Cannabinoid receptor-1 signaling in hepatocytes and stellate cells does not contribute to NAFLD

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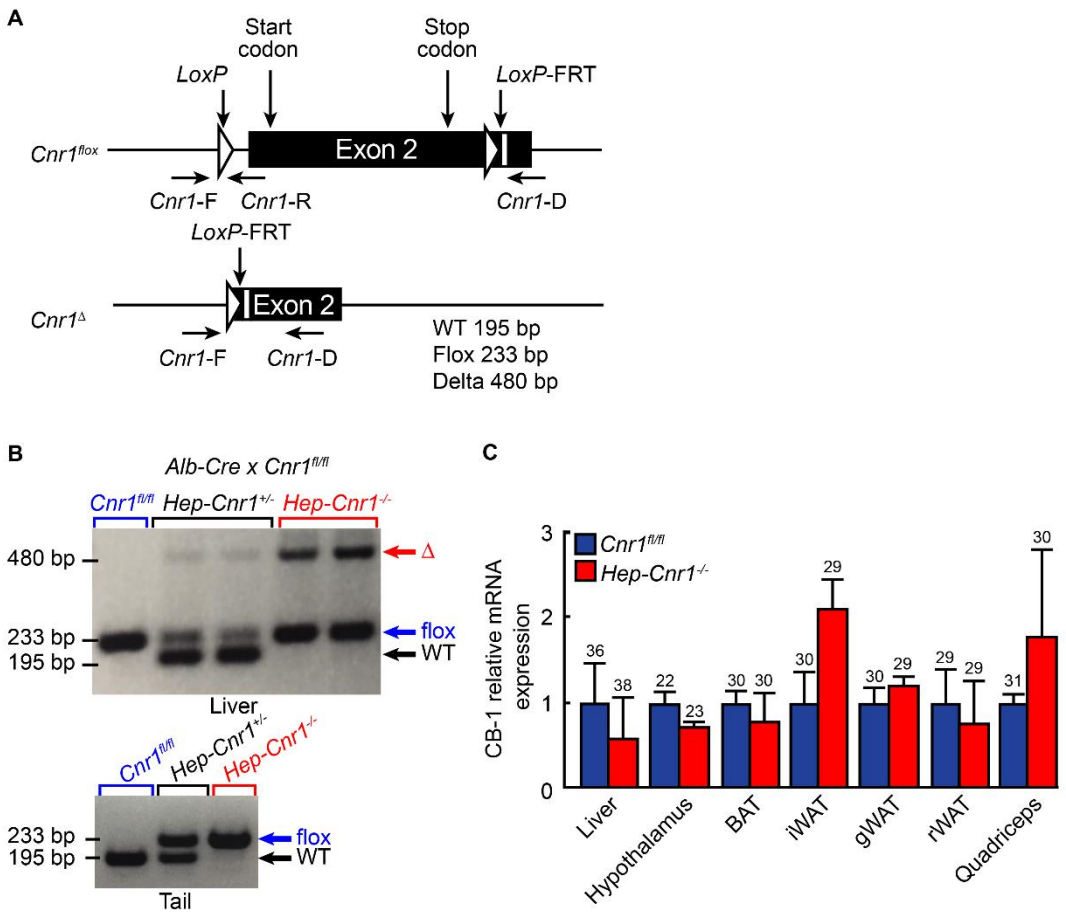
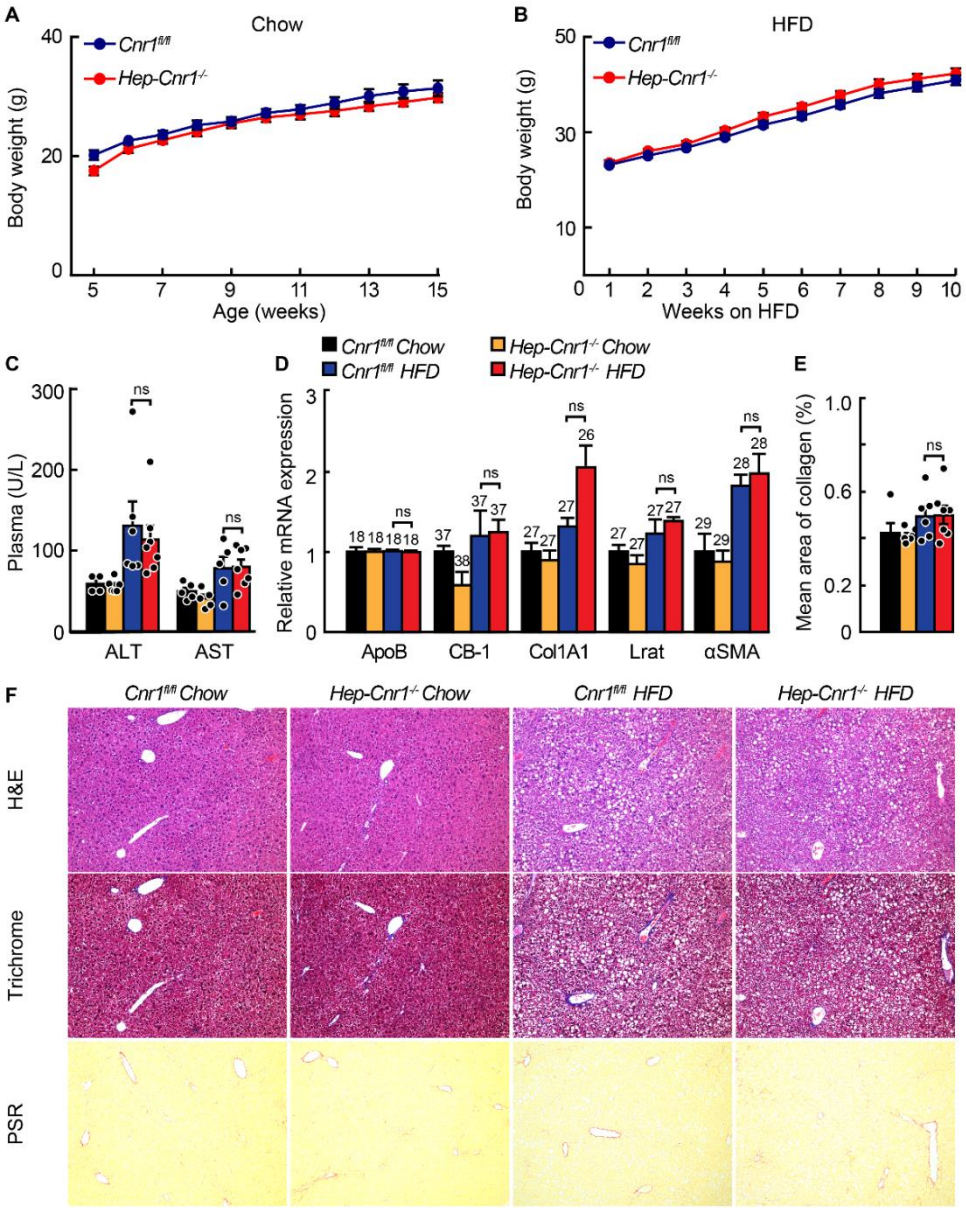
Conflict of interest: Jay D. Horton is on the SAB for Pfizer. Joel K. Elmquist and Guosheng Liang conduct sponsored research with Pfizer.

Supplemental figures and figure legends



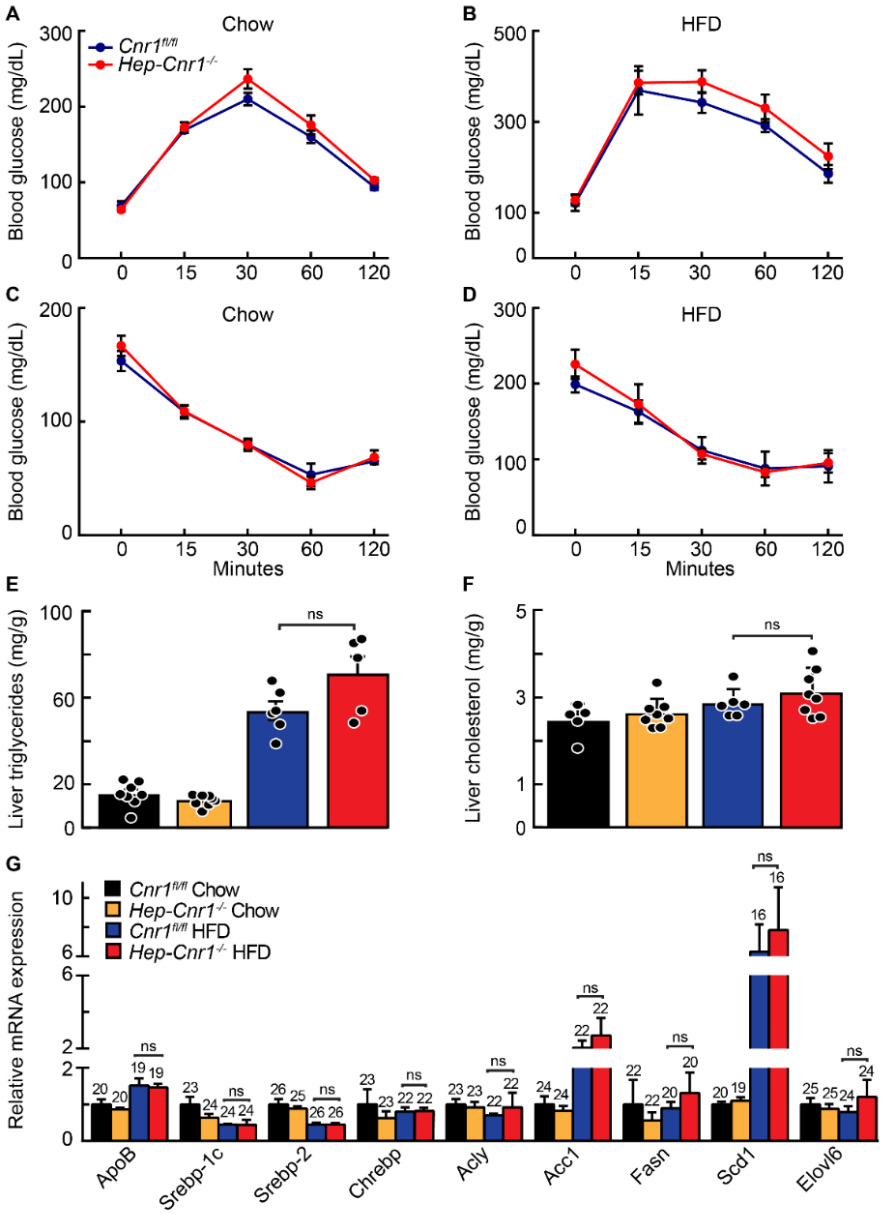
Supplemental Figure 1. **Generation and verification of hepatocyte-specific *Cnr1* knockout mice.** (**A)** Gene-targeting strategy for *Cnr1*$^{fl/fl}$ mice. Two CRISPR-Cas9 sgRNAs were used to target the upstream and downstream of exon 2 of the *Cnr1* allele, flanking the coding sequence. Three sets of primers (WT, flox, and delta were designed for PCR detection of homologous recombination as described in Materials and Methods. (**B**) Validation of hepatocyte-specific *Cnr1* deletion via breeding *Cnr1*$^{fl/fl}$ mice with *Alb-Cre* mice. Offspring (*Hep-Cnr1*$^{-/-}$) resulted in hepatic *Cnr1* deletion with the delta band and homologous recombination with flox band as identified by PCR with liver-derived and tail-derived DNA (Lower). (**C**) Chow-fed *Cnr1*$^{fl/fl}$ and *Hep-Cnr1*$^{-/-}$

mice (n = 5-8/group) were euthanized at 22 weeks of age. Hypothalamus, brown adipose tissue (BAT), iWAT, gonadal white adipose tissue (gWAT) and quadriceps were collected for RNA extraction. CB-1 mRNA expression levels were quantified by real-time PCR. β-actin was used as an invariant control. The values were expressed relative to that of chow fed *Cnr1*$^{fl/fl}$ mice, which was arbitrarily set to 1. Corresponding mean CT values are denoted above. Results shown as mean ± SEM. *$p < 0.05$, **$p < 0.01$ between genotypes, assessed by ANOVA.



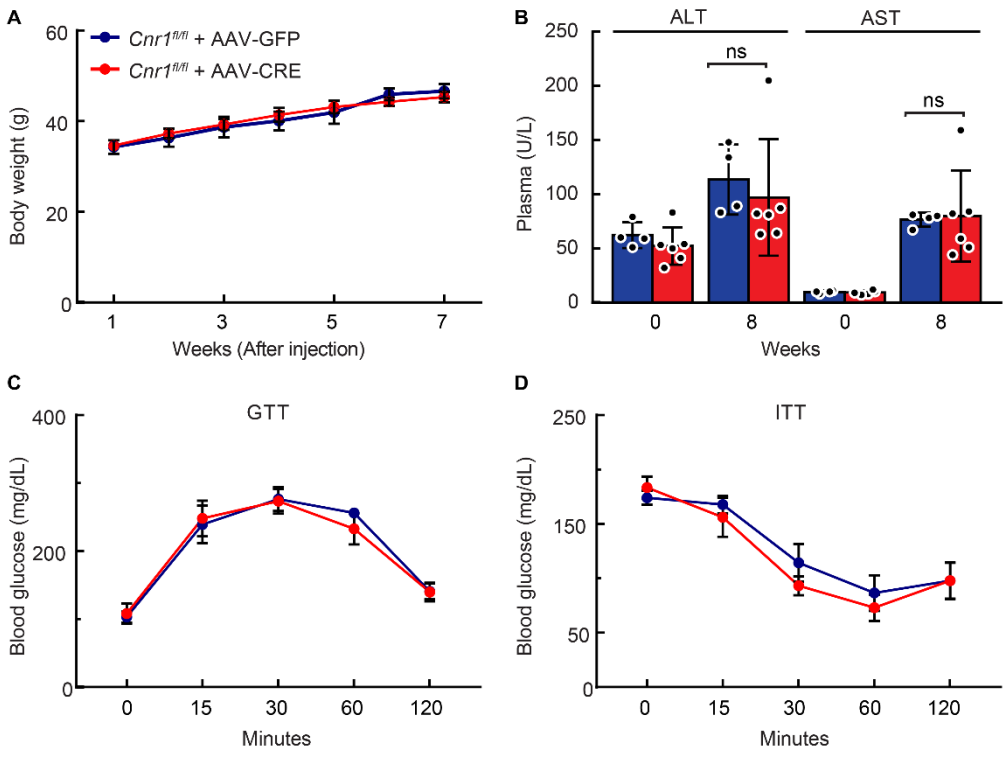
Supplemental Figure 2. *Cnr1* deletion in hepatocytes does not affect body weight, liver function or diet-induced obesity in 16-week old mice fed chow or the HFD. Growth curves of chow-fed (**A**) and HFD-fed (**B**) *Cnr1*$^{fl/fl}$ and *Hep-Cnr1*$^{-/-}$ mice (n = 5-8/group). Body weights were monitored weekly starting at 5 weeks of age. (**C**) Plasma ALT and AST levels. (**D**) Relative mRNA levels of ApoB, CB-1, Col1A1, Lrat and αSMA quantified by qPCR. ApoB was used as an invariant control. Values are expressed relative to chow fed *Cnr1*$^{fl/fl}$ mice, which was arbitrarily

set to 1. Corresponding mean CT values are denoted above. (**E**) Mean area of collagen was obtained by calculating the PSR-stained red area in the image under split green channel, as described in Methods. Results shown as mean ± SEM, assessed by ANOVA. (**F**) H&E, trichrome and PSR staining of liver sections. Scale bar = 100 µm. All experiments (**A**-**F**) were repeated with a separate cohort of mice and with similar results.

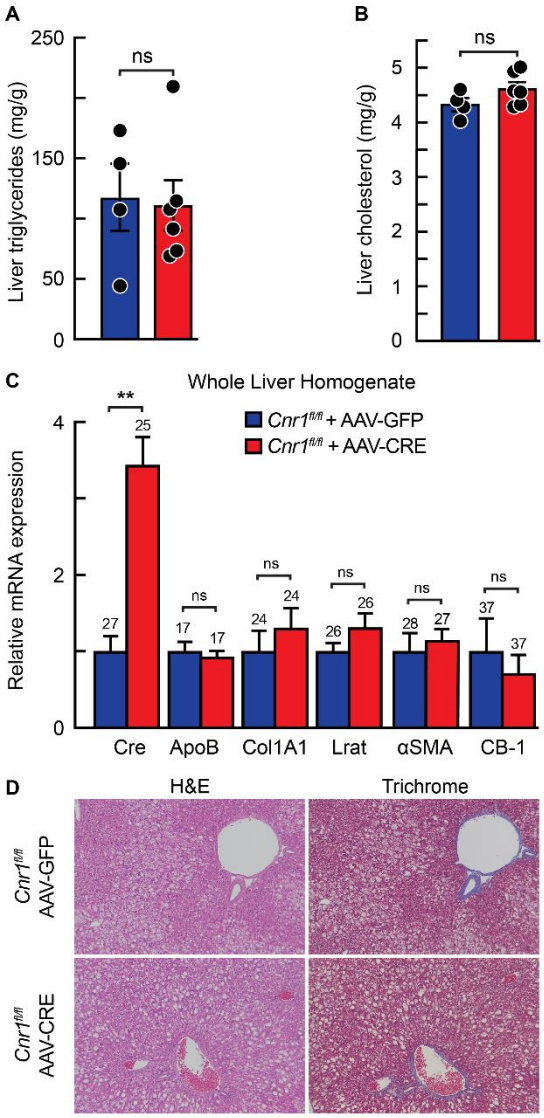


Supplemental Figure 3. Hepatocyte-specific *Cnr1* deletion does not affect glucose tolerance, insulin sensitivity or liver steatosis in mice fed chow or the HFD. Mice used are those described in **Supplemental Figure 2**. Glucose and insulin tolerance tests were carried out 2 and 3 weeks prior to euthanasia, respectively. (**A** and **B**) Blood glucose levels were measured at indicated times after glucose injection. (**C** and **D)** Blood glucose levels were measured at indicated times after insulin injection. (**E** and **F**) Liver TG and cholesterol levels. (**G**) Total RNA was extracted from each mouse liver, and the relative mRNA expression levels of ApoB, Srebp-1c, Srebp-2, Chrebp,

Acly, Acc1, Fasn, Scd1, and Elovl6 were quantified by real-time PCR. β-actin was used as an invariant control. The values were expressed relative to that of chow fed *Cnr1*$^{fl/fl}$ mice, which was arbitrarily set to 1. Corresponding mean CT values are denoted above. Acly, ATP-citrate lyase; Chrebp, carbohydrate response element binding protein; Elovl6, elongation of long chain fatty acids family member 6; Fasn, fatty acid synthase; Scd1, stearoyl CoA desaturase 1. All experiments (**A**-**G**) were repeated in a separate cohort of mice with similar results.

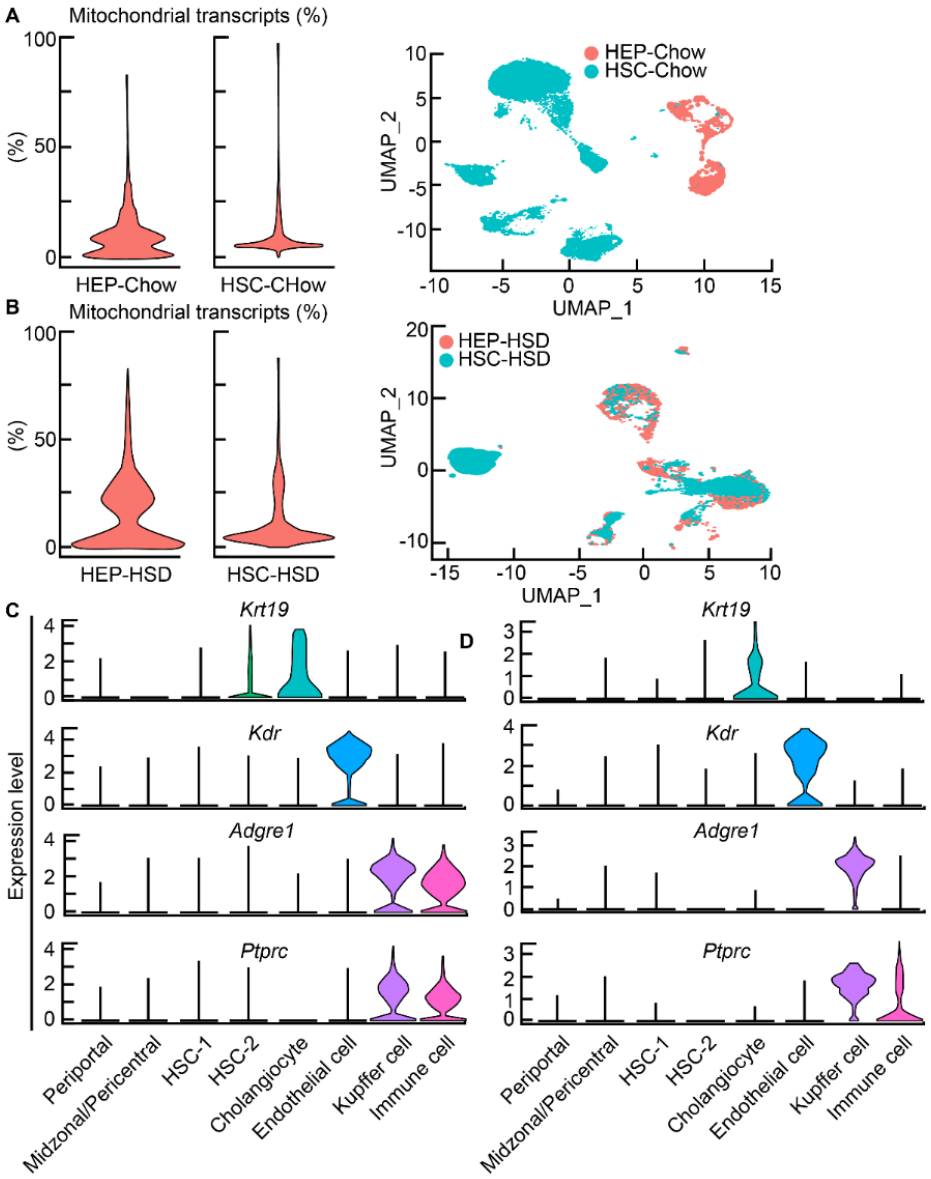


Supplemental Figure 4. *Cnr1* deletion in hepatocytes does not alter body weights, liver function tests, or measures of insulin sensitivity in mice fed the HFD. *Cnr1*$^{fl/fl}$ mice were fed a HFD for 8 weeks and then injected with control AAV-GFP or AAV-Cre (n=4-6 per group). Mice were continued on HFD for another 8 weeks. (**A**) Body weights were monitored weekly following AAV injection. (**B**) Plasma ALT and AST levels were measured before and 8 weeks after AAV injection. The glucose and insulin tolerance tests were carried out 6 and 7 weeks after AAV injection, respectively. (**C**) Blood glucose levels were measured at indicated times after glucose injection. (**D**) Blood glucose levels were measured in mice at indicated times after insulin injection.

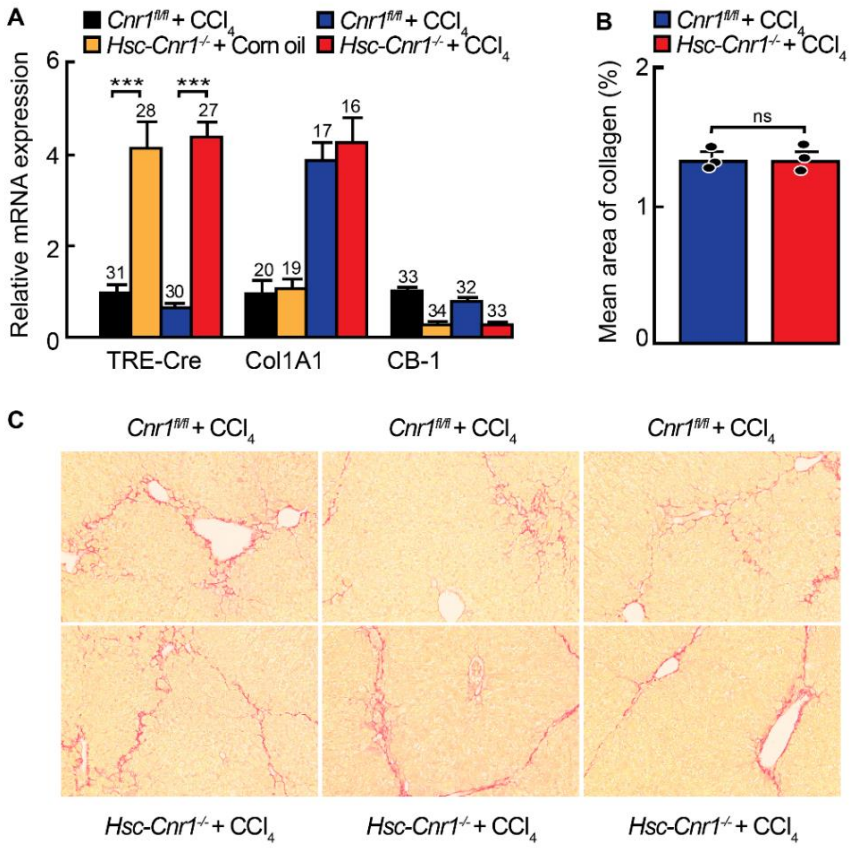


Supplemental Figure 5. *Cnr1* deletion in hepatocytes does not reverse liver steatosis in mice fed the HFD. The mice used here are the same as those described in **Supplemental Figure 4**. *Cnr1*$^{fl/fl}$ mice were fed a HFD for 8 weeks and then injected with control AAV-GFP or AAV-CRE (n=4-6 per group). The mice were continued on HFD for another 8 weeks and sacrificed for analysis. (**A**) Liver TG levels. (**B**) Liver cholesterol levels. (**C**) Real time qPCR analysis. Total RNA was extracted from each mouse liver, and the relative mRNA levels of Cre, ApoB, Col1A1, Lrat, αSMA and CB-1 were quantified by real-time PCR. β-actin was used as an invariant control. The values were expressed relative to that in *Cnr1*$^{fl/fl}$ mice injected with AAV-GFP (Control)

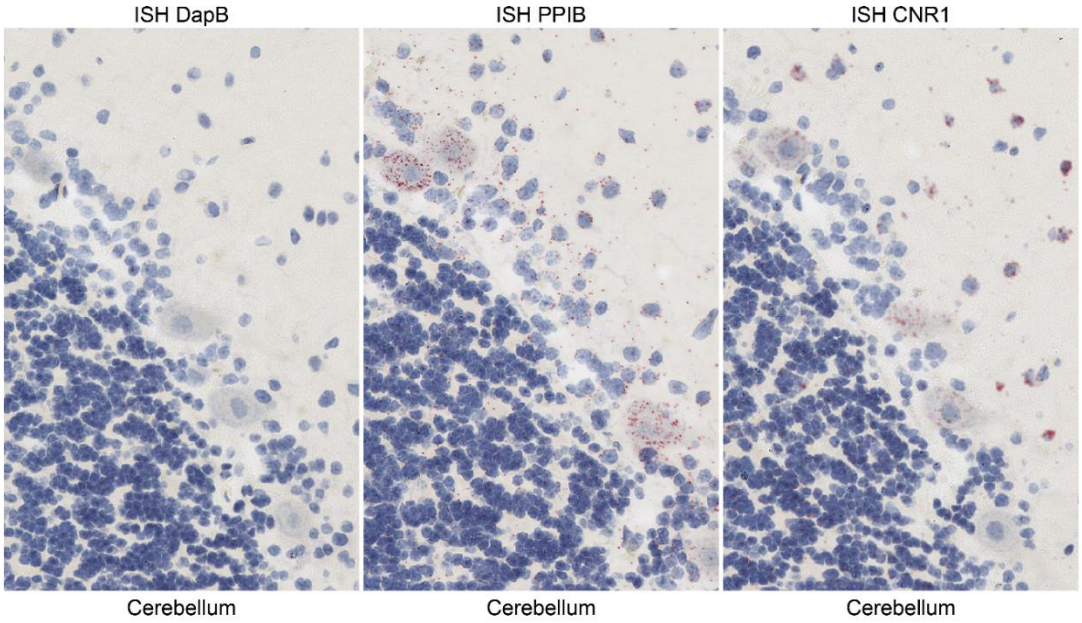
, which was arbitrarily set to 1. Corresponding mean CT values are denoted above. (**D**) Mice were euthanized 8 weeks after AAV injection. Liver sections were processed for H&E and trichrome staining. Magnification of x20. Scale bar = 100 µm. The same experiment was repeated in a separate cohort of mice with similar results.



Supplemental Figure 6. Mitochondrial transcripts and cell cluster identification. Mitochondrial transcript proportion in scRNA-seq data integrated from (**A**) chow-fed wild-type mice or (**B**) wild-type mice maintained on HSD for 17 weeks. Violin plots of cell type-specific signature gene expression in (**C**) chow-fed wild-type mice and (**D**) wild-type mice maintained on HSD for 17 weeks: *Krt19* (cholangiocytes), *Kdr* (endothelial cells), *Adgre1* (Kupffer cells), and *Ptprc* (immune cells).



Supplemental Figure 7. Determination of collagen area using PSR staining. The mice used are those described in **Figure 5.** (**A**) Mice used are those described in **Figure 5E**. Total RNA was extracted from HSCs, and the relative mRNA levels of TRE-Cre, Col1A1, and CB-1 were quantified by real-time PCR. β-actin was used as an invariant control. Values were expressed relative to that of chow-fed doxycycline-treated *Cnr1*$^{fl/fl}$ mice injected with corn oil, which was arbitrarily set to 1. Corresponding mean CT values are denoted above. ***$p < 0.01$. (**B**) Mean area of collagen was obtained by calculating the PSR-stained red area in the image under split green channel, as described in Methods. Results shown as mean ± SEM, assessed by ANOVA. (**C**) PSR staining of liver sections from chow-fed doxycycline-inducible *Cnr1*$^{fl/fl}$ (n=3) and *Hsc-Cnr1*$^{-/-}$ (n=3) mice injected with CCl_4. Scale bar = 100 µm. All experiments (**A**-**C**) were repeated with a separate cohort of mice and the results were similar.



Supplemental Figure 8. CB-1 is highly enriched in human cerebellum. Species-specific positive and negative control probes for a quality control of the automated ISH assay. Human cerebellum is used as positive control tissue for the CNR1 gene. Left panel: Bacillus subtilis dihydrodipicolinate reductase (DapB) gene used as non-specific bacterial negative control probe. Middle panel: Peptidylpropyl isomerase B (PPIB) housekeeping gene as species-specific positive control probe. Right panel: CNR1 detection (red punctate dot in human Purkinje cells and neurons of both the molecular and granular cell layers of the cerebellum. Scale bar = 30 µm.

Tables

Supplementary Table 1. Human Liver Tissue Information

Donors	Gender	Age	Ethnicity	Pathological Diagnosis	Diagnostic Information
Control					
C1	Female	44	Caucasian	Normal	Fibrosis Stage 0, NASH=1
C2	Female	67	African American	Normal	Fibrosis Stage 0, NASH=1
C3	Male	43	Caucasian	Normal	Fibrosis Stage 1, NASH=0
NAFLD/NASH					
N1	Male	3	Caucasian	NAFLD with fibrosis	Fibrosis Stage 2, NASH=2
N2	Male	58	Caucasian	NASH with fibrosis	Fibrosis Stage 2, NASH=8
N3	Female	56	Caucasian	NASH with fibrosis	Fibrosis Stage 2, NASH=6
N4	Female	57	Caucasian	NAFLD with hepatitis and fibrosis	Fibrosis Stage 2, NASH=2
N5	Female	59	Caucasian	NAFLD with hepatitis and fibrosis	Fibrosis Stage 2, NASH=1
N6	Female	46	Caucasian	NAFLD with hepatitis and fibrosis	Fibrosis Stage 2, NASH=4
N7	Female	27	African American	NAFLD with hepatitis and fibrosis	Fibrosis Stage 2, NASH=3

Supplementary Table 2. Histological Assessment and Scoring of *In-situ* Hybridization

Staining Distribution (D)*	Score	Staining Intensity (I)#	Score
-	0	-	0
1-25%	1	1-3	1
26-50%	2	4-25	2
51-75%	3	>25	3
76-100%	4	Many coalesced dots and not countable	4

*staining distribution is determined by the approximate qualitative percentage of positive cells within this cell type population in the whole tissue section examined.

#staining intensity is determined by the number of dots per cell.

Supplementary Table 3. *In-situ* Hybridization of CNR1 mRNA Expression in Human Liver Tissues

	Control			NAFLD/NASH						
Donors	C1	C2	C3	N1	N2	N3	N4	N5	N6	N7
Probes	HsCnr1			HsCnr1						
Liver Cell Types										
Hepatocytes, centrilobular	-	-	-	-	-	-	-	-	-	-
Hepatocytes, midzonal	-	-	-	-	-	-	-	-	-	-
Hepatocytes, periportal	-	-	-	-	-	-	-	-	-	-
Cholangiocytes *	1I/1D	1I/1D	1-2I/1D	1-2I/1D	1I/1D	1I/1D	1-2I/1D	1-2I/1D	1I/1D	1I/1D
Kupffer cells[#] Stellate cells[#] Sinusoidal cells[#]	1I/1D	-	1I/1D	-	-	1I/1D	1I/1D	1I/1D	-	-
Vascular smooth muscle	-	-	-	-	-	-	-	-	-	-
Endothelium	1-3I/1-3D	1-2I/0-2D	1-3I/1-3D	1-2I/0-2D	1-2I/0-2D	1-2I/0-3D	1-3I/1-3D	1-2I/0-2D	1-2I/1D	1I/1D
Mononuclear cells	1I/1D	-	-	1I/1D	1-2I/1D	1I/1D	1-2I/1D	1I/1D	1-2I/1D	-

* low positivity in large ducts.

[#] low abundance of CNR1 mRNA; all present at close proximity in liver parenchyma/sinusoids.