Supplementary Materials for

Hepatic *Hamp* restoration contributes to nicotinamide mononucleotide (NMN)-alleviated hepatic steatosis in chronic alcohol-fed mice

Supplementary Methods

1.1 Human liver samples

This study was approved by the Biomedical Research Ethics Committee of West China Hospital of Sichuan University (REC2019-833) and complied with the ethical guidelines of the Declaration of Helsinki, and written informed consent was obtained from all patients. All ethical regulations relevant to human research participants were followed. Human liver samples were obtained from both healthy individuals and patients with ALD, who underwent treatment or physical examinations at the Physical Examination Center of West China Hospital, Sichuan University (Sichuan, China). Patients with ALD were screened using clinical diagnostic criteria[1]. Specifically, the diagnosis was based on the following widely accepted criteria: (1) a clear history of alcohol consumption, generally defined as long-term drinking for more than 5 years with an average daily ethanol intake of $\geqslant$40 g for men or $\geqslant$20 g for women, or a recent history of heavy drinking within the past two weeks with ethanol intake >80 g/day; (2) liver histopathological evidence showing any one of the following features: hepatic steatosis, hepatitis, fibrosis, or cirrhosis; and (3) exclusion of other causes of liver disease, including current hepatitis virus infection, drug- or toxin-induced liver injury, and autoimmune liver diseases. Baseline clinical and demographic data of human participants are provided in Supplementary Table 6.

1.2 Cell culture

VL-17A, a HepG2-based reformed cell line, was conducted according to previous study [2–4]. HepG2 cell line was obtained from Cell bank of Chinese Academy of Sciences (Shanghai, China). HepG2 cells were stably transfected with both cytochrome P450 2E1 (CYP2E1) and alcohol dehydrogenase (ADH) to construct VL-17A cell line[5]. VL-17A cells were cultured in DMEM (Gibco, Waltham, MA)

supplemented with 10% FBS and 100 U/mL penicillin/streptomycin at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. VL-17A cells were transfected with specific siRNA targeting human *HAMP* or scramble siRNA (NC) for 6 h. To establish an *in vitro* therapeutic model, cells were first exposed to ethanol (100 mM) for 2 h, followed by continuous co-treatment with ethanol and 1 mM NMN for 16 h.

Supplementary Tables

Supplementary Table 1. Chronic alcohol model protocol

Group	Time	Calories from alcohol	Ethanol in the diet (w/v)	Maltodextrin (g/L)	Feed mix (g/L)
PF	Week 1-9	0%	0%	115.20	108.70
AF	Week 1	0%	0%	115.20	108.70
	Week 2	24.50%	3.50%	53.95	108.70
	Week 3-4	25.48%	3.64%	51.50	108.70
	Week 5-6	26.46%	3.78%	49.05	108.70
	Week 7-8	27.44%	3.92%	46.60	108.70
	Week 9	28.42%	4.06%	44.15	108.70

Note: PF represents pair-fed while AF represents alcohol-fed.

Supplementary Table 2. Composition of feed mix

Group	Casein (g/L)	Cellulose (g/L)	Oil mix (g/L)	L-cystine (g/L)	Dl-Methionine (g/L)	Choline Bitartarte (g/L)	Salt mix (g/L)	Suspending agents (g/L)	Vitamin mix (g/L)
PF	41	10	40	0.5	0.5	0.5	7.3	6.8	2.1
AF	41	10	40	0.5	0.5	0.5	7.3	6.8	2.1

Note: PF represents pair-fed while AF represents alcohol-fed.

Supplementary Table 3. Calorie informatioon of the diets

Group	Protein （kcal/L）	Fat （kcal/L）	Carbohydrate （kcal/L）	Ethanol （kcal/L）	Total calorie （kcal/L）
PF	180	360	460	0	1000
AF	180	360	176-460	0-284	1000

Note: PF represents pair-fed while AF represents alcohol-fed

Supplementary Table 4. Primers used in this study.

	Description	Sequence (5'-3')	Species
Sds	Forward	GAAGACCCCACTTCGTGACAG	mouse
	Reverse	TCTTGCAGAGATGCCCAATGC	mouse
Hamp	Forward	CTGAGCAGCACCACCTATCTC	mouse
	Reverse	TGGCTCTAGGCTATGTTTTGC	mouse
Mt-Nd4	Forward	AGCTCAATCTGCTTACGCCA	mouse
	Reverse	GCTGTGGATCCGTTCGTAGT	mouse
Mt-Cytb	Forward	ACGCAAACGGAGCCTCAATA	mouse
	Reverse	CCTCATGGAAGGACGTAGCC	mouse
Mt-Nd4l	Forward	AACCTCACCATAGCCTTCTCA	mouse
	Reverse	TGATGGGGATTGGTATGGAGC	mouse
Cyp2f2	Forward	GGACCCAAACCTCTCCCAATC	mouse
	Reverse	CCGTGAACACCGACCCATAC	mouse
Tmed2b	Forward	CACATCCTACTGAATGGCGGTCT	mouse
	Reverse	AAGTTTCTCCTGACACGCTAGCAA	mouse
Vldlr	Forward	ACGGCAGCGATGAGGTCAACTG	mouse
	Reverse	CAGAGCCATCAACACAGTCTCG	mouse
Fasn	Forward	GGAGGTGGTGATAGCCGGTAT	mouse
	Reverse	TGGGTAATCCATAGAGCCCAG	mouse
Prkaa1	Forward	CAGGCCATAAAGTGGCAGTTA	mouse
	Reverse	AAAAGTCTGTCGGAGTGCTGA	mouse
Scd1	Forward	CGAGGGTTGGTTGTTGATCT	mouse
	Reverse	GCCCATGTCTCTGGTGTTTT	mouse
Pparα	Forward	AGAGCCCCATCTGTCCTCTC	mouse
	Reverse	ACTGGTAGTCTGCAAAACCAAA	mouse
Cpt1	Forward	CTATGCGCTACTCGCTGAAGG	mouse
	Reverse	GGCTTTCGACCCGAGAAGA	mouse
Acc	Forward	GTTCTGTTGGACAACGCCTTCAC	mouse

	Reverse	GGAGTCACAGAAGCAGCCCATT	mouse
Cd36	Forward	ATGGGCTGTGATCGGAACTG	mouse
	Reverse	GTCTTCCCAATAAGCATGTCTCC	mouse
Fatp2	Forward	TCCTCCAAGATGTGCGGTACT	mouse
	Reverse	TAGGTGAGCGTCTCGTCTCG	mouse
Dgat2	Forward	CGAGACACCATAGACTACTTGCT	mouse
	Reverse	GCGGTTCTTCAGGGTGACTG	mouse
Il1b	Forward	GAAATGCCACCTTTTGACAGTG	mouse
	Reverse	TGGATGCTCTCATCAGGACAG	mouse
Tnf	Forward	CCCTCACACTCAGATCATCTTC	mouse
	Reverse	CCCTCACACTCAGATCATCTTC	mouse
Gpx4	Forward	GCCTGGATAAGTACAGGGGTT	mouse
	Reverse	CATGCAGATCGACTAGCTGAG	mouse
Alox12	Forward	TCCCTCAACCTAGTGCGTTTG	mouse
	Reverse	GTTGCAGCTCCAGTTTCGC	mouse
Acsl4	Forward	CCTGAGGGGCTTGAAATTCAC	mouse
	Reverse	GTTGGTCTACTTGGAGGAACG	mouse
Ptgs2	Forward	TTCAACACACTCTATCACTGGC	mouse
	Reverse	AGAAGCGTTTGCGGTACTCAT	mouse
Nox4	Forward	GAAGGGGTTAAACACCTCTGC	mouse
	Reverse	ATGCTCTGCTTAAACACAATCCT	mouse
18s	Forward	AGGTCTGTGATGCCCTTAGA	mouse
	Reverse	GAATGGGGTTCAACGGGTTA	mouse
Pck1	Forward	CTGCATAACGGTCTGGACTTC	mouse
	Reverse	CAGCAACTGCCCGTACTCC	mouse
Fbp1	Forward	ATCAGCCCATCCTGTGGAAC	mouse
	Reverse	TGCAGCTAATCTCTCTAGCACTT	mouse
G6pc	Forward	CGACTCGCTATCTCCAAGTGA	mouse
	Reverse	GTTGAACCAGTCTCCGACCA	mouse

Gck	Forward	CAGACAGCGTCACGACCAT	mouse
	Reverse	CATCAGGCAGGTTTGTGTAGC	mouse
Pfkl	Forward	GGAGGCGAGAACATCAAGCC	mouse
	Reverse	CGGCCTTCCCTCGTAGTGA	mouse
Pklr	Forward	TCAAGGCAGGGATGAACATTG	mouse
	Reverse	CACGGGTCTGTAGCTGAGTG	mouse
Slc2a2	Forward	TCAGAAGACAAGATCACCGGA	mouse
	Reverse	GCTGGTGTGACTGTAAGTGGG	mouse
Tkt	Forward	CTGCGAATCCGTTCCATCAG	mouse
	Reverse	GTCTGGGTTTTCGGGGTCTTC	mouse
Taldo1	Forward	GTAAAGCGCCAGAGGATGGAG	mouse
	Reverse	CTCTTGGTAGGCAGGCATCT	mouse
HAMP	Forward	CTGACCAGTGGCTCTGTTTTC	human
	Reverse	GAAGTGGGTGTCTCGCCTC	human
18s	Forward	GAGGTTCGAAGACGATCAGA	human
	Reverse	TCGCTCCACCAACTAAGAAC	human

Supplementary Table 5. Catalog numbers or clone numbers for antibodies

Antibody	Catalog No.	Source
Rabbit-anti-β-actin	AC026	ABclonal
Rabbit-anti-HEPCIDIN	ab190775	Abcam
Rabbit-anti-HIF-1α	20960-1-AP	Proteintech
Rabbit-anti-C/EBPα	29388-1-AP	Proteintech
Rabbit-anti-LaminB1	12987-1-AP	Proteintech

Supplementary Table 6. Primers used in this study

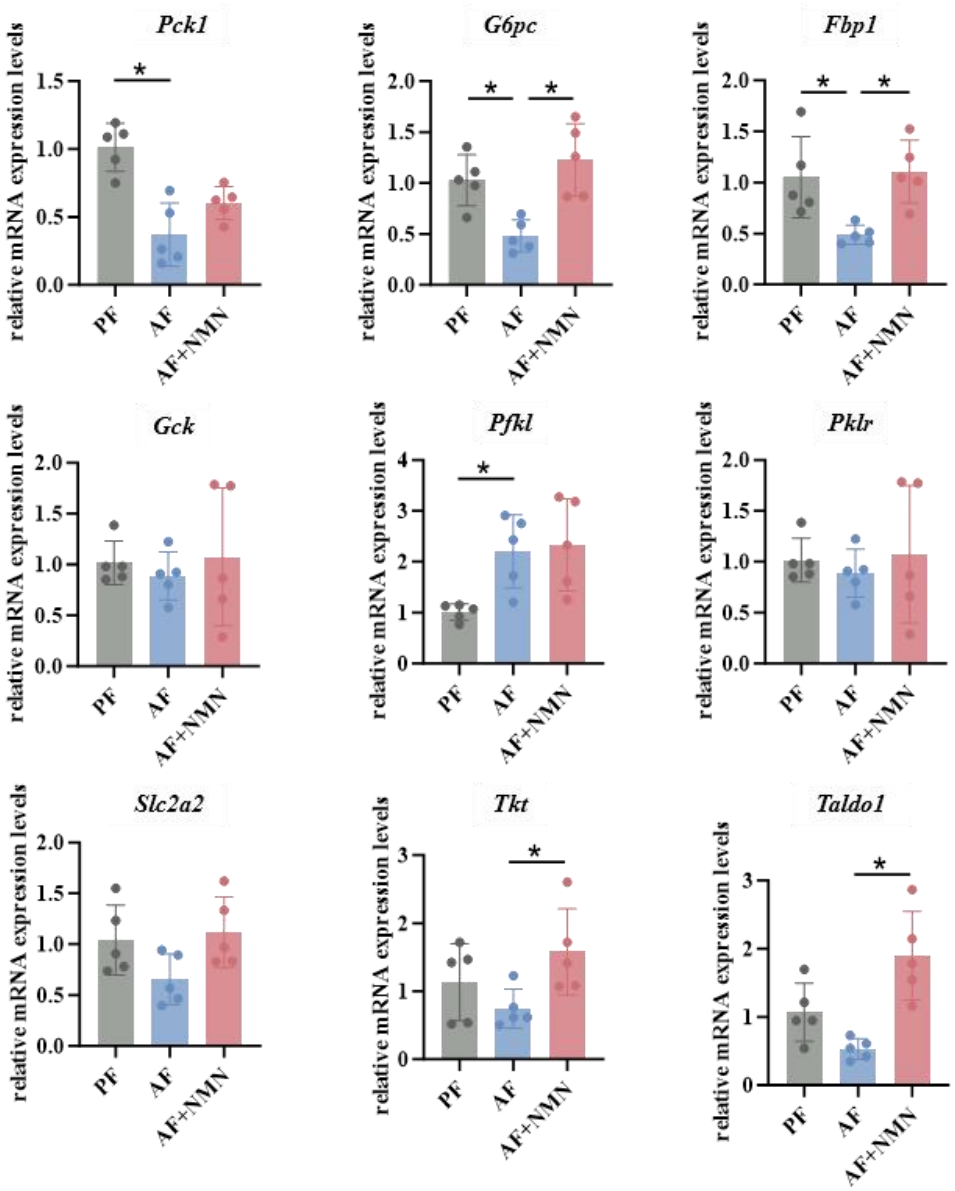
	Sense (5'-3')	Antisense (5'-3')
Negative control（siNC）	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT
siHamp	CCACCUAUCUCCAUCAACATT	UGUUGAUGGAGAUAGGUGGTT

Supplementary Table 7. Information of clinical sample

Variables	Control (N=5)	ALD patients (N=8)
Age (Yrs)	59.40 ± 7.54	64.50 ± 9.64
Sex	3 male/2 female	8 male
AST (U/L)	17.80 ± 3.83	28.86 ± 9.52
ALT (U/L)	12.80 ± 4.02	32.00 ± 12.56
ALP (U/L)	73.20 ± 23.11	130.75 ± 87.99
Average alcohol (g)/ day	none	> 50

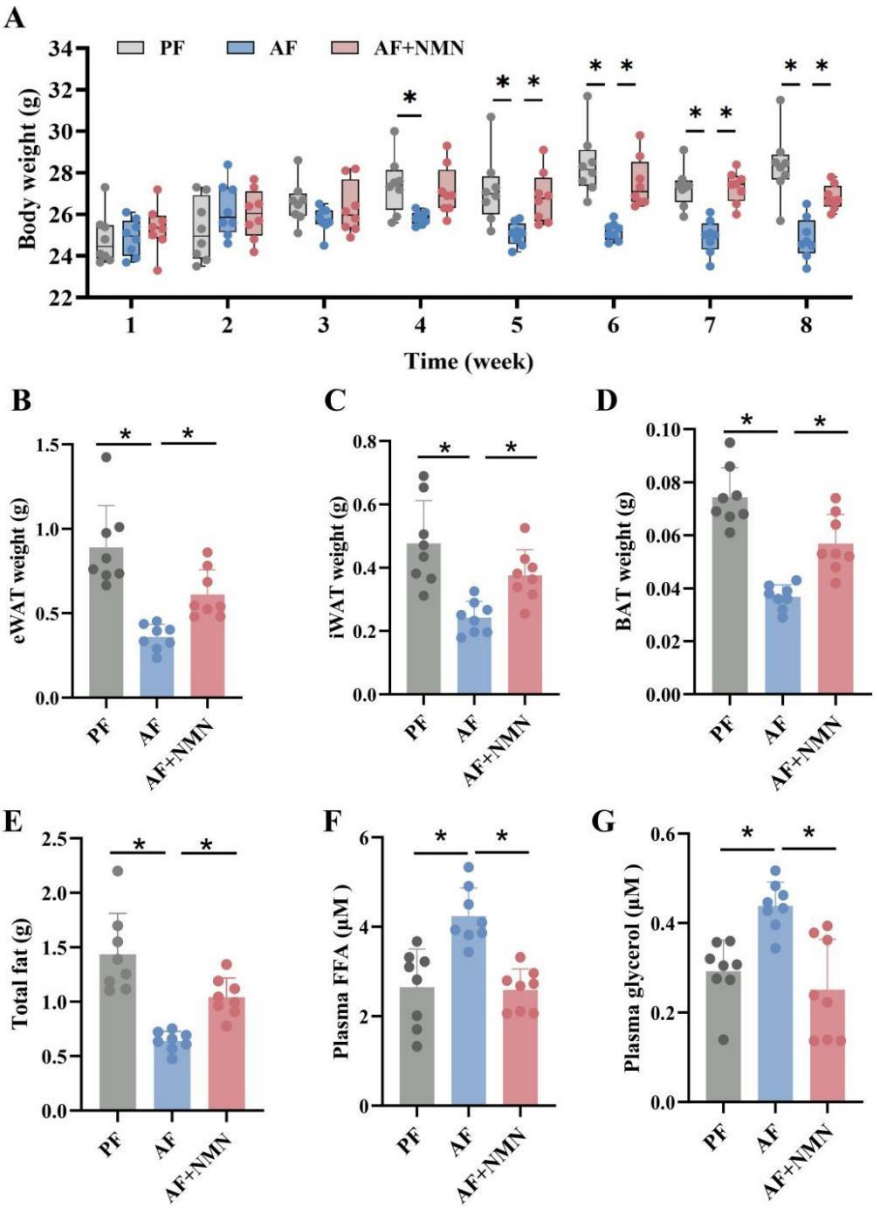
Supplementary Figures

Supplementary Figure 1



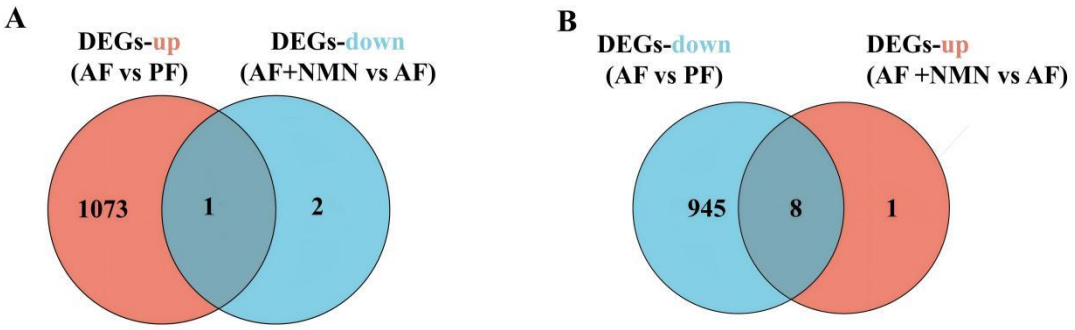
Supplementary Figure 1. Expression levels of hepatic glucose metabolism-related genes in mice. Relative mRNA expression levels of key genes involved in gluconeogenesis, glycolysis, glucose uptake, and pentose phosphate pathways, as determined by qPCR (n = 5 mice/group). Data are presented as means ± SD. *p < 0.05 indicates statistically significant differences. PF, pair-fed; AF, alcohol-fed; AF + NMN, alcohol-fed treated with NMN.

Supplementary Figure 2



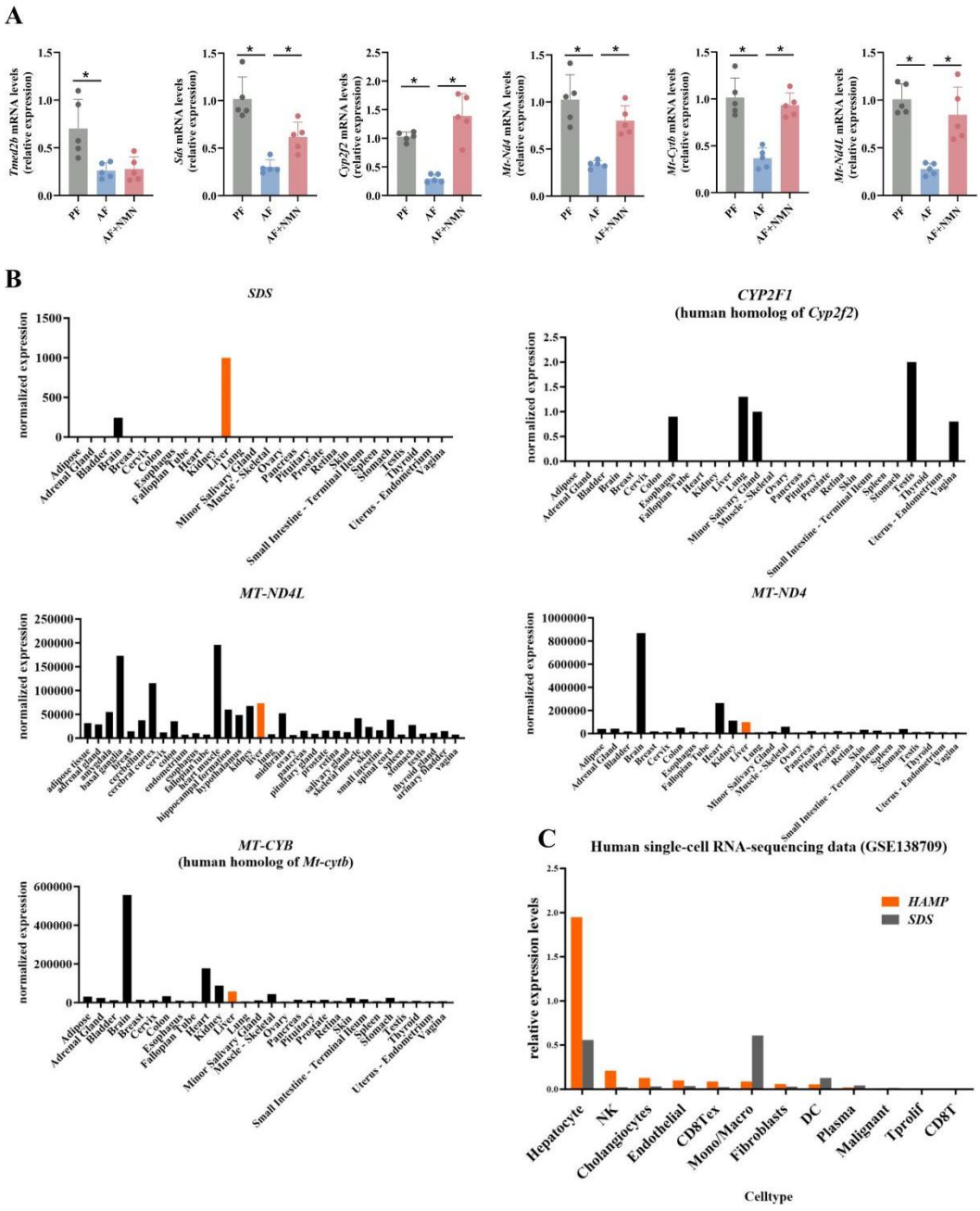
Supplementary Figure 2. NMN supplementation prevents alcohol-induced fat loss and suppresses adipose tissue lipolysis. (A) Body weight changes of mice (n = 8 mice/group). (B - E) The weights of epididymal white adipose tissue (eWAT), inguinal white adipose tissue (iWAT), brown adipose tissue (BAT), and total fat mass (n = 8 mice/group). (F and G) Plasma free fatty acids (FFA) and glycerol contents were measured using commercial enzymatic colorimetric assay kits (n = 8 mice/group). Data are presented as means ± SD. *$p < 0.05$ indicates statistically significant differences. PF, pair-fed; AF, alcohol-fed; AF + NMN, alcohol-fed treated with NMN.

Supplementary Figure 3



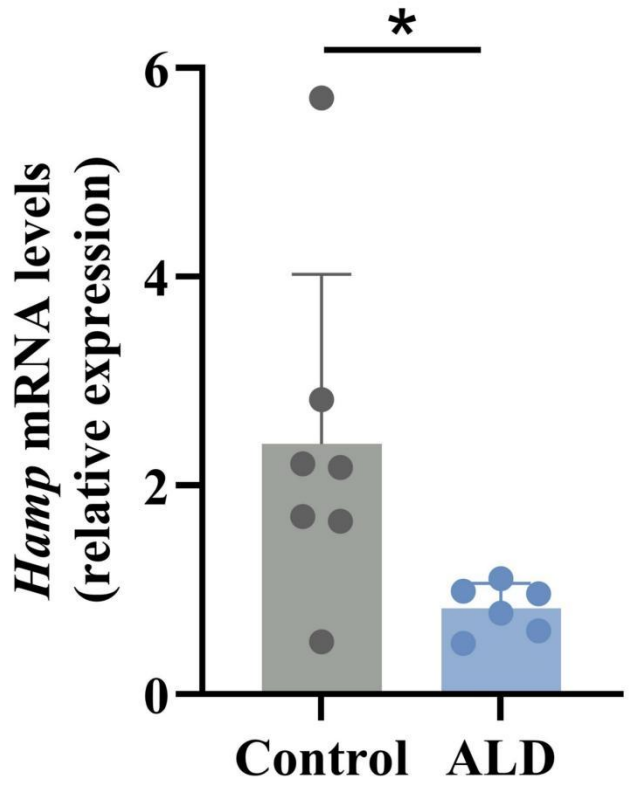
Supplementary Figure 3. Transcriptomic profiling of mouse livers. (A and B) Venn diagrams illustrating the overlap of differentially expressed genes (DEGs) identified by RNA-sequencing among the PF, AF, and AF+NMN groups (n = 3 mice/group). PF, pair-fed; AF, alcohol-fed; AF + NMN, alcohol-fed treated with NMN.

Supplementary Figure 4



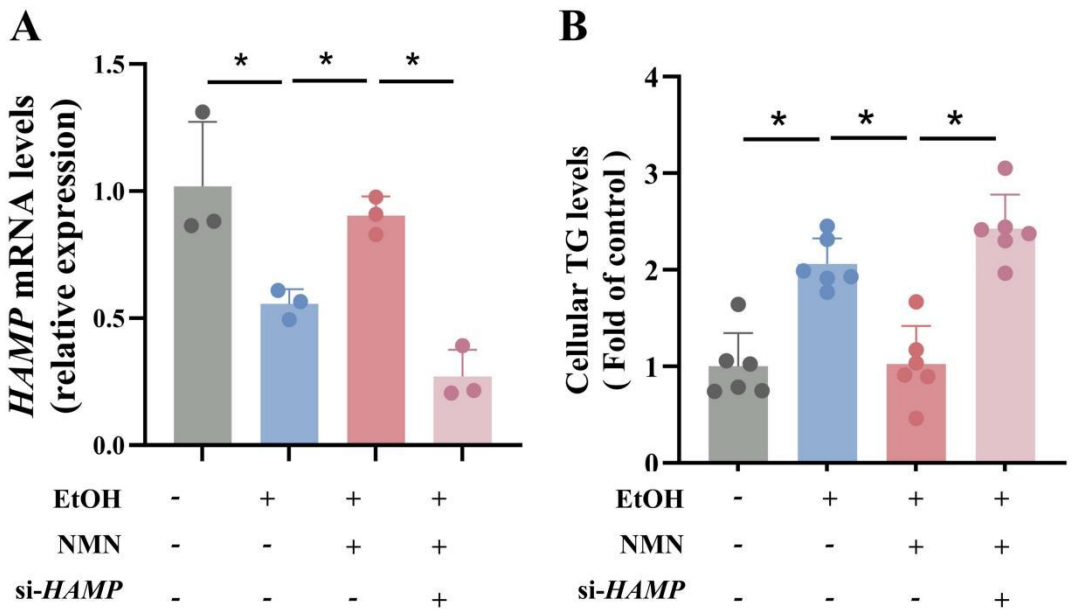
Supplementary Figure 4. Expression profiles of NMN-responsive genes. (A) The mRNA exprssion of *Tmed2b, Sds*, *Hamp*, *Cyp2f2*, *Mt-Nd4*, *Mt-Cytb*, *Mt-Nd4l*, and *Tmed2b* in mice liver (n = 5 mice/group). (B) Expression profiles of five genes across human tissues. Normalized expression (nTPM) for the indicated genes were obtained from the Genotype-Tissue Expression (GTEx) project. (C) *HAMP* and *SDS* expression levels in human liver cells (based on GSE138709). *$p < 0.05$ indicate statistically significant differences. PF, pair-fed; AF, alcohol-fed; AF + NMN, alcohol-fed treated with NMN.

Supplementary Figure 5



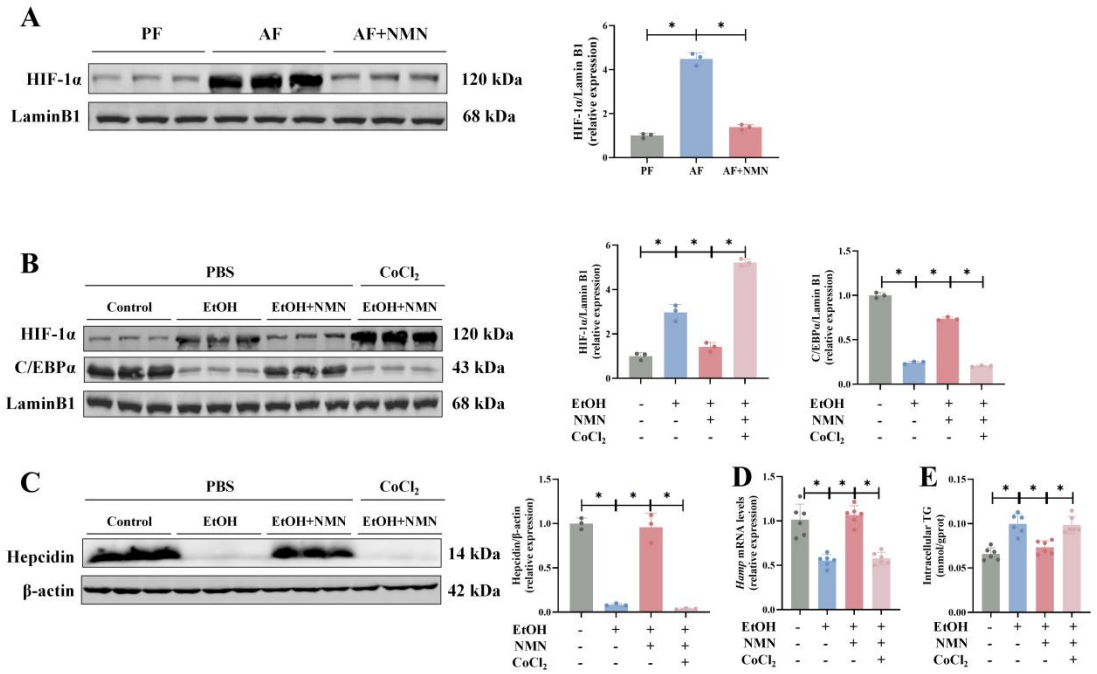
Supplementary Figure 5. The mRNA expressions of Hamp in the liver of ALD patients (n = 6 biologically independent samples) vs. healthy controls (n = 7 biologically independent samples). *$p < 0.05$ indicate statistically significant differences.

Supplementary Figure 6



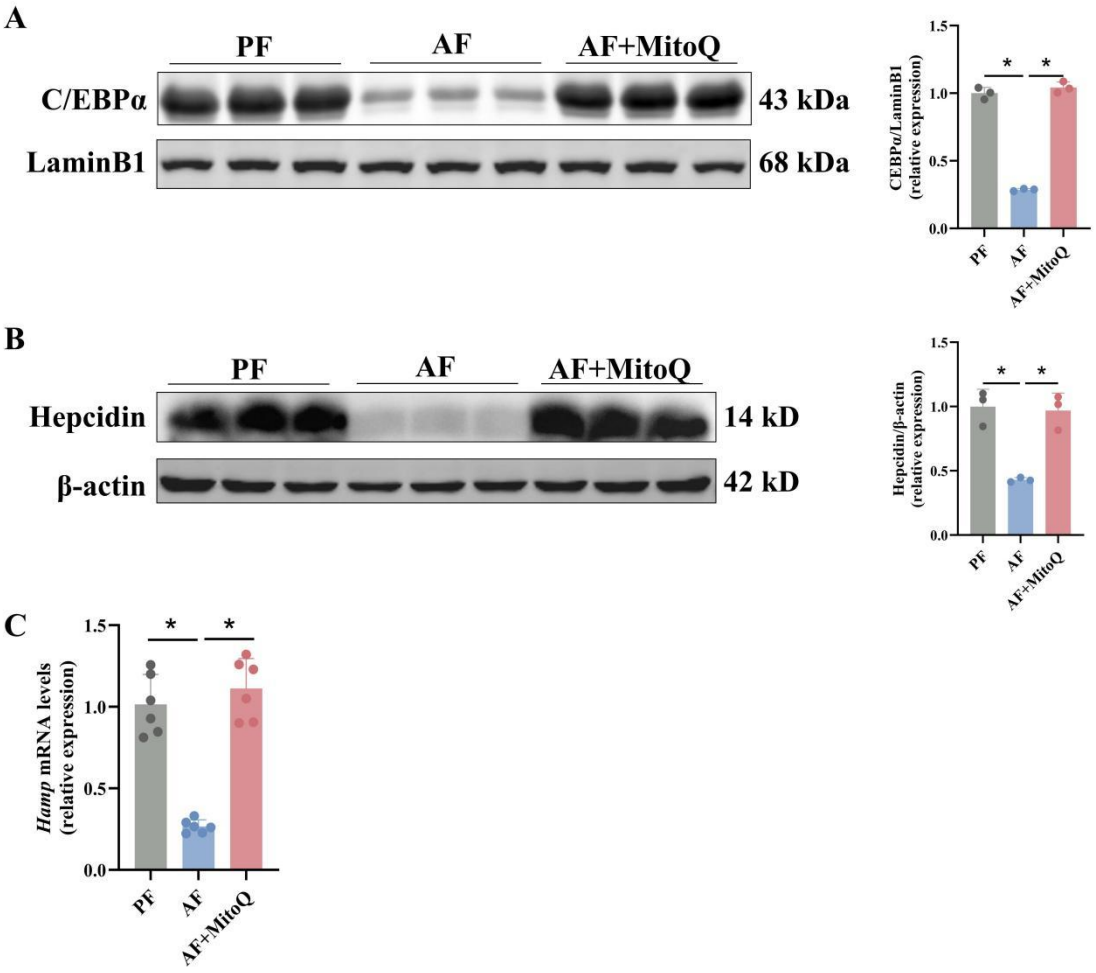
Supplementary Figure 6. The protective effect of NMN against ethanol-induced lipid accumulation is dependent on *HAMP* in human VL-17A hepatocytes. Human VL-17A cells (stably expressing ADH and CYP2E1) were transfected with specific siRNA targeting human *HAMP* or scramble siRNA (NC) for 6 h. To establish an in vitro therapeutic model, cells were first exposed to ethanol (100 mM) for 2 h, followed by continuous co-treatment with ethanol and 1 mM NMN for 16 h. (A) The relative mRNA expression levels of *HAMP* were determined by qRT-PCR (n = 3 biologically independent experiments). (B) Intracellular triglyceride (TG) levels were determined using an enzymatic colorimetric assay (n = 6 biologically independent experiments). Data are presented as means ± SD. *$p < 0.05$ indicates statistically significant differences.

Supplementary Figure 7



Supplementary Figure 7. HIF-1α activation abolishes the protective effect of NMN on the C/EBPα-Hamp pathway. (A) Liver nuclear HIF-1α protein expression (n = 3 mice/group). (B-E) For in vitro experiments, AML-12 hepatocytes were exposed to ethanol (100 mM) for 16 h, and NMN (2 mM) was added 2 h before alcohol treatment, while $CoCl_2$ (100 μmol/L) was added 2 h before NMN intervention. (B) Cell nuclear HIF-1α and C/EBPα protein and (C) nucleus Hepcidin protein expression (n = 3 biologically independent experiments). (D) The *Hamp* mRNA expression (n = 6 biologically independent experiments). (E) Intracellular lipid level was detected by enzymatic methods (n = 6 biologically independent experiments). Data are presented as means ± SD. *p < 0.05 indicate statistically significant differences. PF, pair-fed; AF, alcohol-fed; AF + NMN, alcohol-fed treated with NMN.

Supplementary Figure 8



Supplementary Figure 8. Effect of MitoQ treatment on the C/EBPα-Hamp pathway in ALD mice. Male C57BL/6 mice were subjected to the ALD modeling diet. MitoQ (5 mg/kg body weight) was administered daily via intraperitoneal injection. (A) Liver nuclear C/EBPα protein expression (n = 3 mice/group). (B) Liver nucleus Hepcidin protein expression (n = 3 mice/group). (C) The *Hamp* mRNA expression (n = 6 mice/group). Data are presented as means ± SD. **p* < 0.05 indicate statistically significant differences.

Supplementary Figure 9. Uncropped Blot Images for western blots in Figures.

Figure 4E

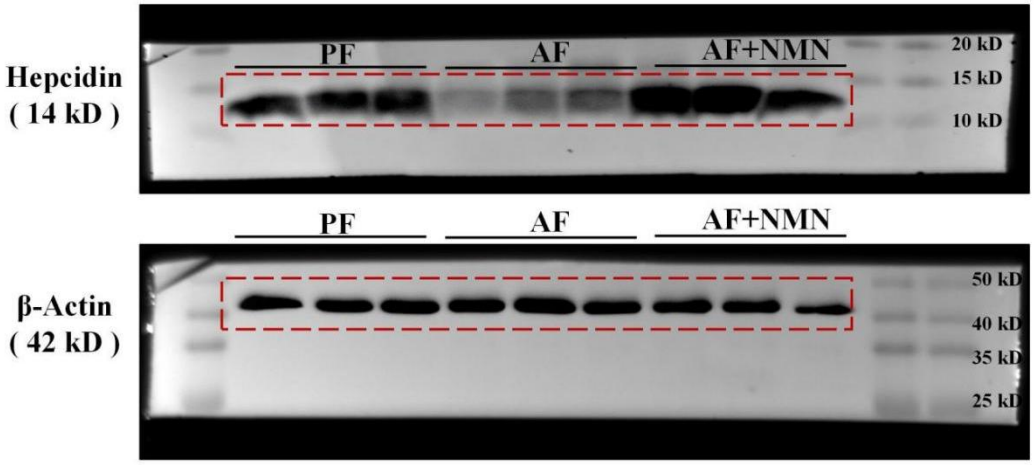


Figure 7D

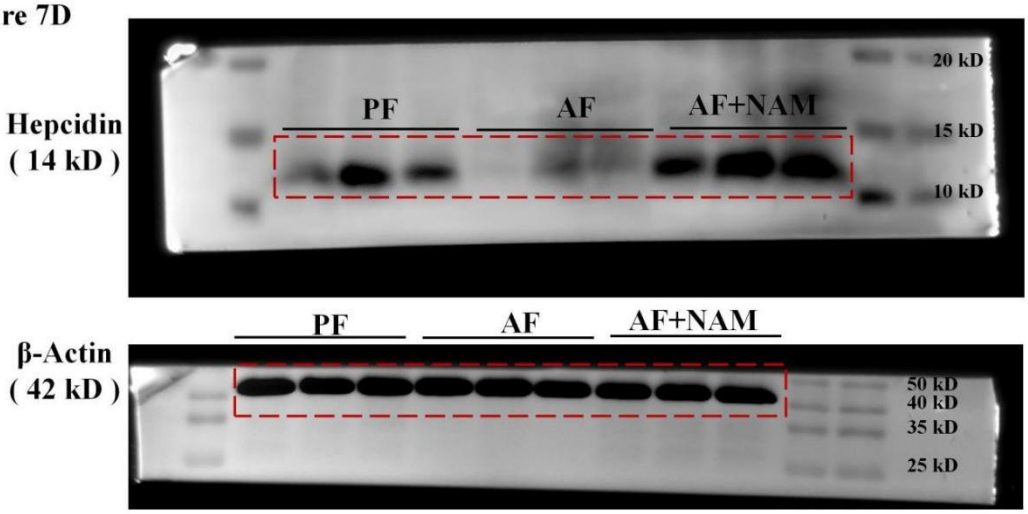


Figure 7E

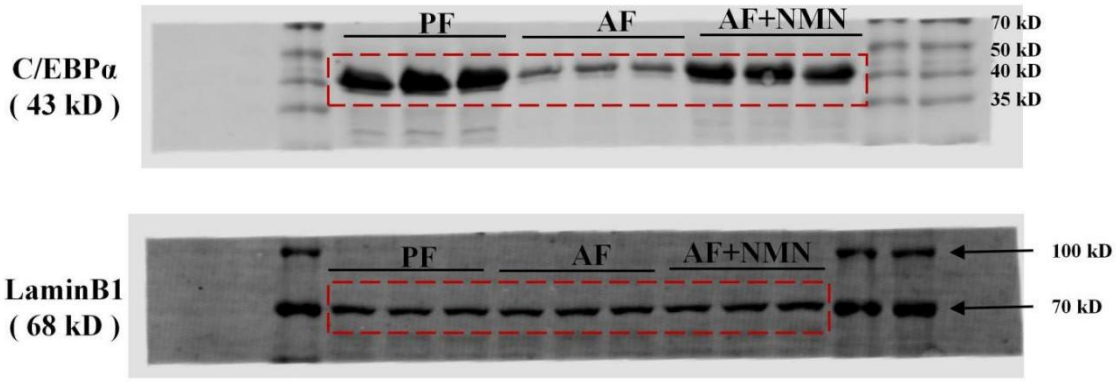


Figure 7F

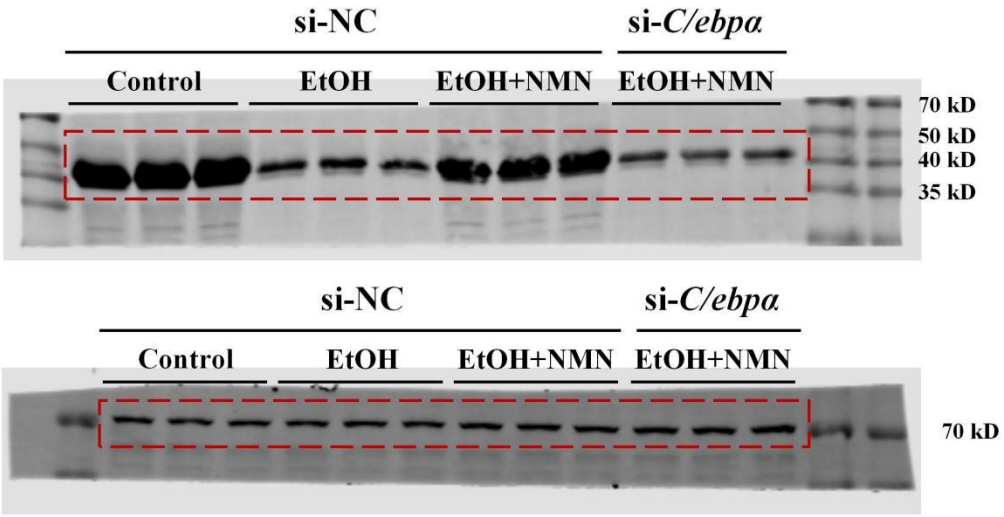


Figure 7H

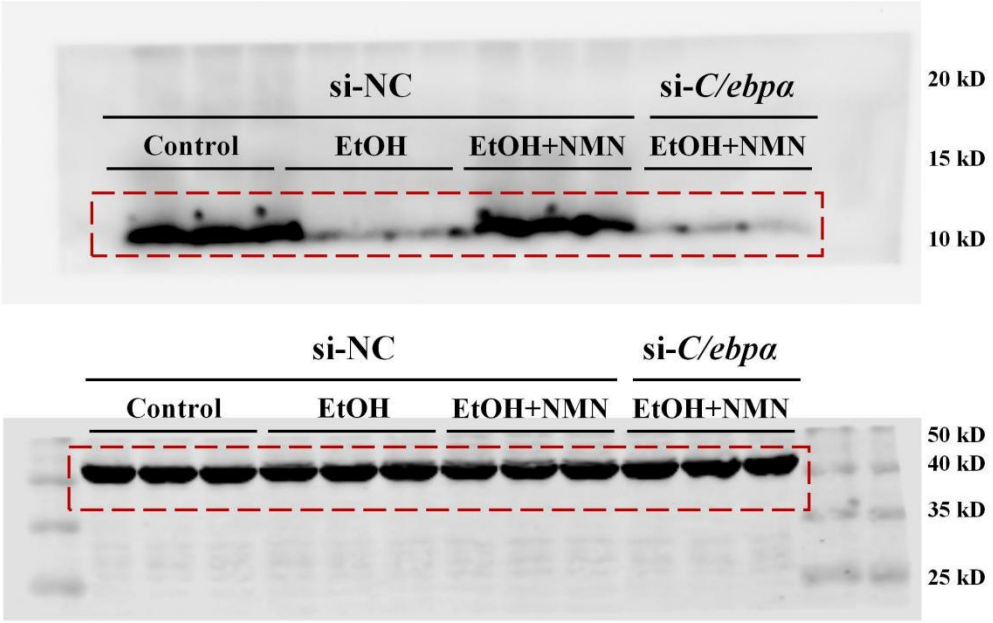


Figure S4A

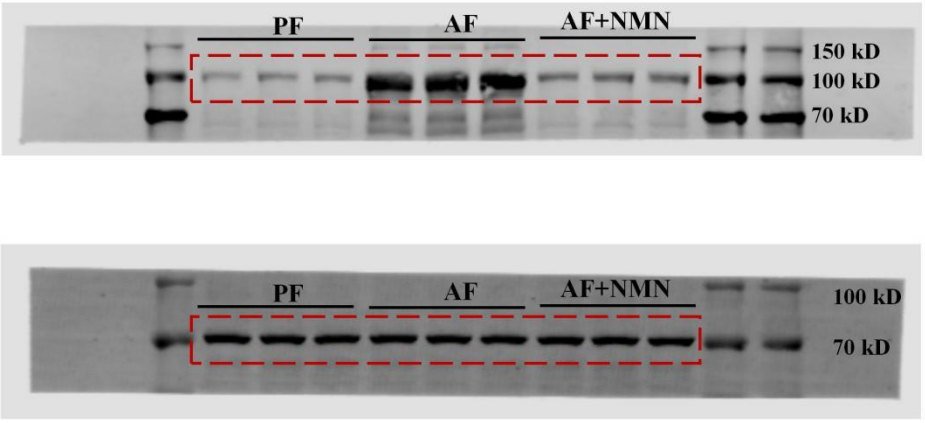


Figure S4B

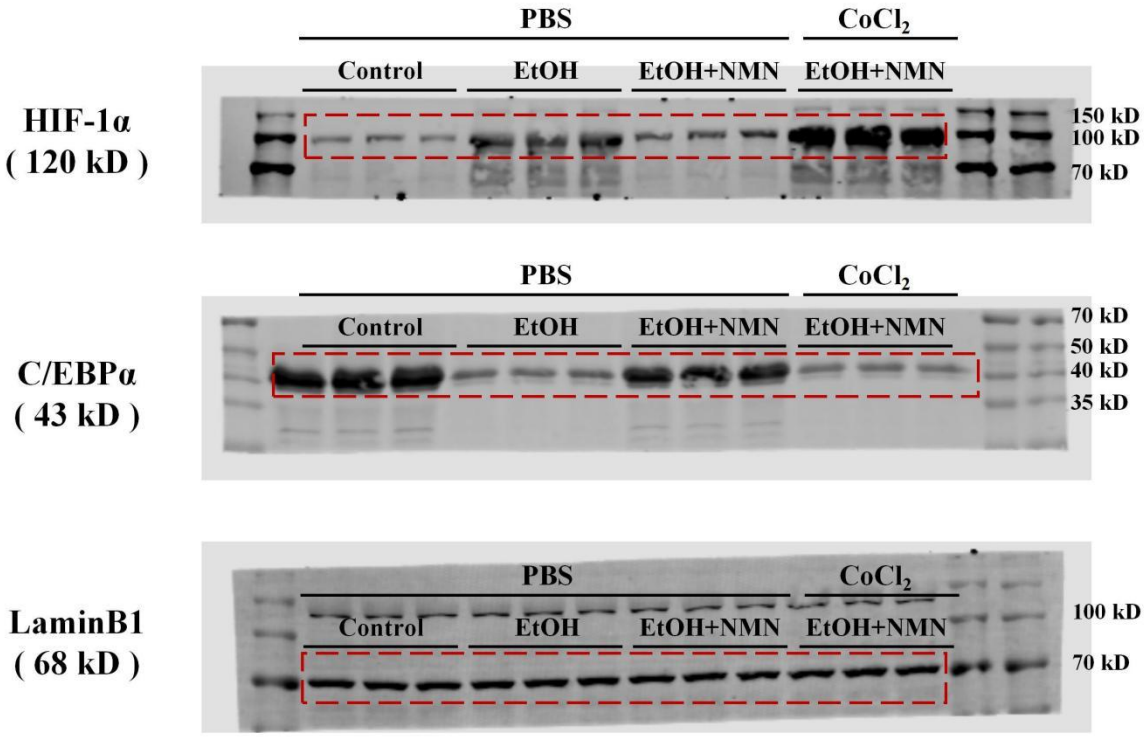


Figure S4C

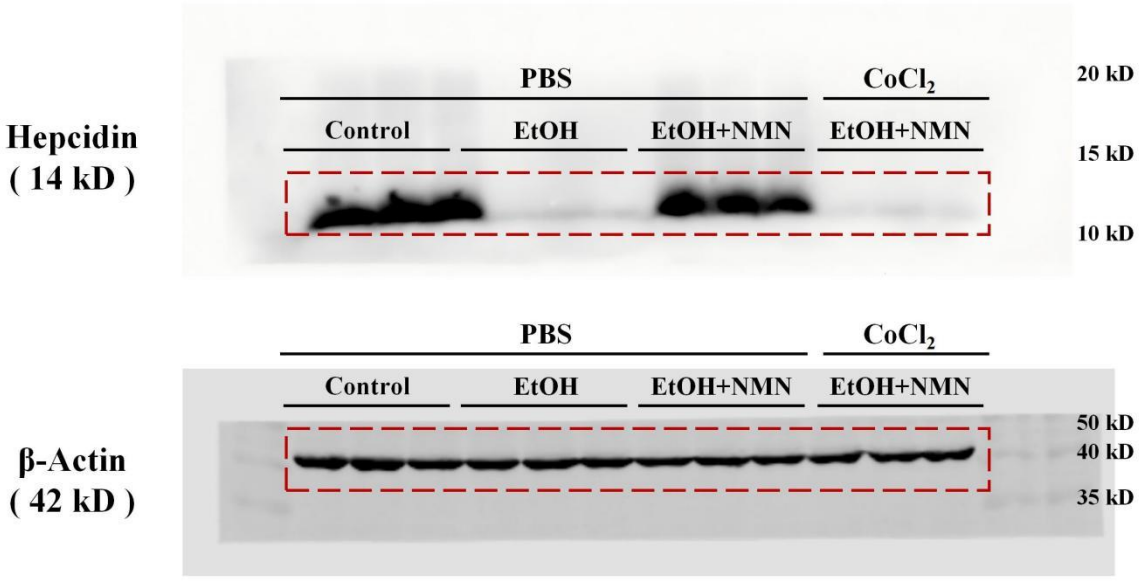


Figure S7A

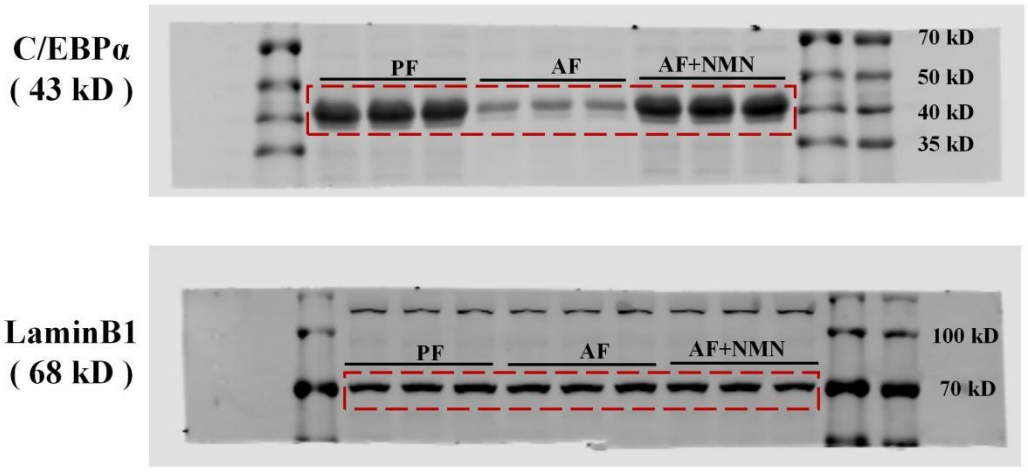
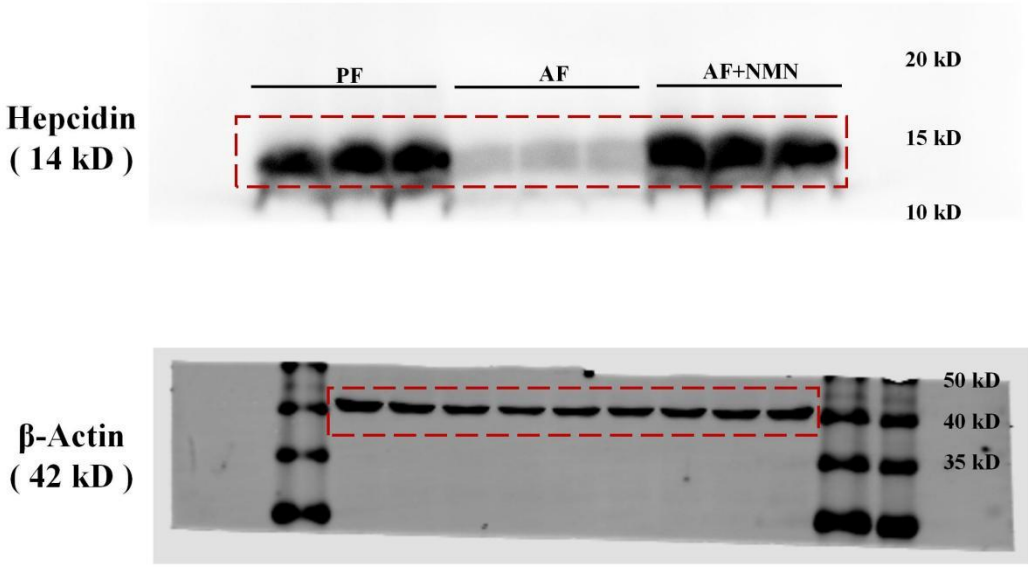


Figure S7B



Reference

1. National Workshop On Fatty Liver And Alcoholic Liver Disease, Chinese Society Of Hepatology, Chinese Medical Association & Fatty Liver Expert Committee, Chinese Medical Doctor Association. [guidelines of prevention and treatment for alcoholic liver disease: A 2018 update]. *Chin. J. Hepatol.* **26**, 188–194 (2018).
2. Ding, Q. *et al.* Hepatic NMNAT1 is required to defend against alcohol-a- ssociated fatty liver disease. *Science Advances* (2025).
3. Hao, L. *et al.* ATF4 activation promotes hepatic mitochondrial dysfunction by repressing NRF1-TFAM signalling in alcoholic steatohepatitis. *Gut* **70**, 1933–1945 (2021).
4. Ding, W.-X. *et al.* Autophagy reduces acute ethanol-induced hepatotoxicity and steatosis in mice. *Gastroenterology* **139**, 1740–1752 (2010).
5. Donohue, T. M., Osna, N. A. & Clemens, D. L. Recombinant hep G2 cells that express alcohol dehydrogenase and cytochrome P450 2E1 as a model of ethanol-elicited cytotoxicity. *Int J Biochem Cell Biol* **38**, 92–101 (2006).