phase animal, stained without the primary anti-γH2AX antibody.

d, Images from control-stained livers, taken at identical settings to Figure 4a images, were quantified. Each dot represents the data from a single technical replicate image. n=1 animal for each control.



Supplementary Figure 9| Inhibition of RNR induced Ser139 phosphorylation of H2AX (γH2AX) *in vitro*

a, Huh7 cells were treated with fludarabine for 16 hours at the indicated concentrations and then total cell lysates were prepared, followed by western blotting analysis of γH2AX. α-tubulin was used as a loading control.

b, Huh7 cells were transfected with RRM1 siRNA and total cell lysates were prepared, followed by western blotting for γ H2AX and α-tubulin 2 days after transfection.

Table 1 **Drugs Reported to affect rAAV Transduction**

Drug reported to increase rAAV transduction	Mechanism	Reference(s)
Torin-1	mTOR inhibitor	Hösel, M. et al.
MG132	proteasome inhibitor	(1) Schreiber, C. A. et al. (2) Johnson, J. S. & Samulski, R. J.
Trichostatin A	HDAC inhibitor	Kia, A., Yata, T., Hajji, N. & Hajitou, A.
FK228	HDAC inhibitor	Okada, T. et al.
Teniposide	topoisomerase inhibitor	Russell, D. W., Alexander, I. E., Dusty Miller, A. & Donnall Thomas, E.
Hydroxyurea	ribonucleotide reductase inhibitor	Marcus-Sekura, C. J. & Carter, B. J.

Table 2 **Animal Health Results**

12 hours post drug treatment

		Treatment					
Normal range		**Fludarabine**	**Fludarabine**	**Fludarabine**		**PBS**	**PBS**
5.5 - 9.3	**WBC**	1.25	1.16	0.76		3.65	6.32
7.0 - 8.8	**RBC**	8.44	8.74	9.32		9.22	8.77
13.7 - 16.4	**HGB**	12.9	13.4	13.9		14	13.8
39.0 - 47.0	**HCT**	44.4	46.8	48.2		48.9	47.6
52.0 - 68.7	**MCV**	52.6	53.5	51.7		53	51.3
18.4 - 19.6	**MCH**	15.3	15.3	14.9		15.2	15.7
34.0 - 36.0	**MCHC**	29.1	28.6	28.8		28.6	29
675 - 1338	**Platelet Count**	1766	1639	1606		1760	1676
	RDW	19.4	19.4	20.5		21.9	20.9
	PDW	7.3	7.2	7.6		7.6	7.4
	MPV	6.4	6.3	6.5		6.3	6.5
	P-LCR	4.4	3.6	4.6		3	4.8
	PCT count	1.13	1.03	1.04		1.11	1.09
1.0 - 2.8	**Reticulocyte count**	2.94	2.85	3.02		8.51	8.1
	IRF	41.4	37.4	40.5		66.1	62
	IFR	58.6	62.6	59.5		33.9	38
	MFR	16.5	17	18.7		19.3	19.2
	Ret. Absolute	248136	249090	281464		784622	710370
	Platelet Estimate	adequate	Adequate	Adequate		Adequate	Adequate
15 - 32	**Neutrophils**	1	3	4		8	1
65 - 83	**Lymphocytes**	95	96	89		91	96
0 - 3	**Monocytes**	2	1	6		1	2
0 - 3	**Eosinophils**	2	0	1		0	1
	Basophils	0	0	1		0	0
	RBC morphology	NORMAL, OCCASIONAL HOWELL JOLLY BOD	Normal, Occasional Howell Joll	Normal Occasional Howell Jolly Bodies		Normal +1 Polychromasia, Occasional Howe	Normal, Occasional Howell J
825 - 2604	**Neutrophils Absolute**	13	35	30		292	63
3685 - 7812	**Lymphocytes Absolute**	1188	1114	676		3322	6067
0 - 279	**Monocytes Absolute**	25	12	46		37	126
0 - 279	**Eosinophils Absolute**	25	0	8		0	63
	Basophils Absolute	0	0	8		0	0
76 -160	**ALT**	126	90	42		41	43

Table 2 **Animal Health Results**

28 days post drug treatment

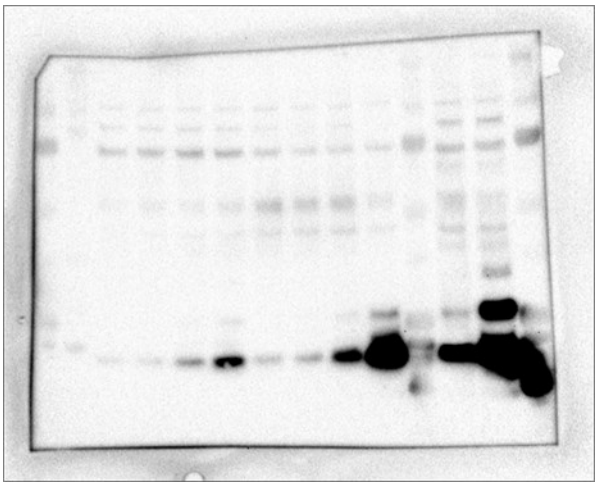
		Treatment					
Normal range		**Fludarabine**	**Fludarabine**	**Fludarabine**		**PBS**	**PBS**
5.5 - 9.3	**WBC**	3.77	6.35	5.23		4.23	3.22
7.0 - 8.8	**RBC**	10.18	10.39	9.95		9.59	10.4
13.7 - 16.4	**HGB**	15.1	15.6	15.1		14.7	15.2
39.0 - 47.0	**HCT**	50.9	51.3	50.1		49	50.4
52.0 - 68.7	**MCV**	50	49.4	50.4		51.1	48.5
18.4 - 19.6	**MCH**	14.8	15	15.2		15.3	14.6
34.0 - 36.0	**MCHC**	29.7	30.4	30.1		30	30.2
675 - 1338	**Platelet Count**	1892	1939	1889		1492	1660
	RDW	19.4	19.9	19.3		18.5	20.1
	PDW	7.8	7.6	7.9		7.4	7.5
	MPV	6.5	6.4	6.6		6.5	6.3
	P-LCR	4.1	3.9	4.6		4.1	3.5
	PCT count	1.23	1.24	1.26		0.97	1.05
1.0 - 2.8	**Reticulocyte count**	5.67	5.71	5.69		6.01	5.17
	IRF	62.8	62	59.5		64.1	62.2
	IFR	37.2	38	40.5		35.9	37.8
	MFR	16.9	17	15.5		19.3	18.2
	Ret. Absolute	577206	593269	566155		576359	537680
	Platelet Estimate	ADEQUATE	ADEQUATE	ADEQUATE		ADEQUATE	ADEQUATE
15 - 32	**Neutrophils**	3	4	4		14	15
65 - 83	**Lymphocytes**	89	89	88		86	77
0 - 3	**Monocytes**	7	6	7		8	7
0 - 3	**Eosinophils**	1	1	1		2	1
	Basophils	0	0	0		0	0
	RBC morphology	NORMAL	NORMAL	NORMAL		NORMAL	NORMAL
825 - 2604	**Neutrophils Absolute**	113	254	209		592	483
3685 - 7812	**Lymphocytes Absolute**	3355	5652	4602		3638	2479
0 - 279	**Monocytes Absolute**	264	381	366		338	225
0 - 279	**Eosinophils Absolute**	38	64	52		85	32
	Basophils Absolute	0	0	0		0	0
76 -160	**ALT**	28	25	37		80	102

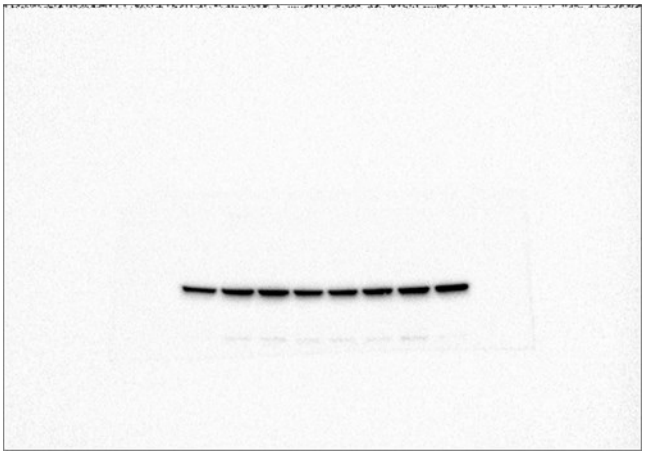
Table 3 **Primer and Oligo information**

	Sequence (5' to 3')
AAV titer for GAPDH-P2A-GFP, Albumin-P2A-GFP	CAAGCTGGAGTACAACTACAA
	GGATCTTGAAGTTCACCTTGA
AAV titer for Albumin-P2A-hF9, Apoe-P2A-hF9	AGCACCAAGTTCACCATCTA
	TTTCTGACCGGCATCATCAG
AAV titer for HLP-hAAT	GCCAGCTTACATTTACCCAAAC
	CCCATTGCTGAAGACCTTAGT
AAV titer for Cas9	aaggatcacccagcctctgc
	cctgctgaagacactcttgcca
mAlb-P2A fusion qPCR	CCTTCCACTCTGATATCTGCAC
	GAAATTGGTGGCGCCGCTTC
Total hF9 qPCR	ATCTACAACAACATGTTCTGCG
	CTGATGATGCCGGTCAGAAA
Total GFP qPCR	CAAGCTGGAGTACAACTACAA
	GGATCTTGAAGTTCACCTTGA
Endogenous mAlb qPCR	CCTTCCACTCTGATATCTGCAC
	CCATGACAGTCTTCAGTTGC
hAAT qPCR	GCCAGCTTACATTTACCCAAAC
	CCCATTGCTGAAGACCTTAGT
Cas9 qPCR	GTACGGCCTGCCCAATGATA
	TGCGTTCTCTTTCCCGGTAG
Fluc qPCR	CACATATCGAGGTGGACATTAC
	TGGTTTGTATTCAGCCCATAG
mAlb-P2A junction PCR	ATCTACAACAACATGTTCTGCG
	ACCACCCATAAGATGGGAGAGTA
mAlb-P2A junction nested PCR	GAGACTCTTGTCAGGGCGATTCTG
	CATTGTGTTGCCCATGTGGAAC
Endogenous mAlb ddPCR	ctgctgtgcaccagttgatgtt
	tctggtgctgaggacacgtagcccagt (HEX Probe)
	TGCTTTCTGGGTGTAGCGAACT
Integrated Alb-GFP ddPCR	gggcaaggcaacgtcatgg
	tggcagtggcatgcttaatcctca (FAM Probe)
	CCAGGGTTCTCTTCCACGTC
Endogenous mActβ qPCR	GTGACGTTGACATCCGTAAAGA
	GCCGGACTCATCGTACTCC

Source Data Files

Supplementary Figure 9a





Source Data Files

Supplementary Figure 9b