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hnRNPU/TrkB Defines a Chromatin Accessibility Checkpoint for Liver Injury and Nonalcoholic Steatohepatitis Pathogenesis

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BACKGROUND AND AIMS: Nonalcoholic steatohepatitis (NASH) is a progressive liver disease that is characterized by liver injury, inflammation, and fibrosis. NASH pathogenesis is linked to reprogramming of chromatin landscape in the liver that predisposes hepatocytes to stress-induced tissue injury. However, the molecular nature of the putative checkpoint that maintains chromatin architecture and preserves hepatocyte health remains elusive.

APPROACH AND RESULTS: Here we show that heterogeneous nuclear ribonucleoprotein U (hnRNPU), a nuclear matrix protein that governs chromatin architecture and gene transcription, is a critical factor that couples chromatin disruption to NASH pathogenesis. RNA-seq and chromatin immunoprecipitation-seq studies revealed an extensive overlap between hnRNPU occupancy and altered gene expression during NASH. Hepatocyte-specific inactivation of hnRNPU disrupted liver chromatin accessibility, activated molecular signature of NASH, and sensitized mice to diet-induced NASH pathogenesis. Mechanistically, hnRNPU deficiency stimulated the expression of a truncated isoform of TrkB (TRKB-T1) that promotes inflammatory signaling in hepatocytes and stress-induced cell death. Brain-derived neurotrophic factor treatment reduced membrane TRKB-T1 protein and protected mice from diet-induced NASH.

CONCLUSIONS: These findings illustrate a mechanism through which disruptions of chromatin architecture drive the emergence of disease-specific signaling patterns that promote liver injury and exacerbate NASH pathogenesis. (Hepatology 2020;71:1228-1246).

The epidemic of metabolic syndrome has greatly increased the risk for type 2 diabetes, cardiovascular disease, and nonalcoholic fatty liver disease (NAFLD). NAFLD is characterized by chronic accumulation of excess fat in the liver and represents the most common metabolic liver disease linked to obesity and insulin resistance.[1-5] NAFLD encompasses a spectrum of liver pathologies ranging from relatively benign hepatic steatosis to nonalcoholic steatohepatitis (NASH), first described by Ludwig et al. in 1980.[6] The latter is a more severe form of fatty liver disease that is associated with progressive liver injury, tissue inflammation, and liver fibrosis. NASH pathogenesis is caused by multifactorial mechanisms that lead to pathological hepatic fat accumulation, stress-induced hepatocyte cell death, and persistently elevated inflammatory and tissue repair response.[2,3] Genetic polymorphisms of several loci have been demonstrated to contribute to NAFLD risk in humans,[1,5,7] underscoring the dysregulations of gene expression and/or

Abbreviations: AAV, adeno-associated virus; ALT, alanine aminotransferase; AMLN, Amylin NASH; BDNF, brain-derived neurotrophic factor; ANOVA, analysis of variance; AST, aspartate aminotransferase; ATAC-seq, Assay for Transposase-Accessible Chromatin using sequencing; CDAHFD, choline-deficient, L-amino acid-defined HFD containing 0.1% methionine; Ctrl, control; DCN, Decorin; H&E, hematoxylin and eosin; ERK, extracellular signal-regulated kinase; FDR, False Discovery Rate; GFP, green fluorescent protein; HFD, high-fat diet; hnRNPU, heterogeneous nuclear ribonucleoprotein U; JNK, c-Jun N-terminal kinase; LKO, liver-specific hnRNPU knockout; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; PA, palmitic acid; qPCR, real-time quantitative PCR; scRNA-seq, single-cell RNA-seq; TNF-α, tumor necrosis factor α; TAG, triacylglycerol; UCSC, University of California, Santa Cruz.

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function as an important pathogenic factor in the initiation and progression of NASH.

The chromatin in eukaryotic cells stores genome information in a highly organized three-dimensional structure. Dynamic regulation of chromatin structure and accessibility is a fundamental aspect of transcriptional control in response to external signals.[8,9] In addition, chromatin-remodeling complexes and histone- and DNA-modifying enzymes act in concert with transcription factors to fine-tune gene expression.[10-12] Hepatic gene expression is exquisitely responsive to nutritional and hormonal signals and undergoes reprogramming in disease states such as NAFLD. Recent transcriptomic and proteomic studies have delineated a core molecular signature of diet-induced NASH in mice[13,14] and in human NASH livers.[15,16] Several features of NASH-associated alterations of hepatic gene expression appear to be conserved during human NASH pathogenesis, including aberrant regulation of metabolic genes and induction of genes involved in immunity, inflammatory response, and fibrosis. The latter reflects a coordinated transcriptional response among multiple liver cell types during NASH development.[2,17] Despite this, the molecular nature of the nuclear factors that govern hepatocyte chromatin accessibility in liver disease remains elusive. Here we show that hnRNPU, a nuclear matrix protein, serves an indispensable role in the control of hepatic chromatin accessibility and gene expression. Hepatocyte-specific inactivation of hnRNPU exacerbates diet-induced NASH pathogenesis in mice, in part, through the aberrant induction of a truncated TrkB isoform that promotes inflammatory signaling in hepatocytes and stress-induced cell death.

Materials and Methods

ANIMAL AND HUMAN STUDIES

All animal studies were performed following the guideline established by the University Committee on Use and Care of Animals at the University of Michigan. Mice containing floxed hnRNPU were generated as described.[18] The Alb-Cre transgenic mice were purchased from the Jackson Laboratory. For chow feeding, mice were fed Teklad 5001 laboratory diet. High-fat diet (HFD) contains 60% kcal% fat (D12492). The Amylin NASH (AMLN; D09100301) diet contains 40% kcal% fat (18% of which was trans fat), 22% fructose, and 2% cholesterol. Choline-deficient, L-amino acid-defined HFD (40% kcal%) containing 0.1% methionine (CDAHFD; A06071309) was used as an alternative NASH-inducing diet. All three special diets were purchased from Research Diets Inc. Human liver samples were obtained from the Liver Tissue Cell Distribution System at University of Minnesota and have been described.[19] Informed consent in writing was obtained from each patient and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the appropriate institutional review committee.

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PRIMARY HEPATOCYTES STUDIES

Primary hepatocytes were isolated using collagenase digestion from wild-type C57BL/6J mice, hnRNPU control (Ctrl), or liver-specific hnRNPU knockout (LKO) mice.[20] Adenoviral infection was performed on the same day of isolation. After 6 hours, transduced hepatocytes were switched to serum-free Dulbecco's modified Eagle's medium (DMEM) and cultured overnight before treatments. Tumor necrosis factor α (TNF-α; 20 ng/mL) was added 2 hours after the treatment of palmitic acid (PA; 200 μM or 400 μM).

qPCR AND IMMUNOBLOTTING ANALYSIS

Real-time quantitative PCR (qPCR) was performed as described,[21] using primers listed in Supporting Table S1. Immunoblotting was performed as described,[19] using antibodies listed in Supporting Table S2. For immunoprecipitation, liver nuclei were isolated from frozen liver tissues from mice fed chow or NASH diet as described.[22] Nuclear extracts were incubated with control immunoglobulin G or antibody specific for hnRNPU in the presence of protein G beads. Proteins associated with beads were analyzed by immunoblotting using antibodies specific for acetyllysine or phosphoserine/threonine.

ISOLATION OF MEMBRANE TRKB-T1

Hepa 1-6 mouse hepatoma cells (American Type Culture Collection, CRL-1830) were used to establish cell lines stably expressing TrkB-T1. Cells were transduced with Murine Stem Cell Virus retroviral vector or a recombinant vector expressing TrkB-T1 followed by selection in the presence of puromycin (5 μg/mL) for at least 2 weeks. Stably transduced cells were switched to serum-free DMEM for 12 hours before treatment with brain-derived neurotrophic factor (BDNF; 248-BD, R&D) for 3 hours. Cell membrane fraction was extracted by ultracentrifugation, as described.[23]

PLASMA ENZYMES AND LIVER TAG ANALYSES

Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) concentrations were measured using kits from Stanbio. Total liver triacylglycerol (TAG) was extracted and measured as described.[19]

HISTOPATHOLOGIC ANALYSIS AND IMMUNOFLUORESCENCE STAINING

Formalin-fixed, paraffin-embedded mouse liver sections were stained with hematoxylin and eosin (H&E) to evaluate steatosis and inflammatory cells infiltration. Liver fibrosis was assessed by Picrosirius (Sirius) red (Polysciences, #24901) staining. For immunofluorescence microscopy, liver tissues were fixed with 10% formalin for 3 hours, 30% sucrose in phosphate-buffered saline (PBS) overnight, embedded into an optimal cutting temperature compound. Cryosections were permeabilized in 0.1% Triton X-100 in PBS for 30 minutes, blocked with 10% horse serum for 1 hour, labeled with antibodies against mouse Decorin (DCN; R&D AF1060), F4/80 (Bio-Rad MCA497G), CD4 (Thermo Fisher 14-0042-82), or CD8a (Thermo Fisher 14-0808-82) overnight, followed by incubation with a fluorophore-conjugated secondary antibody (Jackson Immuno Research) for 1 hour. The stained sections were mounted with 4′,6-diamidino-2-phenylindole-containing mounting medium (VECTASHIELD) and then viewed on a Nikon A-1-A fluorescence microscope.

LIVER ATAC SEQUENCING

Nuclei from frozen liver tissue were isolated as described.[24] A total of 50,000 isolated nuclei in each sample (Ctrl n = 6; LKO n = 6) were used in transposase reaction (Illumina). The libraries were sequenced on NovaSeq-6000 instrument with 50-bp paired-end reads after 2-step PCR amplification.[25] Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq) analysis pipeline was adapted from previous study.[26] The down-regulated (692) and up-regulated (2,045) peaks in LKO mice were independently used for motif-enrichment analysis (HOMER, findMotifs.pl). The University of California, Santa Cruz (UCSC) genome browser was used to visualize the ATAC-seq coverage. Both raw and processed data have been deposited into Gene Expression Omnibus database (GSE131336).

RNA-seq DATA ANALYSIS

Liver RNA sequencing was performed on BGI 500 instrument after polyA-selected mRNA isolation from HFD-fed control and hnRNPU LKO mice. The fastq files were aligned to the mouse reference genome (mm10) using the aligner STAR. All RNA-seq raw data and processed data have been deposited into the GEO database (GSE131336). Gene enrichment analysis was performed using MetaScape (www.metascape.org).

STATISTICAL ANALYSIS

All results are presented as mean ± SEM. Student *t* test was used to analyze the differences between two groups. Two-way analysis of variance (ANOVA) with multiple comparisons was used for statistical analysis of gene expression with the treatment of vehicle or PA plus TNF-α. A *P* value of less than 0.05 was considered statistically significant. Statistical methods and corresponding *P* values are indicated in figure legends.

Results

LIVER-SPECIFIC INACTIVATION OF hnRNPU EXACERBATES DIET-INDUCED NASH PATHOGENESIS

hnRNPU is a nuclear matrix protein that maintains three-dimensional genome architecture in hepatocytes through its association with chromatin.[27,28] These molecular observations led us to postulate that hnRNPU may serve as a chromatin checkpoint for liver metabolism and disease. In support of this, we found that over 20% of hepatic genes exhibiting hnRNPU occupancy were either up-regulated (508) or down-regulated (668) by more than 1.5-fold following AMLN diet–induced NASH (Fig. 1A; GSE75335 and GSE119340). Whereas hnRNPU mRNA and protein expression in the liver remained similar in healthy and NASH livers, serine/threonine phosphorylation of hnRNPU was increased following diet-induced NASH (Supporting Fig. S1). These findings suggest that posttranslational modifications of hnRNPU may play a role in modulating its function during NASH pathogenesis. The extensive overlap between hnRNPU chromatin association and transcriptomic reprograming in the liver prompted us to generate liver-specific hnRNPU knockout mice (LKO) and determine how chromatin structure governed by hnRNPU contributes to NASH pathogenesis. Compared with hnRNPU$^{flox/flox}$ control (Ctrl), mice with hepatocyte-specific inactivation of hnRNPU (Alb-Cre; hnRNPU$^{flox/flox}$) were slightly smaller and exhibited normal blood glucose and plasma TAG levels and liver histology when fed standard rodent chow (Supporting Fig. S2A,B). Whereas plasma ALT was slightly elevated in LKO mice, hepatic expression of genes involved in glucose and lipid metabolism was largely unaffected by hnRNPU deficiency (Supporting Fig. S2C,D).

To probe the role of hnRNPU in the liver under metabolic stress conditions, we fed control and LKO mice CDAHFD to induce NASH pathogenesis. CDAHFD feeding has been shown to elicit the full spectrum of NASH pathologies, including hepatic steatosis, liver inflammation, and fibrosis.[29] Whereas hnRNPU LKO mice weighed slightly less than control, liver-to-weight ratio was higher than control group following 3 weeks of CDAHFD feeding (Supporting Fig. S3A) and appeared more rugged on the surface and rubbery on touch. Whereas liver TAG content and blood glucose levels remained comparable, plasma ALT and AST concentrations were significantly higher in LKO mice than control, indicating that hnRNPU inactivation in hepatocytes sensitized mice to CDAHFD-induced liver injury (Fig. 1B). Consistently, we observed more pronounced immune cell infiltration and liver fibrosis in LKO mice (Fig. 1C-F). Immunofluorescence staining indicated that the abundance of F4/80+ macrophages and CD4+ and CD8+ T cells was markedly elevated in LKO mouse livers (Fig. 1D,F; Supporting Fig. S3B,C). Deposition of pericellular collagen and accumulation of DCN, a proteoglycan secreted by hepatic stellate cells, was markedly enhanced by hnRNPU deficiency (Fig. 1C,E,F). Ductular reaction is a common feature of chronic liver injury in liver diseases including NASH.[30] Analysis of hepatic gene expression indicated that mRNA expression of genes associated with bile duct epithelial proliferation and ductular reaction (*Spp1*, *Gli2*, *Afp*, *Prom1*, *Cd24a*, and *Ncam1*) was significantly increased in hnRNPU

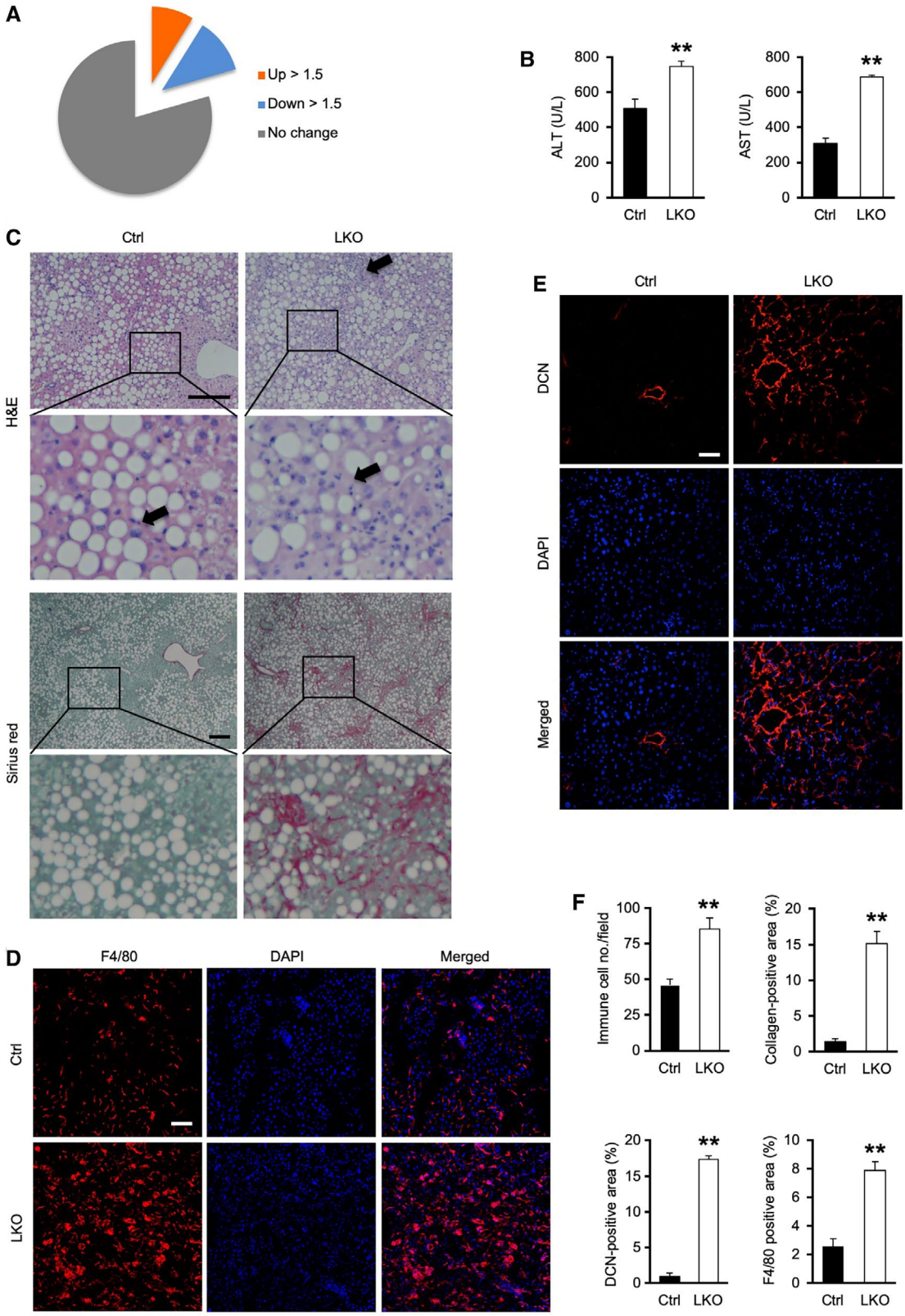
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FIG. 1. Hepatocyte-specific inactivation of hnRNPU exacerbates CDAHFD-induced NASH. (A) Pie chart depicting NASH regulation of the liver genes containing binding sites for hnRNPU. (B) Plasma ALT and AST levels. (C) H&E and Sirius red staining of liver sections from mice fed CDAHFD for 3 weeks. Arrowheads indicate infiltrated immune cells. Scale bars: 200 μm. (D) F4/80 immunofluorescence staining of liver sections. Scale bars: 100 μm. (E) DCN immunofluorescence staining of liver sections. Scale bars: 100 μm. (F) Quantification of immune cell infiltration, collagen deposition, and DCN- and F4/80-positive areas. Data in (B) and (F) represent mean ± SEM (Ctrl n = 8; LKO n = 6). $^{**}P < 0.01$, Ctrl versus LKO, two-tailed unpaired Student *t* test. Abbreviation: DAPI, 4′,6-diamidino-2-phenylindole.

LKO mice following CDAHFD feeding (Supporting Fig. S4A,B). Consistently, we observed increased bile epithelial expansion and osteopontin immunoreactivity near the portal area in LKO mouse livers (Supporting Fig. S4C,D). These findings illustrate that hnRNPU inactivation elicits a full spectrum of NASH-associated liver pathologies.

Chronic HFD feeding promotes obesity, insulin resistance, and hepatic steatosis but fails to induce liver fibrosis in mice. In a separate set of studies, we examined whether hnRNPU deficiency sensitized mice to develop NASH pathologies on HFD feeding. Following 6 months of HFD feeding, hnRNPU LKO mice gained approximately 15% less body weight and exhibited lower liver-to-body weight ratio than control (data not shown). Histological analysis and liver TAG measurements indicated that although LKO mice exhibited slightly less severe hepatic steatosis, they had increased immune cell infiltration in the liver and developed more severe liver fibrosis as revealed by Sirius red staining (Fig. 2A,B). As such, pericellular collagen accumulation was readily detectable in hnRNPU-deficient mouse livers but remained largely absent in control mice. As liver fibrosis is a hallmark of NASH pathogenesis, these findings strongly suggest that hepatic hnRNPU functions to restrict the progression from steatosis to NASH.

REPROGRAMMING OF THE LIVER TRANSCRIPTOME IN RESPONSE TO hnRNPU INACTIVATION

To delineate the effects of hnRNPU deficiency on liver transcriptome, we performed RNA-seq analysis on total liver RNA from HFD-fed control and LKO mice. Our analysis identified a total of 483 genes that were differentially regulated by hnRNPU inactivation (False Discovery Rate (FDR) < 0.1). Gene ontology analysis indicated that down-regulated genes (202) were enriched for monocarboxylic acid and lipid metabolism, whereas up-regulated genes (281) were primarily responsible for host defense, innate immune response, and inflammation (Fig. 2C). Our recent RNA-seq studies comparing healthy and NASH livers defined a molecular signature of diet-induced NASH in mice.[14] Remarkably, 89 of 281 genes elevated in hnRNPU LKO livers were also induced in NASH mouse livers (Fig. 2D). qPCR analysis confirmed the induction of genes involved in type I interferon response (*Ifit1*, *Ifit3*, *Ifi27l2a*, *Ifih1*) and macrophage function (*C1qc*, *Cd5l*, *Gpnmb*, *Trem2*) in LKO livers (Fig. 2E). These results indicate that hnRNPU deficiency elicits transcriptional reprogramming in the liver that is characteristic of NASH pathogenesis.

We recently performed single-cell RNA-seq (scRNA-seq) analysis of liver cells in healthy and diet-induced NASH mice.[31] This scRNA-seq data set contains gene expression data for hepatocytes and nonparenchymal cells at the single-cell resolution. To delineate the cellular origins of hnRNPU-regulated genes in the liver, we obtained expression values for these genes among different liver cell types from the scRNA-seq data set. Clustering analysis revealed that a large proportion of the up-regulated and down-regulated genes were enriched in hepatocytes (Supporting Fig. S5A), supporting the notion that hnRNPU deficiency likely directly perturbed hepatocyte gene expression. Several cell types involved in immune response, including macrophage, T cell, B cell, and dendritic cell, were enriched for genes that were up-regulated in hnRNPU LKO livers (Supporting Fig. S5B). These integrated data analyses illustrate a coordinated transcriptomic response among diverse liver cell types as a result of hepatocyte-specific hnRNPU inactivation.

hnRNPU GOVERNS LIVER CHROMATIN ACCESSIBILITY

We next determined how hnRNPU inactivation alters liver chromatin accessibility and transcriptome during NASH pathogenesis. We performed ATAC-seq on liver nuclei isolated from six pairs of HFD-fed control and LKO mice to delineate the role of

hnRNPU in governing liver chromatin accessibility.[25] We identified a total of 159,578 transposase-accessible chromatin peaks, 2,045 and 692 of which showed decreased and increased accessibility (FDR < 0.1), respectively, as a result of hnRNPU inactivation (Fig. 3A). Using more stringent criteria, we revealed

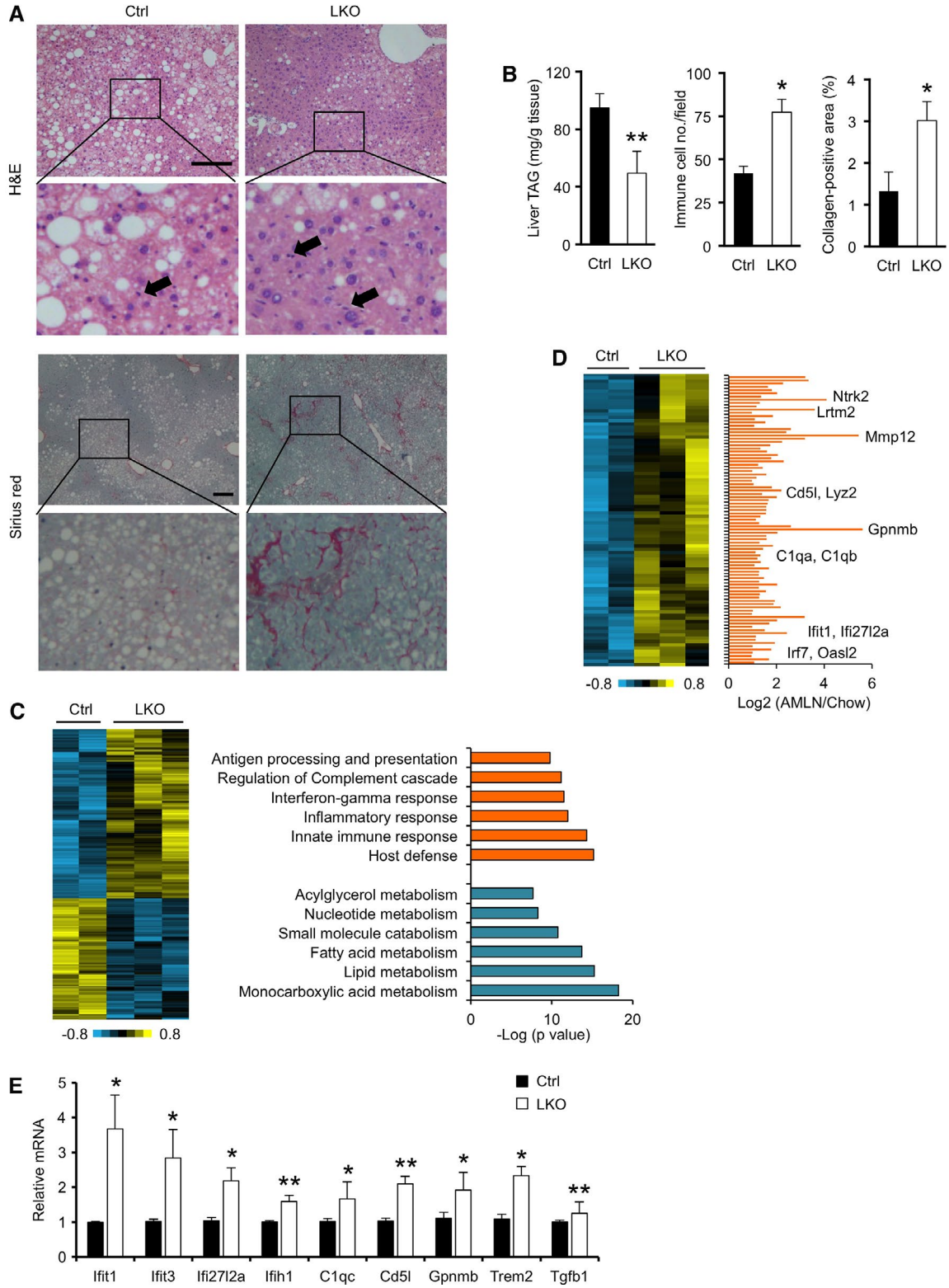


FIG. 2. hnRNPU deficiency worsens NASH pathologies in mice following HFD feeding. (A) H&E and Sirius red staining of liver sections. Arrowheads indicate infiltrated immune cells. Scale bars: 200 μm. (B) Quantification of liver TAG content, immune cell infiltration, and collagen deposition. (C) Heat map of hepatic genes up-regulated or down-regulated in hnRNPU LKO mice (left). Enrichment of biological processes in these two clusters is indicated (right). (D) A cluster of hepatic genes up-regulated in HFD-fed LKO mice (heat map, left) and following AMLN diet–induced NASH (bar graph, right). Liver RNA-seq data from mice fed chow or AMLN diet were used for the bar graph. (E) qPCR analysis of hepatic gene expression in HFD-fed mice (Ctrl n = 6; LKO n = 10). Data in (B) and (C) represent mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$; Ctrl versus LKO, two-tailed unpaired Student *t* test.

a list of ATAC-seq peaks exhibiting differential regulation by over 2-fold in LKO livers with FDR < 0.05 (Fig. 3B). As such, multiple ATAC peaks within the Cyp4a10 locus were significantly lower in hnRNPU LKO livers (Fig. 3C). On the contrary, chromatin accessibility for Ntrk2, which encodes the TrkB receptor tyrosine kinase, was enhanced by hnRNPU deficiency. We next performed transcription factor motif-enrichment analysis on ATAC-seq peaks that were differentially regulated by hnRNPU inactivation. We observed that binding sites predicted for several transcription factors, including ETS, GATA4, SOX9, ERRA, and ZNF711, were highly enriched at genomic locations exhibiting reduced accessibility in hnRNPU LKO mouse livers (Fig. 3D). In contrast, the top five motifs enriched for up-regulated chromatin peaks corresponded to predicted binding sites for HNF1B, RARA, THRB, SOX18, and FOXC1. These results illustrate a global effect of hnRNPU inactivation on liver chromatin accessibility in both directions.

hnRNPU DEFICIENCY ENHANCES INFLAMMATORY SIGNALING AND HEPATOCYTE INJURY

As shown above, hnRNPU LKO mice developed more severe NASH pathologies following CDAHFD feeding. Accordingly, mRNA expression of genes involved in liver fibrosis (*Col1a1*, *Tgfb1*, *Timp1*) and inflammatory response (*Trem2*, *Gpnmb*, *Ms4a7*, *H2-Ab1*, *Ccl2*) was elevated in the LKO mouse livers (Supporting Fig. S6A,B). Immunoblotting analysis revealed that hnRNPU deficiency increased protein expression of TRAF2, a mediator of signaling by the TNF family receptors, and markedly increased protein levels of p52, a key effector of the noncanonical nuclear factor kappa B (NF-kB) pathway.[32] Other factors involved in inflammatory signaling, including RELB and phosphorylated p65, p38, and c-Jun N-terminal kinase (JNK), and phosphorylated extracellular signal-regulated kinase (ERK) were also elevated in hnRNPU LKO mouse livers (Supporting Fig. S6C).

To determine whether hnRNPU regulates hepatocyte inflammatory signaling in a cell-autonomous manner, we performed studies using primary hepatocytes isolated from control and hnRNPU LKO mice. Consistent with *in vivo* results, protein levels of p52, phosphorylated p65, and TrkB-T1 were elevated in hnRNPU-deficient hepatocytes (Fig. 4A). Exposure of hepatocytes to a combination of PA and TNF-α has been used to mimic NASH-associated metabolic stress and inflammatory signaling.[19,33] hnRNPU deficiency further increased p52 and p-JNK protein levels, as well as nuclear p65 level, in hepatocytes in response to acute treatments (15 minutes) with PA plus TNF-α (Fig. 4A,B). Following a more prolonged PA plus TNF-α treatment, hnRNPU-deficient hepatocytes showed increased sensitivity to stress-induced cell death, as indicated by elevated levels of cleaved poly(adenosine diphosphate ribose) polymerase and Caspase 3, markers of apoptotic cell death (Fig. 4C). We previously demonstrated that FADD-like apoptosis regulator plays an important role in protecting hepatocytes from stress-induced cell death.[19] Consistently, protein levels of FADD-like apoptosis regulator were diminished in the absence of hnRNPU (Fig. 4C). Induction of IL1b, Tnf, and Nos2 expression was also augmented in knockout hepatocytes (Fig. 4D). These results demonstrated that hnRNPU deficiency enhances inflammatory signaling in hepatocytes in response to metabolic stress and cytokines, thereby promoting liver injury and NASH progression.

TrkB INDUCTION IN NASH LIVER PREDISPOSES HEPATOCYTES TO STRESS-INDUCED INJURY

The above ATAC-seq and RNA-seq studies revealed a crucial role of hnRNPU in governing liver chromatin architecture and transcriptome. Notably,

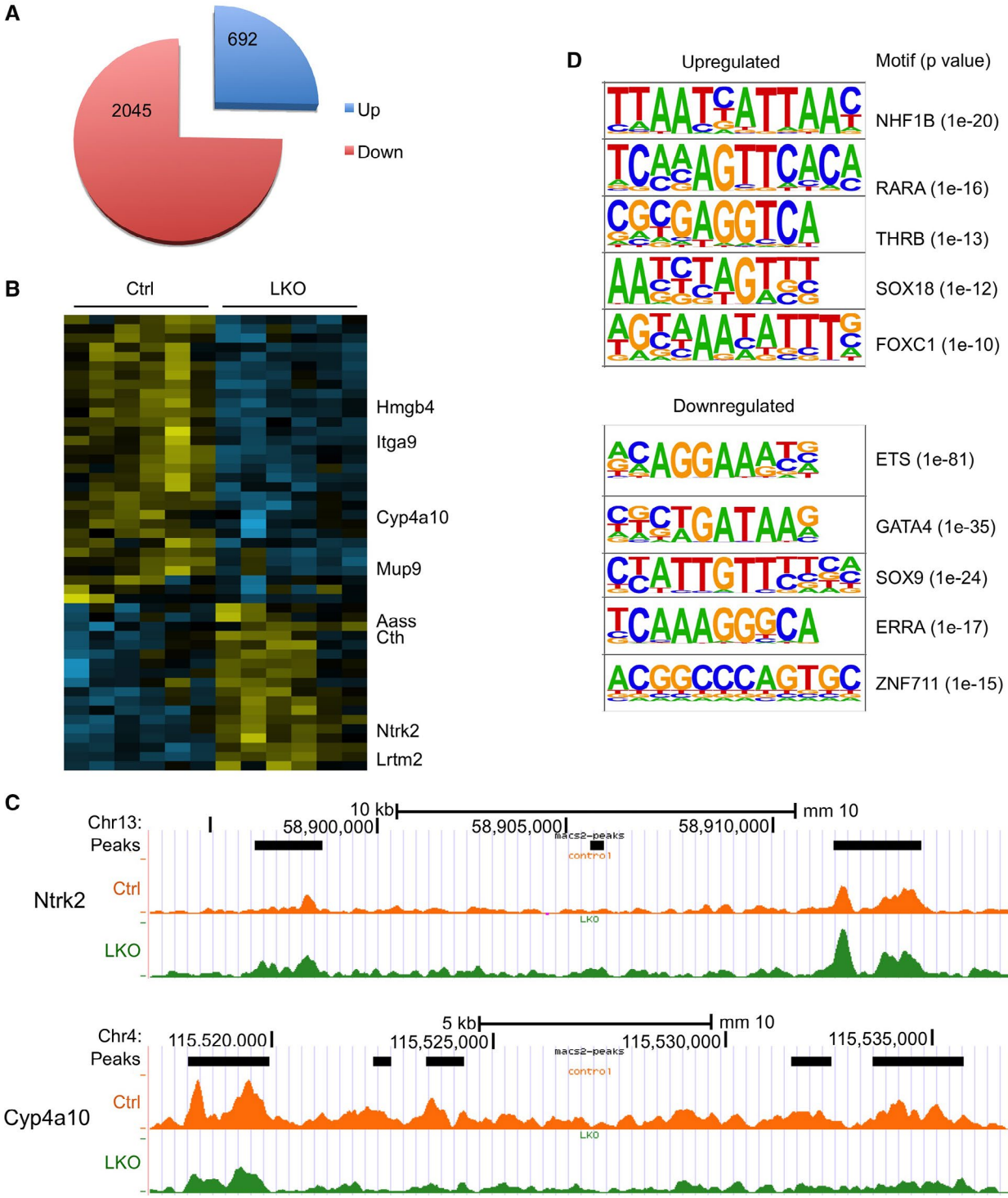


FIG. 3. ATAC-seq analysis of liver chromatin accessibility. ATAC-seq was performed on liver nuclei isolated from Ctrl (n = 6) and LKO (n = 6) mice after 6 months of HFD feeding. (A) Pie chart showing ATAC-seq peaks altered by hnRNPU deficiency. (B) Heat map representation of peaks increased or decreased by over 2-fold in hnRNPU LKO mouse livers. (C) UCSC genome browser tracks near Ntrk2 and Cyp4a10. (D) Motif-enrichment analysis of the up-regulated and down-regulated ATAC-seq peaks.

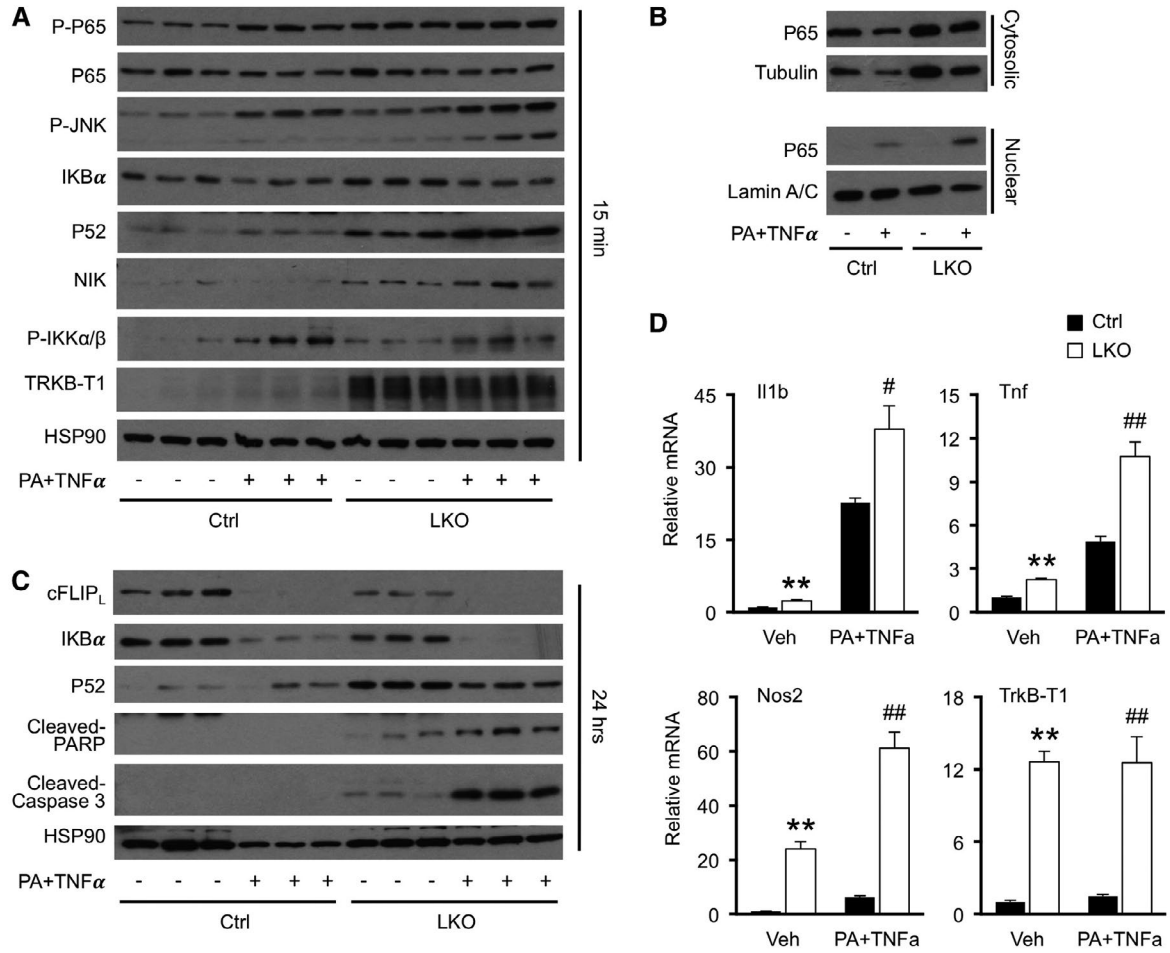
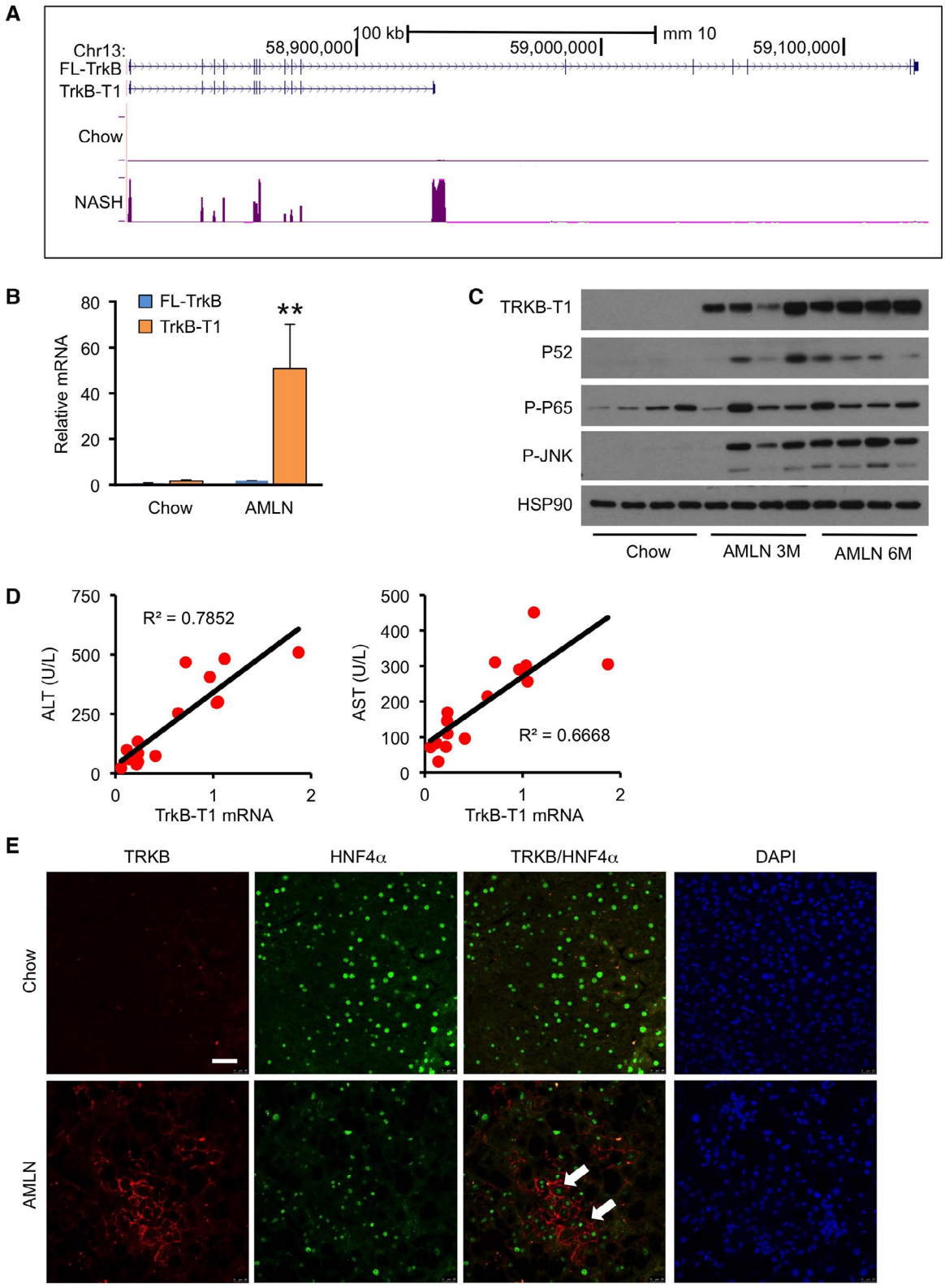


FIG. 4. Cell-autonomous regulation of hepatocyte signaling and gene expression by hnRNPU. The following experiments were performed in primary hepatocytes prepared from Ctrl or LKO mice. Hepatocytes were cultured in serum-free media overnight before treatments. PA (200 μM) was added 2 hours before to the addition of TNF-α (20 ng/mL). (A) Immunoblots of total lysates from hepatocytes treated with vehicle (-) or PA + TNF-α (+) for 15 minutes. (B) Immunoblots of cytosolic or nuclear extracts from treated hepatocytes. (C) Immunoblots of total lysates from hepatocytes treated with vehicle (-) or PA + TNF-α (+) for 24 hours. (D) qPCR analysis of gene expression from hepatocytes treated with vehicle or PA + TNF-α (+) for 6 hours (n = 3). Data represent mean ± SEM. $^{**}P < 0.01$, Ctrl versus LKO in vehicle-treated cells. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, Ctrl versus LKO in hepatocytes treated with PA + TNF-α. Two-way ANOVA with multiple comparisons. Abbreviation: Veh, vehicle.

hnRNPU deficiency increased chromatin accessibility at the Ntrk2 locus and its mRNA expression (Figs. 2E, 3B,C). Two predominant isoforms of TrkB correspond to the full-length isoform (FL-TrkB) and a truncated isoform (TrkB-T1) that lacks most of the intracellular domains.[34,35] The TrkB-T1 transcript originates from the use of an alternative 3′ exon, resulting in premature termination of transcription and production of a truncated protein. Inspection of liver RNA-seq tracks from mice fed chow or AMLN diet revealed that NASH increased the expression of TrkB in an isoform-specific manner (Fig. 5A). qPCR analysis using isoform-specific primers indicated that TrkB-T1, but not FL-TrkB, was markedly increased during AMLN diet–induced NASH (Fig. 5B). Consistent with these qPCR results, we observed strong induction of TRKB-T1 protein expression in NASH liver lysates following 3 and 6 months of AMLN diet feeding (Fig. 5C). The induction of TrkB-T1 expression was associated with elevated P52 levels and increased phosphorylation of P65 and JNK, molecular markers of inflammatory cytokine signaling during NASH pathogenesis.

We next examined whether hepatic TrkB-T1 mRNA expression is associated with severity of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Chow
AMLN

FIG. 5. Induction of TrkB-T1 expression in mouse NASH. (A) UCSC genome browser view of Ntrk2 liver RNA-seq reads from mice fed chow or AMLN diet (n = 3). (B) qPCR analysis of TrkB isoforms in the liver from mice fed chow or AMLN diet (n = 3) using isoform-specific primers. Data represent mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$; AMLN versus chow, two-tailed unpaired Student *t* test. (C) Immunoblots of total liver lysates from chow or ALMN diet–fed mice. (D) Correlation of liver TrkB-T1 mRNA expression with plasma ALT and AST in a cohort of AMLN diet–fed wild-type mice exhibiting different severity of liver injury (n = 15). (E) Representative TrkB immunofluorescence in chow- and AMLN diet–fed mouse livers. Arrowheads indicate HNF4α-positive hepatocytes. Scale bars: 100 μm. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; HNF4α, hepatocyte nuclear factor 4α.

liver injury in NASH. In a cohort of wild-type mice fed AMLN diet exhibiting different degree of liver injury, we observed that hepatic TrkB-T1 expression strongly correlated with plasma ALT and AST levels (Fig. 5D). Using immunofluorescence staining, we confirmed that TrkB induction was restricted to hepatocytes, which were marked by nuclear staining of hepatocyte nuclear factor 4α, a hepatocyte-specific transcription factor (Fig. 5E). Importantly, increased *TrkB-T1* mRNA and protein expression was observed in human NASH liver biopsies (Fig. 6A,B). Similar to diet-induced NASH in mice, increased *TrkB*-T1 expression was associated with induction of JNK and P65 phosphorylation and increased P52 protein expression, suggesting that both the canonical and noncanonical NF-kB pathway is enhanced during human NASH pathogenesis. Interestingly, protein levels of RIPK3 and phosphorylated MLKL, two markers of necroptotic cell death, and cleaved Caspase 3 were increased in human NASH livers (Fig. 6B). We performed qPCR and immunoblotting analyses on control and hnRNPU LKO mouse livers using isoform-specific primers and confirmed that hnRNPU inactivation significantly increased hepatic expression of TrkB-T1 under both HFD and CDAHFD feeding conditions (Fig. 6C,D). The induction of TRKB-T1 expression was also observed in hnRNPU-deficient hepatocytes, indicating that this is a cell-autonomous effect of hnRNPU (Fig. 4A). These results illustrate that *TrkB*-T1 induction is a common molecular feature of NASH pathogenesis in mice and humans. In addition, hnRNPU regulates the expression of TrkB in an isoform-specific manner.

The biological function of the truncated TrkB isoform in hepatocytes remains unexplored. TRKB-T1 stimulation has been shown to trigger ERK phosphorylation and regulates actin dynamics in glia cells.[36-38] However, BDNF appeared to have modest effects on ERK phosphorylation and actin cytoskeletal organization in primary hepatocytes (Supporting Fig. S7). To investigate whether TrkB-T1 plays a protective or detrimental role in stress signaling, we transduced cultured primary hepatocytes with recombinant adenoviral vectors expressing green fluorescent protein (GFP) or TrkB-T1 and examined their response to PA/TNF-α treatments. At the baseline, we observed that adenoviral overexpression of TrkB-T1 led to slightly elevated levels of P52 and phosphorylated P65 and JNK in transduced hepatocytes. On PA/TNF-α treatments for 15 minutes, TrkB-T1 overexpression augmented the stimulation of JNK and P65 phosphorylation (Fig. 6E), and nuclear translocation of P65 (Fig. 6F), suggesting that TrkB-T1 facilitates activation of signaling pathways in response to metabolic stress and cytokine stimulation. Compared with GFP control, the levels of cleaved Caspase 3 were markedly higher in hepatocytes transduced with adenoviral TrkB-T1 following prolonged exposure to PA/TNF-α. Analysis of gene expression in treated hepatocytes indicated that TrkB-T1 overexpression augments the induction of Tnf, Il1b, Nos2, and Il6 expression under PA/TNF-α treated condition (Fig. 6G). These studies demonstrate that TrkB-T1 overexpression is sufficient to enhance inflammatory signaling in hepatocytes in response to stress signals relevant for NASH pathogenesis.

BDNF REDUCES MEMBRANE TRKB-T1 AND PROTECTS MICE FROM DIET-INDUCED NASH

TRKB-T1 contains an extracellular ligand-binding domain identical to FL-TRKB, yet it lacks the intracellular receptor tyrosine kinase domain. As a result, TRKB-T1 is unable to trigger the canonical downstream signaling pathway in response to its ligand BDNF. Interestingly, we found that BDNF treatment of mouse hepatoma cells stably overexpressing TRKB-T1 diminished membrane TRKB-T1 protein levels (Fig. 7A), whereas the levels of epidermal growth factor receptor remained largely unchanged by BDNF. Importantly, BDNF treatment decreased endogenous TRKB-T1 protein levels in primary hepatocytes isolated from hnRNPU LKO mice and

ameliorated PA/TNF-α-induced Casp3 cleavage and hepatocyte cell death (Fig. 7B,C). These results suggest that BDNF may directly modulate stress-induced hepatocyte injury through its regulation of TRKB-T1 protein levels. Although the exact mechanisms through which BDNF reduces membrane TRKB-T1 remain unknown, a likely scenario is that ligand binding triggers endocytosis and subsequent

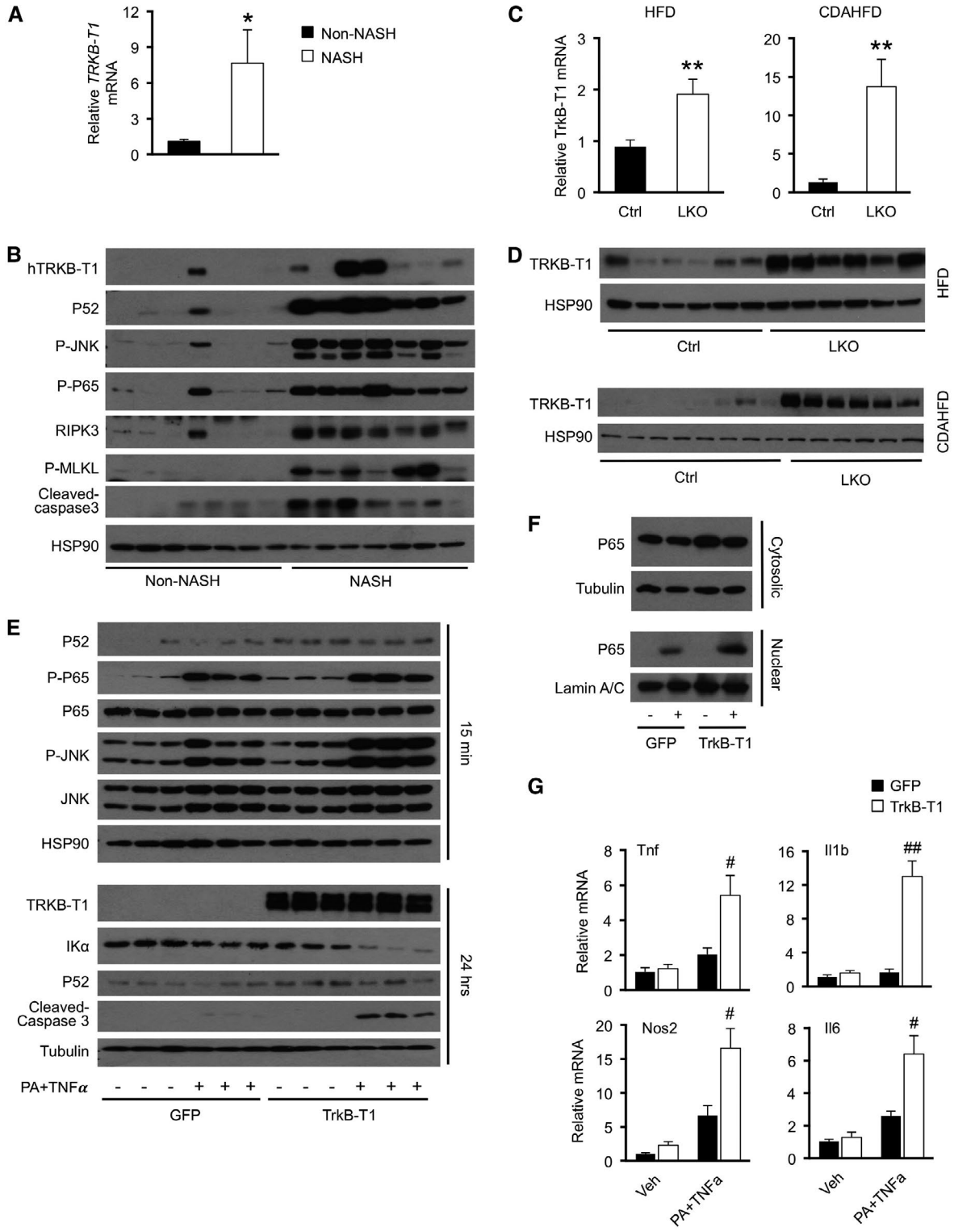

FIG. 6. Regulation and functional role of TRKB-T1 in hepatocyte stress response. (A) qPCR analysis of *TRKB-T1* expression in liver biopsies obtained from non-NASH control (n = 7) and patients with NASH (n = 7). Data represent mean ± SEM. $^{*}P < 0.05$, NASH versus control, two-tailed unpaired Student *t* test. (B) Immunoblots of total liver lysates from non-NASH and NASH individuals. (C) qPCR analysis of liver TrkB-T1 expression in control and hnRNPU LKO mice fed HFD (Ctrl n = 6; LKO n = 10) or CDAHFD (Ctrl n = 8; LKO n = 6). Data represent mean ± SEM. $^{**}P < 0.01$, LKO versus Ctrl, two-tailed unpaired Student *t* test. (D) Immunoblots of total liver lysates from mice fed HFD or CDAHFD. (E) Immunoblots of total cell lysates from primary hepatocytes transduced with GFP or TrkB-T1 adenovirus followed by treatment with vehicle (-) or PA + TNF-α (+) for 15 minutes (top) or 24 hours (bottom). (F) Immunoblots of cytosolic or nuclear extracts from transduced hepatocytes treated with vehicle (-) or PA + TNF-α (+) for 15 minutes. (G) qPCR analysis of gene expression in transduced hepatocytes treated with vehicle or PA + TNF-α for 6 hours (n = 3). Data represent mean ± SEM. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, TrkB-T1 versus GFP in hepatocytes treated with PA + TNF-α. Two-way ANOVA with multiple comparisons. Abbreviation: Veh, vehicle.

lysosomal degradation of TRKB-T1 in hepatocytes. In fact, BDNF has been reported to bind TRKB and promote its endocytosis and degradation.[39]

A prediction of the above findings is that BDNF overexpression may diminish TRKB-T1 protein levels in the liver, thereby alleviating diet-induced NASH pathogenesis. To test this, we generated a recombinant adeno-associated virus (AAV) overexpressing BDNF under the control of liver-specific thyroid hormone-binding globulin promoter. As expected, tail vein injection of AAV-BDNF resulted in robust BDNF mRNA and protein expression in the liver (Fig. 7D; Supporting Figs. S8D and S9A). BDNF overexpression had modest effects on plasma parameters, liver histology, and hepatic gene expression when mice were fed chow diet (Supporting Fig. S8). Following AMLN diet feeding, mice transduced with AAV-BDNF exhibited significantly lower plasma ALT and AST levels and reduced P52 and RIPK3 protein levels and P65, P38, and JNK phosphorylation in the liver than control (Fig. 7E). mRNA expression of proinflammatory genes (*Ccl4*, *Il10*, *Cd74*, *H2-Ab1i Gpnmb*, and *Trem2*) and fibrosis markers (*Col1a1* and *Col1a2*) was significantly decreased by BDNF overexpression (Fig. 7F). Although TrkB-T1 mRNA levels remained similar between two groups, its protein expression was notably lower in the AAV-BDNF group (Fig. 7D). Histological staining indicated that hepatic steatosis, liver fibrosis, and DCN accumulation were markedly improved in mice transduced with AAV-BDNF (Fig. 8A,B). Liver inflammation, as indicated by the abundance of macrophages and T cells, was greatly attenuated in response to BDNF overexpression (Fig. 8C,D; Supporting Fig. S9B,C). Together, these results demonstrate that elevating BDNF levels is sufficient to reduce TRKB-T1 protein expression and ameliorate diet-induced NASH pathologies in mice.

Discussion

The liver transcriptome is highly responsive to nutritional, hormonal, and circadian cues. Reprogramming of the liver transcriptome as a consequence of altered epigenetic state and chromatin accessibility in hepatocytes is a fundamental feature of NAFLD.[14,40,41] Despite this, the molecular nature of the regulatory network that governs chromatin architecture in the liver remains obscure. In this study, we uncovered hnRNPU as a regulator of chromatin accessibility across the genome that functions to protect hepatocytes from stress-induced cell injury (Fig. 8E). Hepatocyte-specific inactivation of hnRNPU exacerbated diet-induced NASH pathogenesis in mice, including liver injury, inflammation, and fibrosis. hnRNPU is a nuclear matrix protein that regulates three-dimensional chromatin structure and gene transcription.[27,28] In the heart, hnRNPU is required for postnatal development and cardiac function.[18] The expression levels of hnRNPU in the liver remained largely unchanged during diet-induced NASH in mice and in human NASH, suggesting that other mechanisms are likely at work to regulate the dynamic chromatin response. In support of this, hnRNPU phosphorylation at serine/threonine residues appeared to be enhanced in diet-induced NASH. However, the exact residues, their upstream kinases, and the effects of hnRNPU phosphorylation on its biological functions remain currently unknown. Previous studies have demonstrated that hnRNPU interacts with a number of nuclear proteins including heterochromatin protein 1 alpha, the transcription factor Zbtb7b, and the long noncoding RNA Blnc1.[21,42,43] It is likely that regulation of these hnRNPU-interacting factors may also underlie its effects on chromatin structure in response to physiological and pathophysiological stimuli.

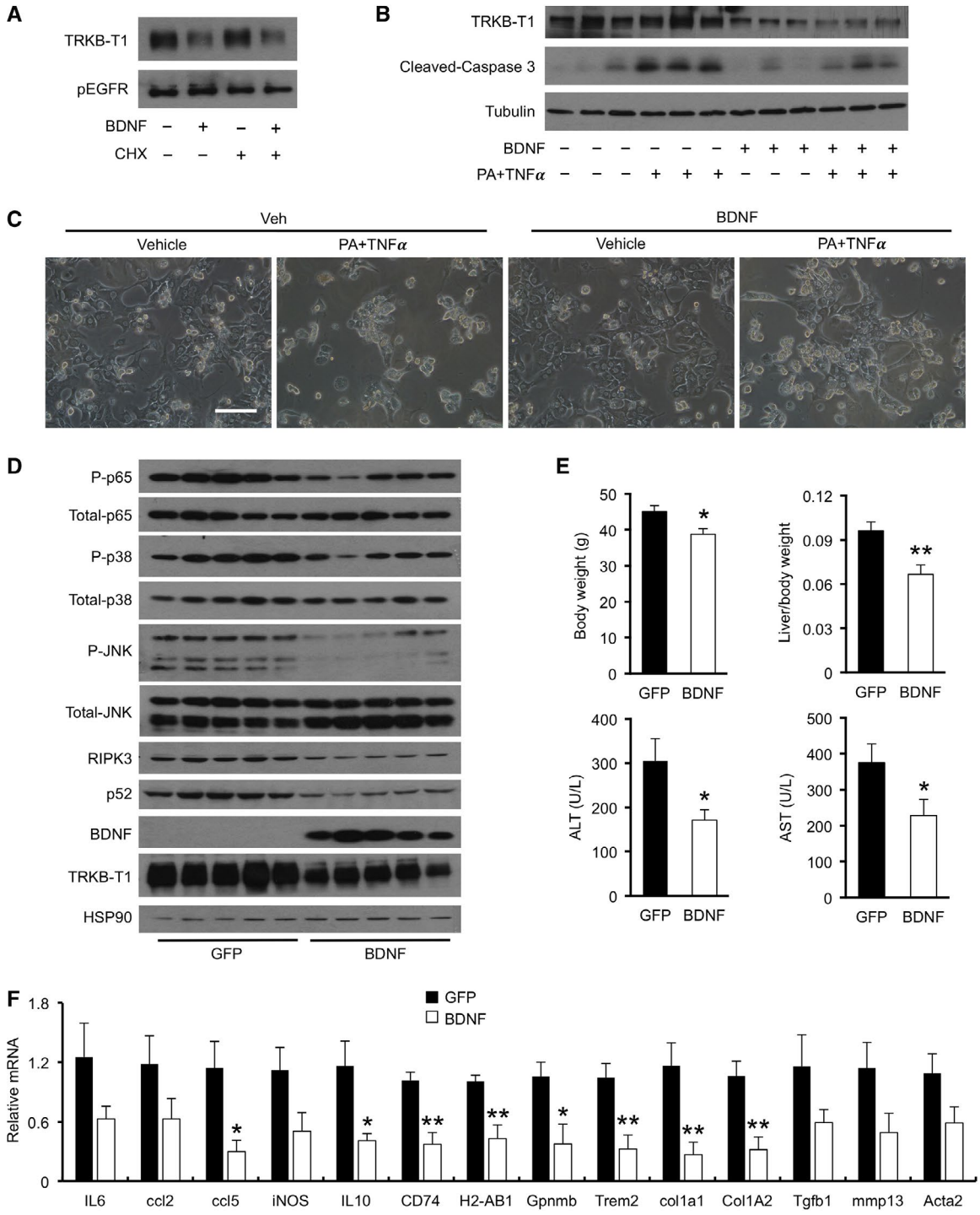

FIG. 7. BDNF decreases membrane TRKB-T1 and protects mice from diet-induced NASH. (A) Immunoblots of membrane proteins isolated from Hepa 1-6 cells stably expressing TrkB-T1. Transduced cells were treated with vehicle (-) or BDNF in the presence or absence of cycloheximide (4 μM). (B) Immunoblots of total cell lysates from primary hepatocytes isolated from hnRNPU LKO mice. Hepatocytes were pretreated with BDNF (100 ng/mL) for 2 hours before addition of PA (200 μM) and TNF-α (20 ng/mL). (C) Morphology of treated hepatocytes. Hepatocytes were pretreated with BDNF (100 ng/mL) for 2 hours before addition of PA (400 μM) and TNF-α (20 ng/mL). (D) Immunoblots of total liver lysates from mice transduced with AAV-GFP or AAV-BDNF (GFP n = 7; BDNF n = 6). (E) Metabolic parameters and plasma ALT/AST levels in transduced mice. (F) qPCR analysis of hepatic gene expression. Data in (E) and (F) represent mean ± SEM. *$P < 0.05$, **$P < 0.01$; BDNF versus GFP, two-tailed unpaired Student t test. Abbreviation: CHX, cycloheximide.

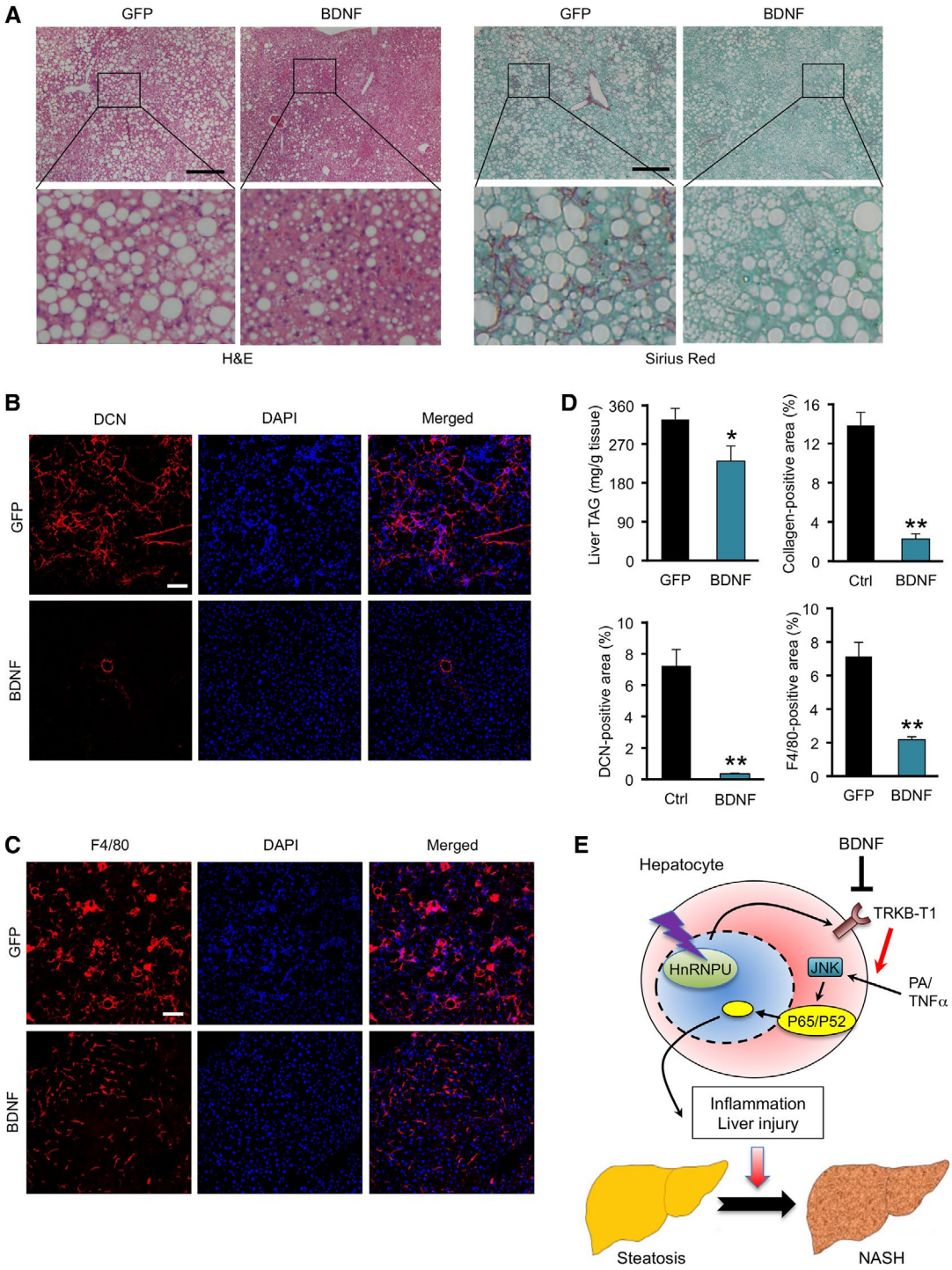

FIG. 8. AAV-mediated BDNF overexpression protects mice from diet-induced NASH pathogenesis. (A) H&E and Sirius red staining of liver sections from mice transduced with AAV-GFP or AAV-BDNF. Arrowheads indicate infiltrated immune cells. Scale bars: 200 μm. (B) F4/80 immunofluorescence staining of liver sections. Scale bars: 100 μm. (C) DCN immunofluorescence staining of liver sections. Scale bars: 100 μm. (D) Quantification of immune cell infiltration, collagen deposition, and DCN and F4/80 positive areas. Data represent mean ± SEM (GFP n = 7; BDNF n = 6). $^{*}P < 0.05$, $^{**}P < 0.01$; GFP versus BDNF, two-tailed unpaired Student *t* test. (E) Model depicting the role of hnRNPU/TRKB-T1 as a chromatin accessibility checkpoint for NASH pathogenesis. Abbreviation: DAPI, 4′,6-diamidino-2-phenylindole.

Unlike transcriptional coactivators and repressors, the effects of hnRNPU inactivation on chromatin accessibility appeared to be bidirectional, suggesting that hnRNPU acts in a context-dependent manner to influence hepatic gene expression. Integration of the RNA-seq data from the hnRNPU LKO mouse livers with single-cell RNA-seq data revealed an interesting feature of liver gene regulation by hnRNPU (Supporting Fig. S4A). Although many differentially expressed genes exhibit highest expression in hepatocytes, among different liver cell types, mRNA expression of a large number of genes altered by hnRNPU deficiency appeared to be enriched in nonparenchymal cells. Remarkably, many genes up-regulated in hnRNPU LKO livers are restricted to cells involved in immunity and inflammation, including macrophage, T cell, B cell, and dendritic cell (Supporting Fig. S4B). These findings illustrate the prevalence of intercellular crosstalk between hepatocytes and other cell types in the liver. Although the exact mechanisms that mediate immune cell gene expression remain unknown, a likely scenario is that hnRNPU deficiency sensitizes hepatocytes to stress-induced injury, leading to enhanced inflammatory response and stellate cell activation.

The receptor tyrosine kinase TrkB is a high-affinity receptor for neurotrophins that is known to promote neuronal differentiation and survival.[44,45] A number of TrkB transcripts have been described as a result of alternative splicing. The full-length isoform contains the extracellular ligand-binding domain and intracellular tyrosine kinase domain, whereas TrkB-T1 is truncated immediately after the transmembrane domain and lacks the kinase domain.[46] Our RNA-seq study comparing healthy and NASH mouse livers revealed that TrkB-T1, but not the FL-TrkB isoform, was among the most highly induced genes in the liver following diet-induced NASH (Fig. 5A). It is notable that TrkB expression is largely absent in healthy liver. As such, the induction of TrkB-T1 represents an emergent property of NASH hepatocytes that may be functionally linked to NASH pathogenesis. This isoform-specific induction of TrkB expression was observed in human NASH liver biopsies, illustrating that dysregulation of TrkB expression is likely a conserved feature of NASH (Fig. 6A,B). Interestingly, a recent study showed that expression of TrkB and its ligand BDNF was induced in the liver following bile duct ligation[47]; however, the exact isoform(s) involved was not determined. TrkB-T1 has been shown to regulate calcium signaling and actin dynamics[36-38] and may act as a dominant negative receptor for BDNF, thereby attenuating BDNF signaling through TrkB.[46] Our functional studies demonstrate that TRKB-T1 promotes stress-induced hepatocyte death whereas its ligand BDNF diminishes membrane TRKB-T1 protein levels and protects mice from diet-induced NASH.

TRANSLATIONAL AND CLINICAL POTENTIAL OF THE STUDY

This work delineates a molecular pathway that links chromatin regulation to NASH pathogenesis. The aberrant induction of TRKB-T1 expression in mouse and human NASH is particularly intriguing. Our results support a deleterious effect of membrane TRKB-T1 on hepatocyte health, whereas BDNF-mediated down-regulation elicits protective activities. As such, the BDNF/TRKB-T1 axis may present unique molecular targets for the therapeutic intervention of NASH.

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Author Contributions: J.D.L. and J.X. conceived the project and designed research. J.X., T.L., L.M., H.K., X.X., Z.C., and S.L. performed the experiments and analyzed the data. T.L., J.X., and J.D.L. performed RNA-seq and ATAC-seq data analyses. J.D.L. and J.X. wrote the manuscript.

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Supporting Information

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