PNAS
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Supplementary Information for

Targeting liver aldehyde dehydrogenase-2 prevents heavy but not moderate alcohol drinking

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This PDF file includes:

www.pnas.org/cgi/doi/10.1073/pnas.1908137116

SUPPLEMENTARY METHODS

Assessment of tissue-specific *Aldh2* deletion. Tissue-specific *Aldh2* deletion was confirmed at the protein levels by Western blot. Briefly, total protein concentration was evaluated by using a BCA Protein Assay kit (Pierce, Carlsbad, CA), and 30 µg total proteins were loaded per well in a 4-12% Bis-Tris gel (Bio-Rad, Hercules, CA). ALDH2 protein was detected using an anti-ALDH2 antibody (Abcam, Cambridge, MA). Antibodies raised against β-actin (Abcam Cambridge, MA) and GAPDH (Cell Signaling, Danvers, MA) were used as protein loading controls.

Serum sample extraction procedure and gas chromatograph-mass spectrometer (GC-MS) analysis. Mice were deeply anesthetized one-hour or three-hours after 5 g/kg ethanol gavage by a lethal dose of intraperitoneal pentobarbital injection. Blood was collected by traumatic avulsion of the orbital globe and kept for 10 minutes at room temperature in tubes containing a serum gel with clotting activator (Sarstedt, Newton, NC). Blood samples were then centrifuged at 4,000 g for 10 minutes at 4°C. Fifty microliters (µL) of fresh mouse sera (on ice) was added to 15 µL of an extraction solution (1.2% Triton X-100 in 1.8 g/L acetonitrile in H_2O) as described by Pontes and colleagues (1) to which was added 15 µL of labeled internal standards (ethanol-d2, 537.5 mM, acetaldehyde-d4 solution 803 µM from Cambridge Isotope Labs [Andover, MA]). Samples were vortexed for one min and filtered through conical 0.5 mL Amicon microcon 30-micron YM-3 filter devices (Millipore, Bedford MA) using an Eppendorf 5417R centrifuge equipped with a F45-30-11 rotor at 12,000 rpm at 4°C for 12 min. Finally, 1.2 µL of solution was taken for analysis on an Agilent 5973 gas chromatograph-mass spectrometer in the electron impact mode. Samples were injected using the splitless procedure with a 0.2 min delay. The injection port was maintained at 50°C and the oven temperature was programed from 45-120 °C at 8 °C/min. Analysis was carried out in the electron impact mode with a 70-eV ionization energy. The acetaldehyde eluted at 2.6 min and was quantified using the ratio of the d4-molecular ion (m/z 48) to that non-labeled acetaldehyde (m/z 44). Ethanol eluted at 5.2 min and the endogenous compound were quantified using the ratio of the d2-fragment ion (m/z 48) of the labeled ethanol to that non-labeled compound (m/z 46).

Drinking in the dark (DID). Mice were individually housed for a week before starting the experiment (acclimation period) and given free access to a water-containing bottle during

daylight. Three hours after lights were turned off in the animal holding room, the water bottle was replaced by an ethanol-containing bottle (20% vol/vol) for two hours (days 1-3) or for four hours (day 4). Ethanol intake was measured at the end of each period. Bottle replacement was performed while a red-lamp was temporarily turned on to prevent disruption of the mice' circadian rhythm.

Chronic binge model. The E10d + 1B feeding model was described previously (Bertola et al., Nat Protoc. 2013 Mar; 8(3): 627–637). Briefly, mice were initially fed a control Lieber-DeCarli liquid diet (catalog F1259SP, Bio-Serv, Flemington, NJ) *ad libitum* for 5 days during acclimatization. Subsequently, mice were allowed free access to an ethanol diet (catalog F1258SP, Bio-Serv) containing 5% (v/v) ethanol for 10 days. On day 11, they received a single dose of ethanol (5 g/kg body weight) via oral gavage and were killed 9 hours later.

Biochemical assays. Serum alanine aminotransferase (ALT), and aspartate aminotransferase (AST) activities were determined using a Catalyst Dx Chemistry Analyzer (IDEXX Laboratories, Westbrook, ME).

Serum cytokine levels. Cytometric bead array (CBA) test was performed using LEGENDplex bead-based immuoassays (BioLegend, San Diego, CA), following manufacturer's instructions.

Blood cell count. The anticoagulated blood was collected from mice. Cell count test was performed with an Hemavet 950 FS Hematology Analyzer (Drew Scientific, Dallas, TX).

Tissue staining. Tissue samples were collected and fixed in 10% formalin and paraffin-embedded following standard procedure. Embedded liver tissues were cut to 4 μm thickness and subjected to staining with hematoxylin and eosin (H&E), or subjected to immune-histochemical staining for GFP, F4/80 or myeloperoxidase (MPO). Rabbit anti-GFP (Cell Signaling), anti-F4/80 (Bio-Rad, Hercules, CA) and anti-MPO polyclonal antibody (Biocare Medical, LLC, Concord, CA), and a rabbit ABC staining kit (Vector Laboratories, Inc., Burlingame, CA) were used according to the manufacturer's instruction.

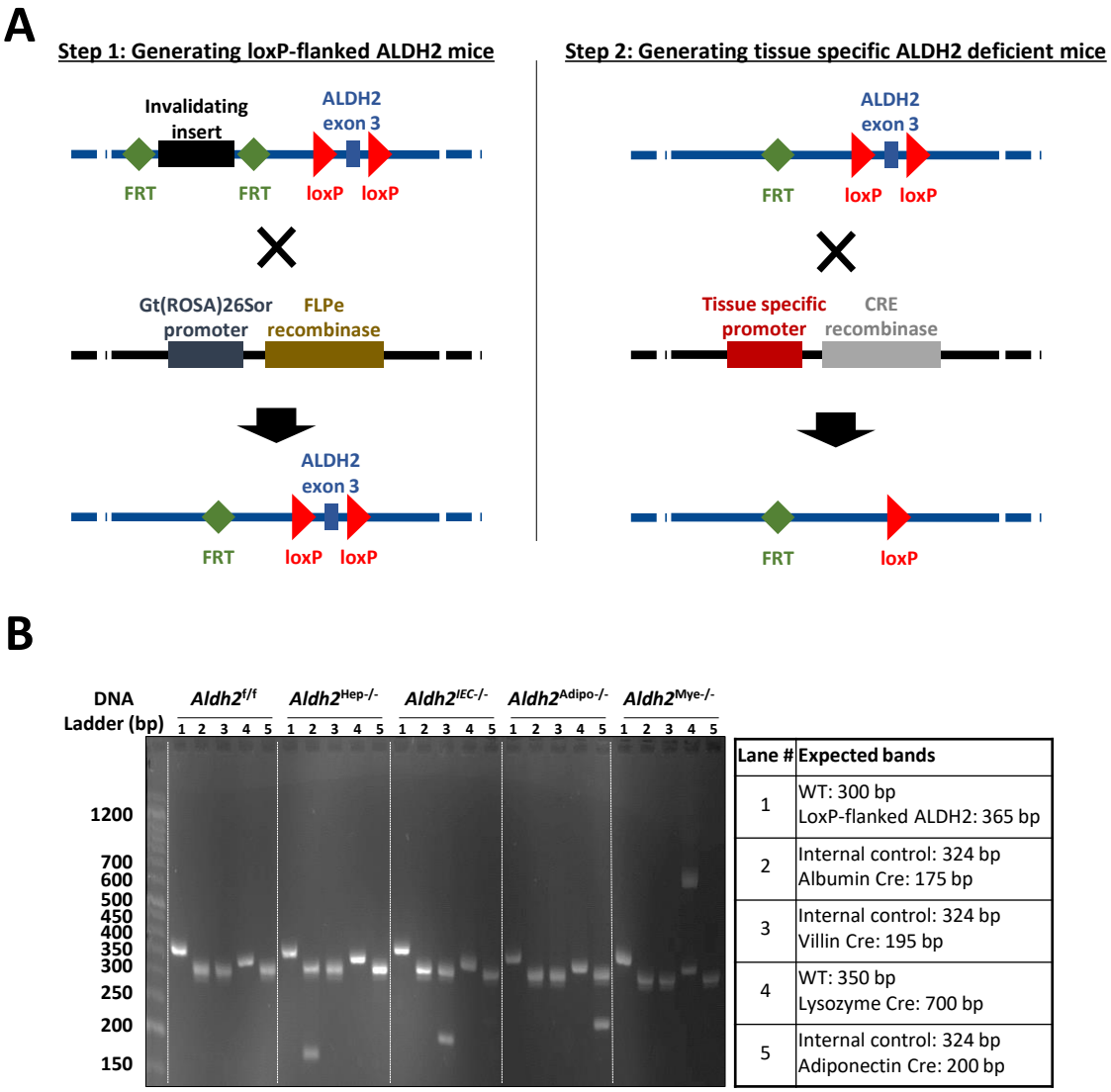
Quantitative RT-PCR. Total RNA was extracted from snap-frozen liver tissue or cell lysates using the RNeasy Mini Kit (Qiagen, Germantown, MD), following manufacturer's instructions. High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific, Carlsbad, CA) was used for reverse transcription. Real-time PCR was performed on a QuantStudio 6 Flex Real-Time PCR System (ThermoFisher Scientific, Carlsbad, CA), using BrightGreen 2X qPCR MasterMix-ROX (Applied Biological Materials Inc., Richmond, BC). Oligonucleotides used for PCR amplification are listed in Supporting Table 1.

SUPPLEMENTARY REFERENCE

1. Pontes H*, et al.* (2009) GC determination of acetone, acetaldehyde, ethanol, and methanol in biological matrices and cell culture. *J Chromatogr Sci* 47(4):272-278.

Supporting Table 1. Primer sequences for RT-PCR

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
Ccl2	TCTGGACCCATTCCTTCTTGG	TCAGCCAGATGCAGTTAACGC
Cd68	CCATCCTTCACGATGACACCT	GGCAGGGTTATGAGTGACAGTT
Cxcl1	TCTCCGTTACTTGGGGAC	CCACACTCAAGAATGGTCGC
Cxcl2	TCCAGGTCAGTTAGCCTTGC	CGGTCAAAAAGTTTGCCTTG
F4/80	CTTTGGCTATGGGCTTCCAGTC	GCAAGGAGGACAGAGTTTATCGTG
Ifng	TAGCCAAGACTGTGATTGCGG	AGACATCTCCTCCCATCAGCAG
Il6	TCCATCCAGTTGCCTTCTTG	TTCCACGATTTCCCAGAGAAC
Ly6g	TGCGTTGCTCTGGAGATAGA	CAGAGTAGTGGGGCAGATGG
Tnfa	AGGCTGCCCCGACTACGT	GACTTTCTCCTGGTATGAGATAGCAAA
18s	ACGGAAGGGCACCACCAGGA	CACCACCACCCACGGAATCG



Lane #	Expected bands
1	WT: 300 bp LoxP-flanked ALDH2: 365 bp
2	Internal control: 324 bp Albumin Cre: 175 bp
3	Internal control: 324 bp Villin Cre: 195 bp
4	WT: 350 bp Lysozyme Cre: 700 bp
5	Internal control: 324 bp Adiponectin Cre: 200 bp

Figure S1: Generation of tissue-specific ALDH2 knock-out mice

The *Aldh2* gene was disrupted by inserting SA-beta gal-Neo that was flanked by FRT sequence (*Aldh2*$^{tm1a(EUCOMM)Wtsi}$ mice [kindly provided by Dr. Patel]). This *Aldh2* gene was also flanked with two loxP sites outside of exon 3. (*A*) To generate conditional *Aldh2*$^{-/-}$ mice, we first activated *Aldh2* gene in *Aldh2*$^{-/-}$ mice by crossing *Aldh2*$^{-/-}$ mice with FLPe recombinase expressing mice to remove the FRT-flanked invalidating insert from the *Aldh2* gene. This led to the generation of mice having a functional *Aldh2* gene with its exon 3 flanked by two loxP sequences. In a second step, exon3-floxed mice were bred with homozygous *Aldh2*-floxed and heterozygous tissue-specific Cre-expressing mice, to obtain Cre^{+}*Aldh2*$^{f/f}$ mice, thus generating tissue-specific *Aldh2*-deficient mice and littermate controls (*Aldh2*$^{f/f}$). By using this breeding method, we generated liver (*Aldh2*$^{Hep-/-}$), intestinal epithelial cell (*Aldh2*$^{IEC-/-}$), adipose tissue (*Aldh2*$^{Adipo-/-}$), myeloid cell (*Aldh2*$^{Mye-/-}$), glial cell (*Aldh2*$^{Gfap-/-}$), and neuron (*Aldh2*$^{Camk2a-/-}$) specific *Aldh2*-deficient mice. (*B*) Mice genotypes were confirmed by PCR product gel migration. Table represents expected band sizes.

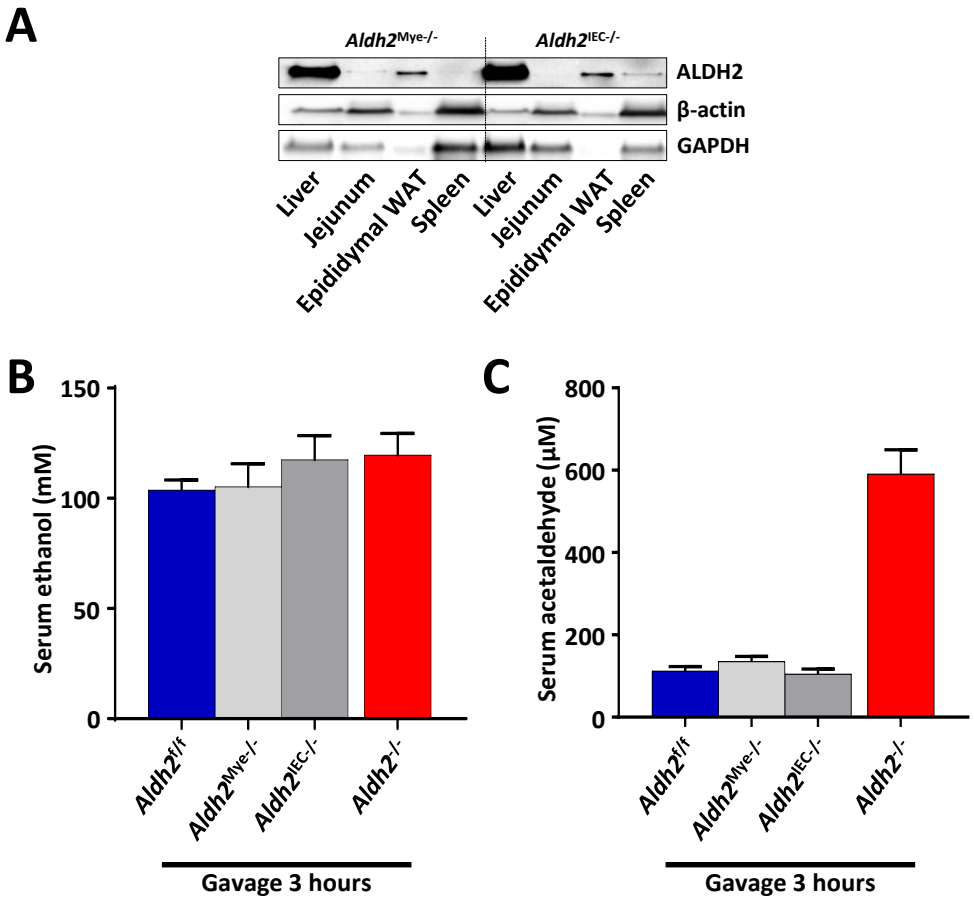


Figure S2: Intestinal epithelial cell- and myeloid-cell *Aldh2*-deficiency does not impact acetaldehyde clearance

Myeloid cell and intestinal epithelial cell- *Aldh2*-deficient mice were generated as described in Figure S1. (*A*) Tissue specific *Aldh2*-deficiency was verified by Western blot. (*B*) Serum ethanol and (*C*) acetaldehyde levels were measured after a 5 g/kg ethanol gavage, as described in Figure 2. Data is represented as mean ± S.E.M. (n=9-14 mice per group).

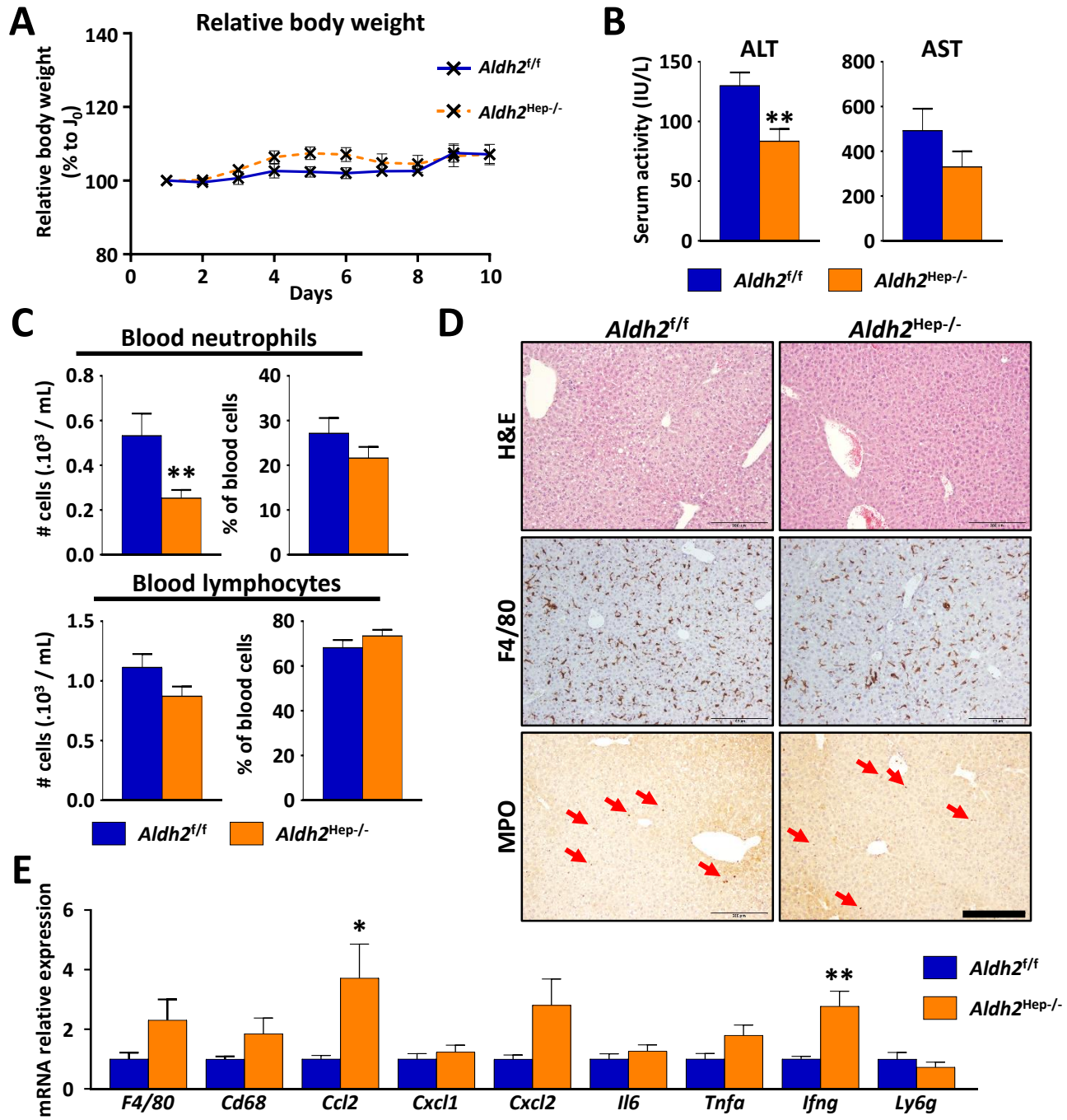


Figure S3: Liver ALDH2-deficiency leads to reduced circulating immune cell numbers and liver injury, but increased liver inflammation in the chronic plus ethanol binge model

Aldh2$^{f/f}$ and *Aldh2*$^{Hep-/-}$ mice were fed with an ethanol diet for 10 days, then received a single ethanol gavage and were killed 9 hours later. (*A*) Body weights were recorded from the start of the ethanol feeding (Day 0). (*B*) Serum alanine aminostransferase (ALT) and aspartate aminotransferase (AST) were measured. (*C*) The number and percentage of circulating blood neutrophils and lymphocytes were measured by an haematology counter. (*D*) Liver sections were performed and stained with hematein-eosin (H&E), anti-F4/80 or anti-myeloperoxidase (MPO) antibodies. Red arrows indicate MPO^{+} neutrophils. Scale bar: 200 µm. (*E*) Quantitative RT-PCR was performed on liver homogenates in the chronic plus binge model. Data is represented as mean ± S.E.M. (n=12-14 mice per group), unpaired Student's t test (*B, C, E*).

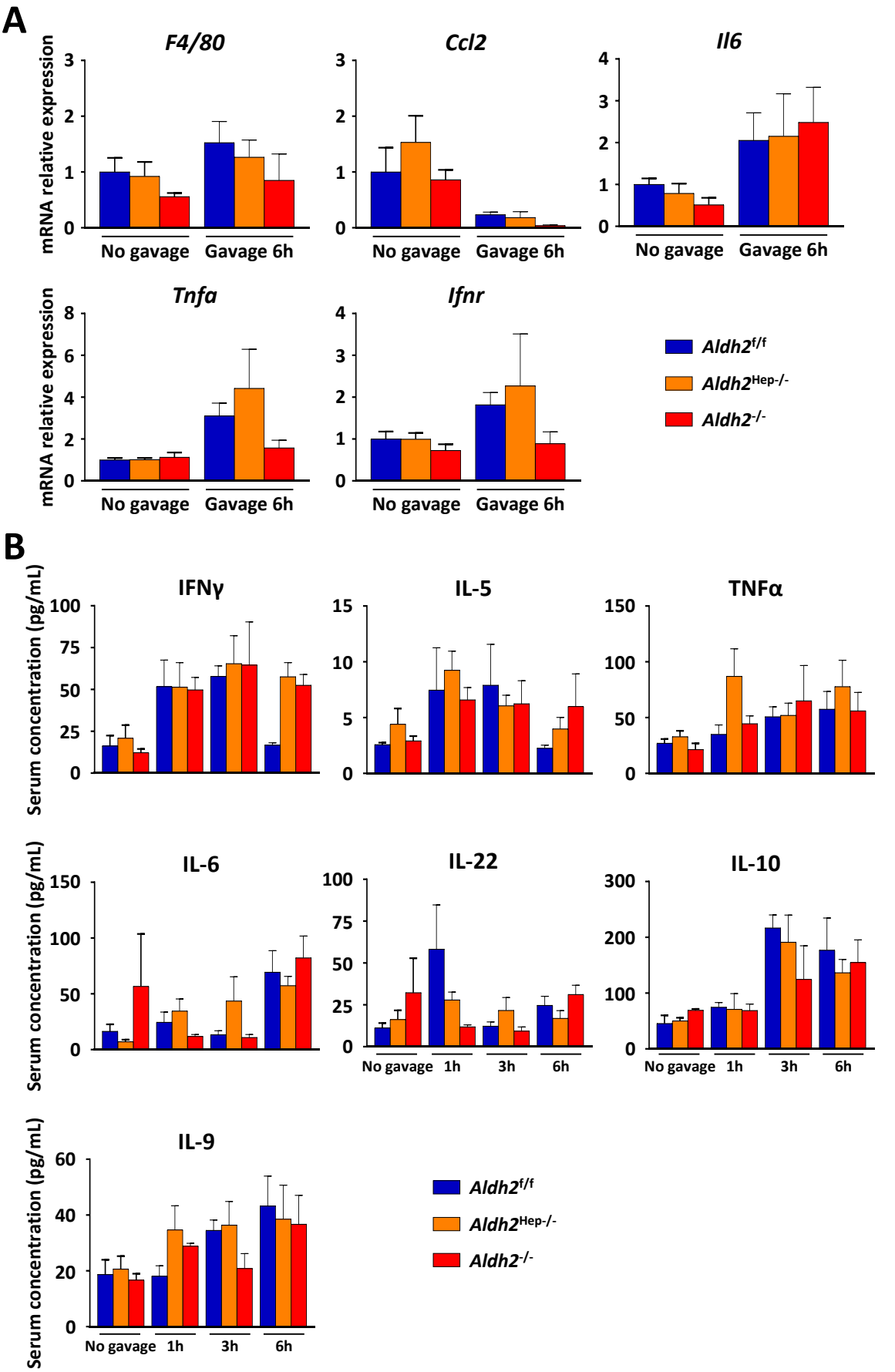

A
F4/80
Ccl2
Il6
Tnfa
Ifnr
mRNA relative expression
No gavage
Gavage 6h
Aldh2f/f
Aldh2Hep-/-
Aldh2-/-
B
IFNγ
IL-5
TNFα
IL-6
IL-22
IL-10
IL-9
Serum concentration (pg/mL)
No gavage
1h
3h
6h

Figure S4: Liver ALDH2-deficiency does not affect hepatic or systemic inflammation after ethanol gavage

Aldh2$^{f/f}$, *Aldh2*$^{Hep-/-}$ and *Aldh2*$^{-/-}$ mice received a single ethanol gavage (5 g/kg) and were killed at the indicated times. (*A*) Quantitative RT-PCR was performed on liver homogenates. (*B*) Serum cytokine levels were measured by Cytometric bead array . Data is represented as mean ± S.E.M. (n=3-5 mice per group), 1-way ANOVA followed by multiple comparison test was performed (no relevant statistical differences were observed between the experimental groups).

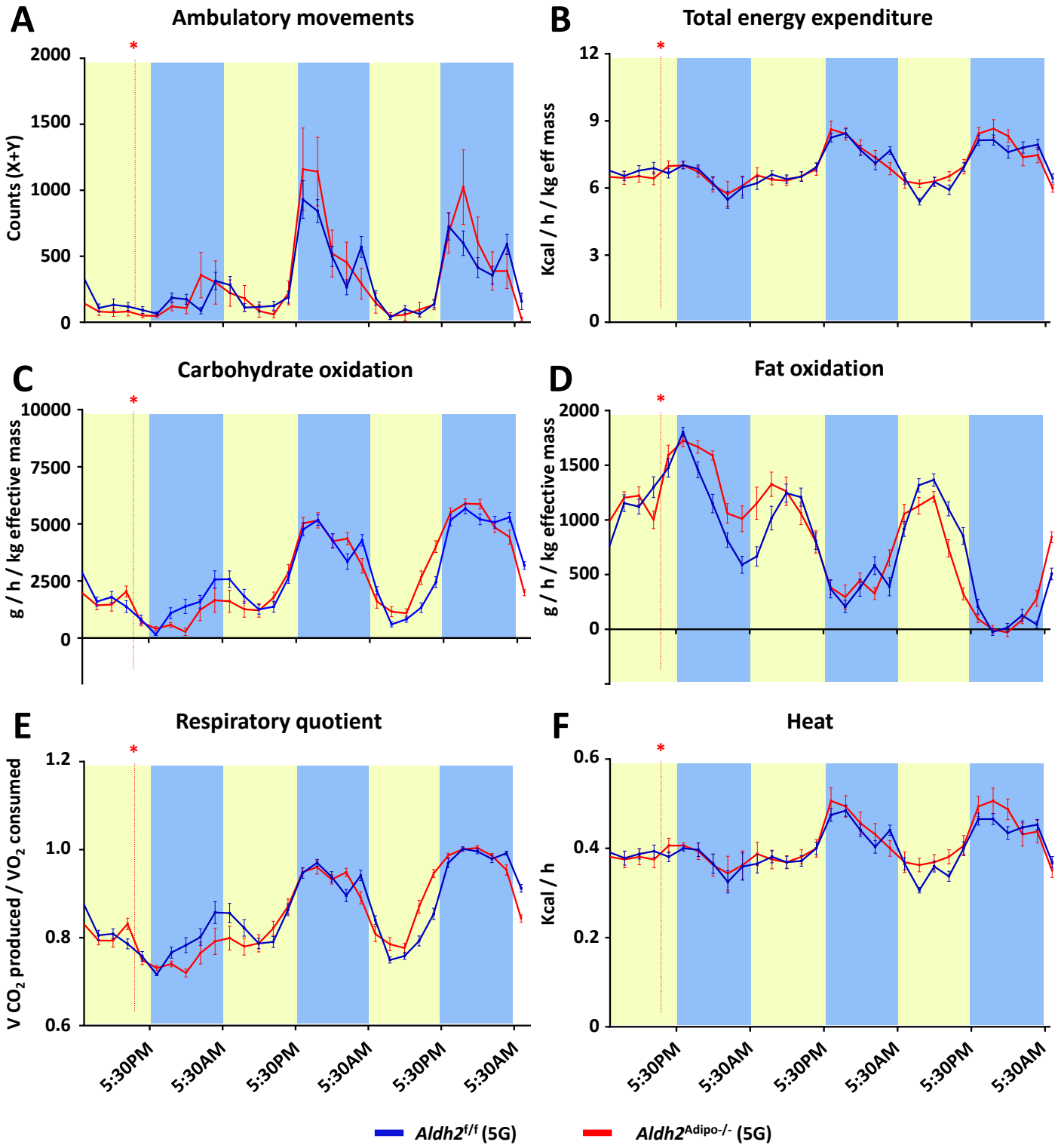


Figure S5: Adipose-tissue specific *Aldh2* deficiency does not affect metabolic rates after acute ethanol gavage

(*A-F*) Metabolic parameters were measured in control, or adipose tissue Aldh2 deficient mice (*Aldh2*$^{Adipo-/-}$) after a single 5 g/kg ethanol gavage. The red line indicates the time at which the gavage was given to the mice. Yellow and blue bars represent 12-hour day- and night-time, respectively. Data is represented as mean ± S.E.M. (n=4 mice per group).

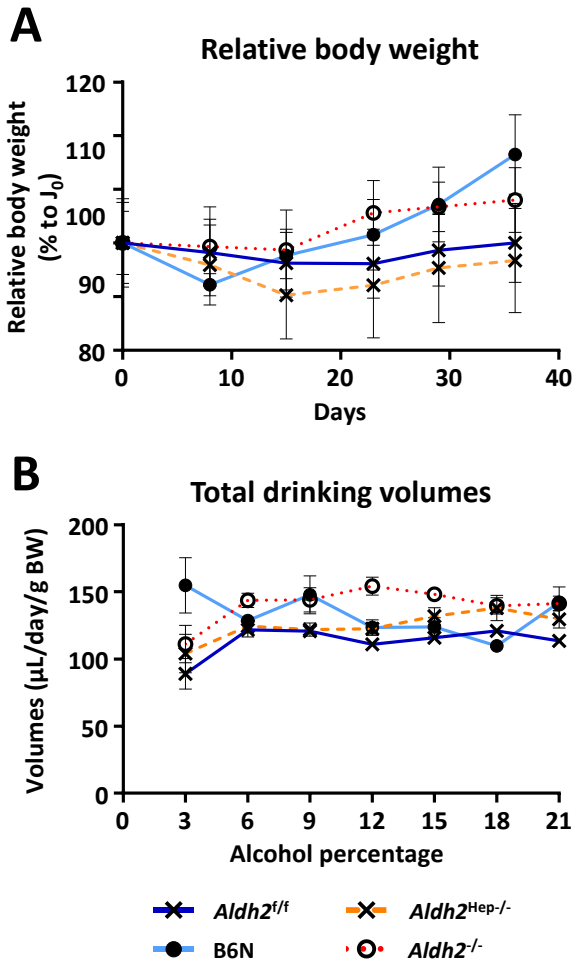


Figure S6: Body weight remains constant during two-bottle choice experiment, and all groups had similar total drinking volumes

(*A*) Mouse body weight was measured and remained unchanged during the two-bottle experiment in all groups. (*B*) Mice of all genotypes drank similar cumulated volumes of water and ethanol all along the protocol. Data is represented as mean ± S.E.M. (n=5-7 mice per group).

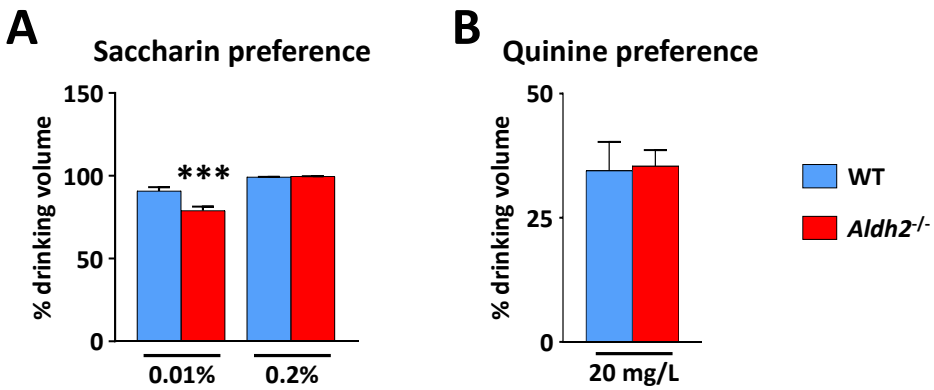


Figure S7: Global Aldh2-deficiency does not affect sweet and bitter taste preference

WT and *Aldh2*$^{-/-}$ mice were subjected to a two-bottle paradigm in which they were offered a bottle of regular drinking water, and a bottle containing either (*A*) 0.01% or 0.2% saccharin, or (*B*) 20 mg/L quinine. Drinking preference of the test bottle is indicated as the percentage of daily total liquid intake from the bottle containing saccharin or quinine. Data is represented as mean ± S.E.M. (n=6-7 mice per group), ***p<0,005, unpaired Student's t test.

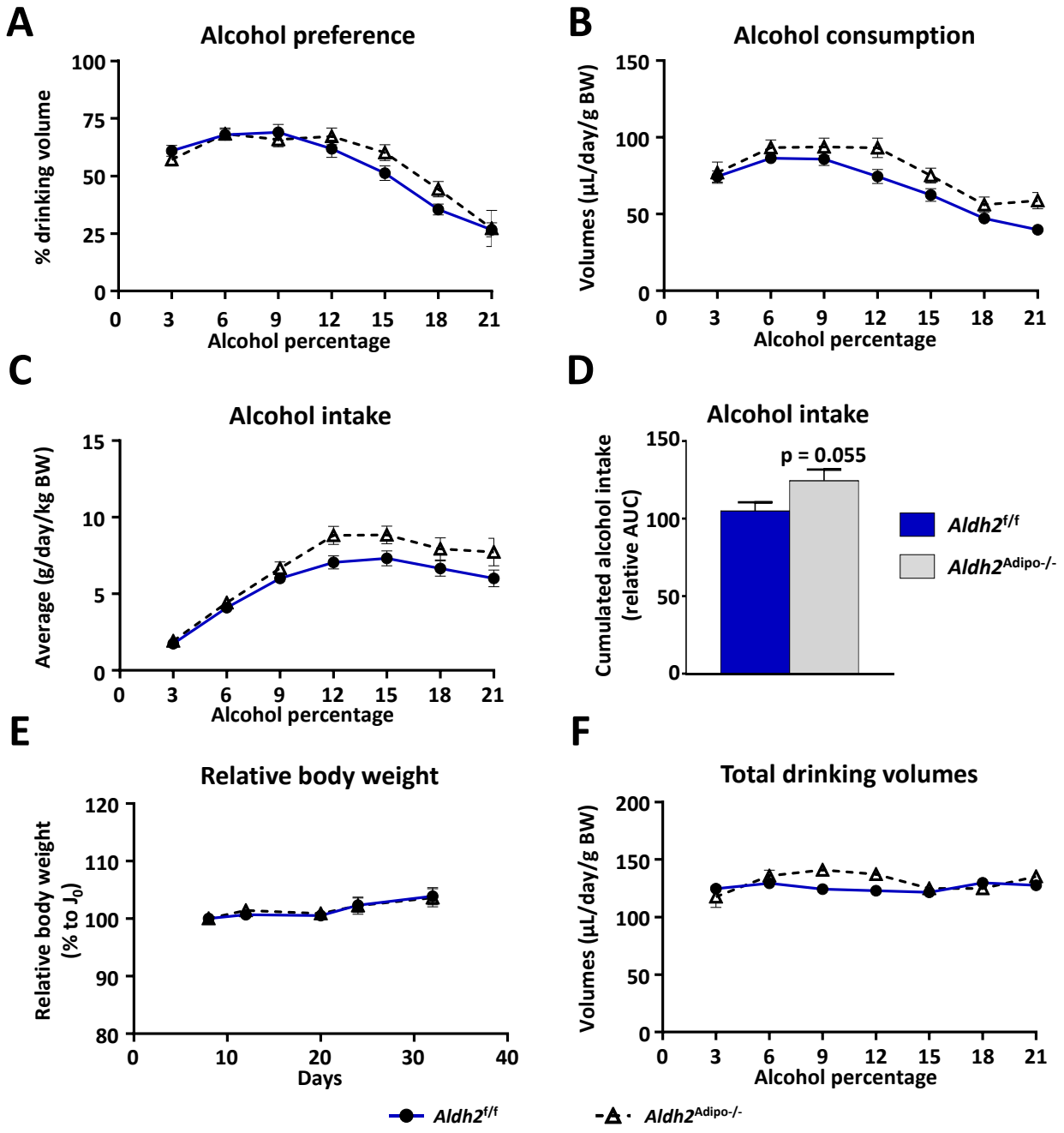


Figure S8: Adipose tissue ALDH2-deficient mice tend to have a higher alcohol preference than control animals

Concomitantly with mice presented in Figure 4, *Aldh2*$^{Adipo-/-}$ mice were subjected to the two-bottle paradigm. (*A*) Alcohol preference, (*B*) consumption, and (*C*) intake were measured. (*D*) Cumulated area under curve representing total alcohol intake during the experiment. (*E*) Relative body weight and (*F*) total drinking volumes were recorded. Data is represented as mean ± S.E.M. (n=7 mice per group), unpaired Student's t test.

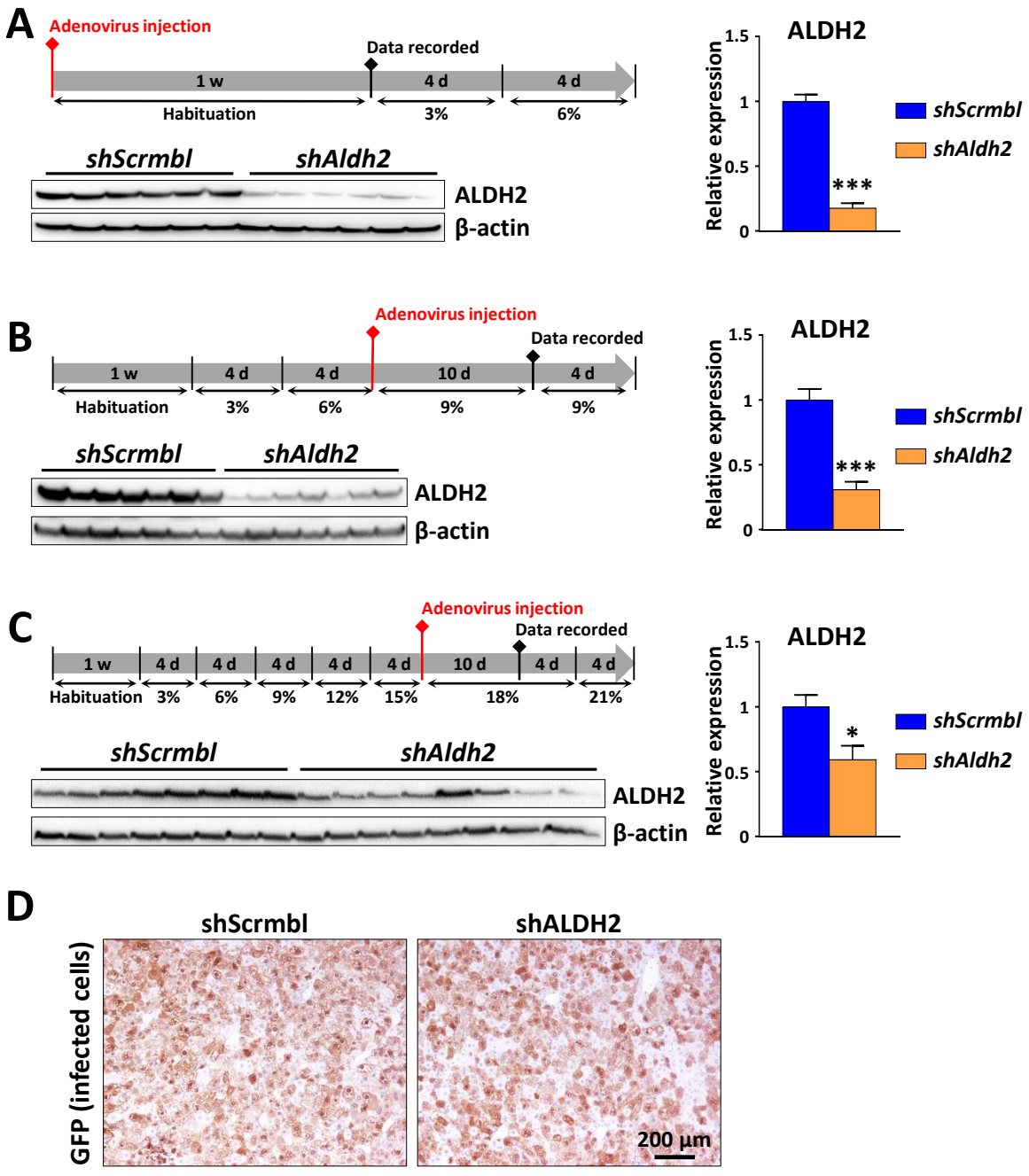


Figure S9: Successful hepatocyte infection and *Aldh2* silencing by using *shAldh2* adenovirus

Mice were injected with scramble or *shAldh2* adenovirus as indicated in Figure 6. (*A-C*) At the end of each set of measurements, mice were killed and liver ALDH2 expression was assessed by Western blot. Panels *A*, *B*, and *C* correspond to the data presented in Figure 6 panels *A*, *B*, and *C*, respectively. (*D*) Hepatocyte infection was verified by GFP immunostaining (brown) on liver sections.