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TRIM24 suppresses development of spontaneous hepatic lipid accumulation and hepatocellular carcinoma in mice

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Abstract

Background and Aims—Aberrantly high expression of TRIM24 occurs in human cancers, including hepatocellular carcinoma. In contrast, TRIM24 in the mouse is reportedly a liver-specific tumor suppressor. To address this dichotomy and uncover direct regulatory functions of TRIM24 *in vivo*, we developed a new mouse model that lacks expression of all *Trim24* isoforms, as the previous model expresses normal levels of *Trim24* lacking only exon 4.

Methods—To produce germline-deleted $Trim24^{dlE1}$ mice, deletion of the promoter and exon 1 of *Trim24* was induced in $Trim24^{LoxP}$ mice by crossing with a zona pellucida 3-*Cre* line for global deletion. Liver-specific deletion ($Trim24^{hep}$) was achieved by crossing with an Albumin-*Cre* line.

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Phenotypic analyses were complemented by protein, gene-specific and global RNA expression analyses and quantitative chromatin immunoprecipitation.

Results—Global loss of *Trim24* disrupted hepatic homeostasis in 100% of mice with highly significant, decreased expression of oxidation/reduction, steroid, fatty acid and lipid metabolism genes, as well as increased expression of genes in unfolded protein, endoplasmic reticulum stress and cell cycle pathways. *Trim24*$^{dlE1/dlE1}$ mice have markedly depleted visceral fat and, like *Trim24*$^{hep/hep}$ mice, spontaneously develop hepatic lipid-filled lesions, steatosis, hepatic injury, fibrosis and hepatocellular carcinoma.

Conclusions—TRIM24, an epigenetic co-regulator of transcription, directly and indirectly represses hepatic lipid accumulation, inflammation, fibrosis and damage in the murine liver. Complete loss of *Trim24* offers a model of human nonalcoholic fatty liver disease, steatosis, fibrosis and development of hepatocellular carcinoma in the absence of high-fat diet or obesity.

Keywords

NAFLD; NASH; steatosis; hepatic lesions; HCC; histone reader

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease in the Western world [1]. NAFLD occurs with an excessive accumulation of triglycerides (TG) in the liver (steatosis) of individuals who do not consume excessive amounts of alcohol. A subset of these patients develops non-alcoholic steatohepatitis (NASH), due to the presence of chronic inflammation and hepatocellular injury. These individuals have an increased risk of cirrhosis, liver failure and hepatocellular carcinoma (HCC), compared to individuals with NAFLD [2]. A greater understanding of the molecular mechanisms involved in progression of the disease from a metabolic disorder to cancer is needed for better prevention and treatments.

Here, we report a new mouse model that recapitulates development of HCC following spontaneous hepatic lipid accumulation, inflammation, and damage of liver tissue in the absence of *Trim24* expression. The Tripartite motif (TRIM) protein family is defined by conserved N-terminal domains of RING, B- box and coiled-coil and variable C-terminal domains, which contribute to TRIM protein functions in differentiation, development, oncogenesis and apoptosis [3]. TRIM24 (previously known as TIF1α) is a multi-functional protein: an E3-ubiquitin ligase targeting p53 for degradation, a co-regulator of nuclear receptors, and a Plant Homeodomain (PHD)/Bromodomain histone “reader” [4–7]. TRIM24 is highly expressed in multiple types of human cancers, including breast [5, 6], head and neck [8], non-small cell lung [9], glioblastoma [10], and HCC [11].

In contrast to apparent oncogenic function of TRIM24 in humans, analysis of a mouse model with *cre*-mediated excision of exon 4 of *Trim24* (*Trim24*$^{dlE4/dlE4}$) suggested that TRIM24 is a liver-specific, retinoid-dependent tumor suppressor [12]. However, recent evidence shows that the *Trim24*$^{dlE4/dlE4}$ mouse is not null for *Trim24* in the liver, as it retains normal levels of *Trim24* RNA lacking exon 4 [13]. HCC occurs in the

Trim24$^{dlE4/dlE4}$ mouse without apparent progression from NAFLD, due to activation of retinoid-dependent enhancers present in endogenous, murine VL30-retroviral transposons inserted across the mouse genome [13]. These long terminal repeat (LTR) enhancers are repressed by TRIM24/TRIM33 heteromeric complexes, similar to silencing of proviral DNA expression by TRIM28 [14]. In this mouse model, loss of TRIM24/TRIM33-mediated repression causes aberrantly high expression of inflammatory pathway genes linked to neoplastic inflammation in the liver [7].

The expression of *Trim24* RNA lacking exon 4 (E4), as observed in *Trim24*$^{dlE4/dlE4}$ mice [13], leaves unresolved whether this aberrant RNA or encoded protein isoforms exhibit dominant negative or gain-of-function, contributing to the observed HCC phenotype. These unknowns and, importantly, the documented over expression of TRIM24 in human cancers, including HCC [11], led us to create a conditional knockout mouse that is null for *Trim24* expression by genetic excision of the promoter and first exon (*Trim24*$^{dlE1/dlE1}$ and *Trim24*$^{hep/hep}$). This mouse model revealed a previously unknown role for TRIM24 in hepatic homeostasis, as an epigenetic regulator of oxidation/reduction, lipid, steroid and fatty acid metabolism, as well as unfolded protein response and ER-stress pathways. These alterations were accompanied by inflammation and fibrosis, progressing to HCC; all without manipulation of dietary fat or chemical induction.

Materials and Methods

Generation of germline Trim24^{dlE1} deficient mice

To generate *Trim24*-null and hepatic-deleted mice, the linearized targeting vector (Fig. 1A, Supplemental methods) was electroporated into embryonic stem cells (ES, TC-1, MD Anderson Cancer Center) and positive ES cell clones were used to generate chimeric mice. Progeny were backcrossed to C57BL6/J mice (The Jackson Laboratory) for *Trim24*$^{LoxPNeo/+}$ mice. *Trim24*$^{LoxPNeo/+}$ mice were crossed to ROSA26-FLPeR mice (The Jackson Laboratory) to delete the Neomycin cassette: *Trim24*^{LoxP} mice. *Trim24*^{LoxP} mice were crossed to the zona pellucida 3 promoter-driven *Cre*-line (*Zp3-Cre*, The Jackson Laboratory). The *Trim24*$^{dlE1/+}$ offspring were intercrossed to yield *Trim24*$^{+/+}$, *Trim24*$^{+/dlE1}$ and *Trim24*$^{dlE1/dlE1}$, and monitored for survival over a time-course of 585 days. Similarly, *Trim24*$^{hep/hep}$ were generated by crossing *Trim24*^{LoxP} mice with Albumin promoter-driven Cre line (B6.Cg-Tg(Alb-cre)21Mgn/J, The Jackson Laboratory). All animal experiments were approved by the IACUC of the University of Texas MD Anderson Cancer Center.

Histological and biochemical studies

Mouse tissues were fixed/stained with hematoxylin/eosin (H&E) or Oil Red O (ORO) using standard procedures [15]. AST, ALT, total plasma cholesterol (TPC), plasma TG, non-HDL-C, and HDL-C were quantitated using an Olympus clinical analyzer (Olympus) [16]. Hepatic lipid profiles were assessed using the Folch liver lipid extraction method [17].

See Supplementary Appendix for a description of additional methods.

Results

A Trim24$^{-/-}$ mouse generated by deletion of promoter and exon 1

We engineered a conditional knockout mouse of *Trim24* by genetic targeting of the transcription start site and first exon (Fig. 1A–B, Supplemental Fig.1); progeny were confirmed by PCR analysis (Fig. 1B) and were viable and fertile in both male and female. Deletion of *Trim24* occurs without allelic compensation, shown by protein and RNA analyses of mouse embryonic fibroblasts (MEFs), from *Trim24*$^{+/+}$, *Trim24*$^{+/dlE1}$ and *Trim24*$^{dlE1/dlE1}$ E12.5 embryos (Fig. 1B), adult liver and hepatocytes (Supp. Fig. 1). Deep sequencing of RNA (RNA-seq), from *Trim24*$^{+/+}$ and *Trim24*$^{dlE1/dlE1}$ liver (2 mos), showed no detectable expression across the *Trim24* locus in *Trim24*$^{-/-}$ (Fig. 1C). Quantitative RT-PCR analysis of all three, potential protein-coding isoforms of *Trim24* (http://useast.ensembl.org/) with primers covering *Trim24* exon 2/3, exon 7/8, exon 11/13, and the 3'UTR (Supplemental Table 2) confirmed complete loss of *Trim24* RNA expression in MEFs (Fig. 1D, E). Since our analyses of *Trim24* expression show that the *Trim24*$^{dlE1/dlE1}$ mouse is truly null for *Trim24* RNA and protein expression (Supp. Fig. 1E), it is further discussed as *Trim24*$^{-/-}$.

TRIM24 belongs to the TRIM protein TIF1 subfamily (C-VI) of which there are three members: TRIM24, TRIM28 and TRIM33 [18]. These proteins have high homology and form heteromeric complexes that vary with cell type [18]. We determined if other TIF1 subfamily members potentially compensate for loss of *Trim24* [3][19], and analyzed *Trim28* and *Trim33* RNA and protein from wild type (WT) and *Trim24*$^{-/-}$ MEFs (Fig. 1D, E). These showed no significant differences; therefore, it is unlikely that any phenotype of the *Trim24*$^{-/-}$ mouse is due to compensatory functions of TRIM28 or TRIM33.

Trim24$^{-/-}$ mice develop hepatocellular lesions, steatosis and HCC

A major phenotype of *Trim24*$^{-/-}$ mice is development of macroscopic white lesions in the liver by 4–6 months of age (Fig. 2A; a), compared to the normal morphology of *Trim24*$^{+/-}$ liver and *Trim24*$^{+/+}$ liver (Fig. 2A; b, Supplemental Fig. 2). H&E staining showed that *Trim24*$^{-/-}$ liver lesions are composed of both micro- and macro-vesicular steatosis (Fig. 2A; c, d). Oil Red O (ORO) staining revealed that hepatocytes within the lesions of *Trim24*$^{-/-}$ liver are filled with lipid (Fig. 2A; e, f) in contrast to *Trim24*$^{+/+}$ liver (Supplemental Figs. 2 and 3). Further, hepatic lipid accumulation can be seen as early as 2 weeks of age (Supplemental Fig. 3A) with significant accumulation of lipid in foci at 2 and 4 months of age (Supplemental Fig. 3B, C). Trichrome staining to assess fibrotic progression was negative for collagen and mucin in *Trim24*$^{-/-}$ liver at 3 and 10 weeks of age (Supplemental Fig 4A,B), when lipid accumulation is already obvious, but was positive for fibrosis by 6 months (Fig. 2A; g, h). Consistent with positive trichrome staining, significantly increased RNA levels of several collagen and matrix metallopeptidase (*Mmp*) genes and transforming growth factor-β receptor 2 (*Tgfbr2)* indicate fibrosis and damage at 10 weeks of age (Fig. 2B).

We saw increased liver disease with age: the liver to body weight ratio (liver index) of *Trim24*$^{-/-}$ mice increased 2.7 fold compared to *Trim24*+/+ and *Trim24*$^{+/-}$ mice (Fig. 2C).

Numerous macroscopic hepatic tumors (>2mm) were present in all *Trim24*$^{-/-}$ mice (29/29), regardless of gender (Fig 2D; a). In contrast, there was no evidence of hepatic tumors or anomalies in age- and background-matched *Trim24*$^{+/+}$ (0/17, 12–15 months) and *Trim24*$^{+/-}$ (0/11, 12–18 months) mice (Supplemental Table 4, Supplemental Fig. 3). At 18 months, *Trim24*$^{-/-}$ liver showed a spectrum of hyperplastic lesions, preneoplastic foci of cellular alteration (FCA) and neoplastic lesions of hepatocellular adenoma (HCA) with and without fatty changes (Fig. 2D; a–i). The non-nodular or nodular HCA lesions had a vacuolated cell mass compressing the non-tumor parenchyma (Fig. 2D; b,c). ORO staining revealed lipid accumulation in vacuolated cells of the tumor (Fig. 2D; d, e). Locally invasive hepatocellular carcinoma (HCC) with nodules (Fig. 2D; f, g) or without nodules (Fig. 2D; h, i) was seen in terminal mice. The *Trim24*$^{hep/hep}$ liver had highly similar lipid accumulation, steatosis, fibrosis and tumor development, less apparent prior to 10–12 months but equivalent to *Trim24*$^{-/-}$ liver after this age (Supplemental Fig. 5). The distribution of tumor pathologies in *Trim24*$^{-/-}$ (Supplemental Figs. 5 and 6) and *Trim24*$^{hep/hep}$ mice did not differ significantly (Supplemental Fig. 5B). *Trim24*$^{-/-}$ mice have a median survival time of 409 days as compared to *Trim24*$^{+/+}$ (no deaths at 585 days) (Supplemental Fig. 7).

Inflammation, injury and degeneration are increased in adult Trim24$^{-/-}$ mice

Key components of NASH in humans are aberrant plasma and intrahepatic lipid levels with accumulation of lipids, mainly triglycerides (TG), and increased hepatocelluar inflammation and steatosis, with subsequent injury and degeneration [20]. We quantified indicators of liver damage and NASH in plasma. Both alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were significantly increased in *Trim24*$^{-/-}$ and *Trim24*$^{hep/hep}$ mice consistent with liver damage (Fig. 3A and Supp. Fig. 8). In human NASH, there is dysfunction in lipase-mediated hydrolysis of TG in the liver to yield fatty acid and glycerol. Human patients have elevated plasma and hepatic TG levels, often associated with obesity [20]. In contrast, *Trim24*$^{-/-}$ mice are not obese: hepatic TG levels and visceral fat significantly decreased (Fig. 3B and Supplemental Fig. 9). Additionally, plasma cholesterol and lipoprotein levels decreased, while plasma TG and hepatic lipids increased.

In further assessments of potential NASH, we performed histological analyses and quantified expression of genes associated with hepatocellular steatosis, injury and degeneration. Histological analysis of young adult *Trim24*$^{-/-}$ liver (10 weeks) revealed degenerated or damaged hepatocytes throughout (Fig. 3C). Swollen, vacuolated cells (Fig. 3C; b, arrows), Mallory bodies (Fig. 3C; c, arrow), and nuclear inclusion bodies (Fig. 3C; d, arrow and insert) are characteristic of *Trim24*$^{-/-}$, unlike *Trim24*$^{+/+}$ liver at 10 weeks (Supplemental Fig. 10). An increase in cleaved-Caspase-3-positive hepatocytes suggested hepatocellular damage induced cell death in the *Trim24*$^{-/-}$ liver by 10 weeks (Fig. 3C; e, f, arrow; quantified in Fig. 3D). Consistent with these observations, expression of pro-apoptotic genes *Bax* and *Caspase12* and ER stress gene *Ddit3* (DNA-damage inducible transcript 3) are significantly increased in 10-week *Trim24*$^{-/-}$ liver (Fig. 3E). These signs of serious liver damage accompanied significant changes in specific pro-inflammatory gene expression (Fig. 3F). Similar patterns of expression were seen in *Trim24*$^{hep/hep}$ liver, pinpointing hepatic functions of TRIM24 (Supplemental Fig. 14). Taken together, lack of

Trim24 in mice recapitulates the parameters of human NAFLD, and a majority of NASH symptoms with age, although mice are lean (Supplemental Fig. 5, 10).

Genome-wide analysis of RNA expression in Trim24$^{-/-}$ liver

To assess the global impact of TRIM24 on gene expression, we performed deep sequencing of RNA (RNA-seq). Three biological RNA replicates were isolated from male *Trim24*$^{+/+}$ and *Trim24*$^{-/-}$ liver at 10 weeks of age and sequenced (Fig. 1C). A total of 763 genes showed statistically significant changes and differential expression at a false discovery rate (FDR) adjusted p-value (q-value) of 1%, calculated by EdgeR RNASeq analysis [21]. An approximately equal number of genes were activated or repressed in *Trim24*$^{-/-}$ (Supplemental Table 5), consistent with TRIM24 functions as a co-repressor or -activator of transcription [22–25].

The top five biological functions identified by DAVID analysis [26] of up-regulated genes in *Trim24*$^{-/-}$, reflecting loss of TRIM24 function as a co-repressor, are protein folding, defense response, RNA metabolism, cell cycle regulation and ER-nuclear signaling (Fig. 4A) (Supplemental Table 5). Genes with decreased expression in *Trim24*$^{-/-}$, reflecting loss of TRIM24 function as a co-activator, were grouped into biological functions of oxidation/ reduction, steroid metabolism, lipid biosynthesis, fatty acid metabolism and steroid biosynthesis (Fig. 4A).

Lipid metabolism and inflammation are aberrantly regulated in Trim24$^{-/-}$ liver

We assessed key genes associated with biological functions significantly impacted by loss of *Trim24*, as well as aberrantly regulated in human NAFLD and NASH [2, 27]. Hepatic lipases, lipid transport/receptors and pro-inflammatory factor genes were significantly up-regulated, consistent with both the *Trim24*$^{-/-}$ phenotype and human NAFLD to NASH progression (Fig. 4B, Supplemental Table 6). Inconsistent with human NASH, genes associated with endogenous fatty acid synthesis (i.e. *FASN*, *Scd1* and *Acaca*), were generally and significantly down-regulated (Fig. 4B, Supplemental Table 6).

Overall, complete loss of *Trim24*, as opposed to lower levels in heterozygous animals or isoforms found in *Trim24*$^{dl4/dl4}$, decreased expression of genes and proteins involved in a) *de novo* lipid synthesis, b) apolipoproteins and lipid droplet/lipid storage, and c) VLDL transporter and receptors; as well as, increased expression of genes d) encoding lipases and apolipoprotein receptors and e) involved in proinflammation/ fibrosis (Fig. 4, Supplemental Figs. 11–17). Expression changes and levels of individual genes differ in levels and time of induction, likely reflecting developmental and epigenetic regulation during aging.

TRIM24 directly regulates genes in lipid metabolic, inflammation and damage pathways

As a histone reader, TRIM24 does not bind DNA in a sequence-specific manner but rather interacts with specific histone post-translational modifications via a C-terminal PHD/ bromodomain [6]. TRIM24 enrichment at chromatin may recruit specific TRIM24-interacting transcription factors, *e.g.* nuclear receptors RARα or estrogen receptor, to DNA binding sites [6, 12]. Ingenuity Pathway Analysis [28] of the most significant, differentially expressed, down-regulated gene pathways (by p-value): LPS/IL-1 inhibition of RXR

function, FXR/RXR activation, super-pathway of cholesterol biosynthesis, PXR/RXR activation, and maturity onset diabetes of young (MODY) signaling, further supported TRIM24/nuclear receptor regulatory interactions. Thus, we used nuclear receptor DNA binding motifs (GGTCA half-sites) [29, 30] to focus ChIP-PCR analysis (Fig. 4D) of TRIM24-chromatin interactions and assess direct regulation of genes altered in *Trim24*$^{-/-}$ liver.

Among the apolipoprotein genes (Fig. 4C), we found significant TRIM24 binding at *Apoa1*, *Apoc3* and *Apod1* (Fig. 4D). Several *de novo* lipid synthesis genes downregulated in the *Trim24*$^{-/-}$ liver, such as *Acacb*, were bound by TRIM24 at verified RARα binding sites [31]. Additionally, TRIM24 bound and directly regulated lipase *Pnpla3*, as well as VLDL transporter *Mttp* and the *Vldlr* gene (Fig. 4C). Increased expression of several lipases in *Trim24*$^{-/-}$ liver (Fig. 4B) suggests an imbalance in energy use or feedback response to accumulation of lipid [32].

TRIM24 was also enriched at genes, associated with inflammation and liver damage, with significantly altered expression (Figs. 3 and 4). Pro-inflammatory factors *Ccr2*, *Icam* and *Il-1a*, as well as liver damage and apoptosis genes, *Ddit3*, *Bax* and *Casp12*, had TRIM24 enrichment, consistent with changes in gene expression and liver damage in *Trim24*$^{-/-}$. TRIM24 binding at the RARE/ERE consensus sites of *Apoc3*, at - 150, and *Ccr2*, at −100, proved insignificant, reinforcing that not all predicted RARE/ERE sites show TRIM24 recruitment in liver tissue. These studies of TRIM24-chromatin interactions and direct regulation of target gene expression support a significant role for TRIM24 in homeostasis by regulating genes of the lipid metabolic, inflammatory, and apoptotic pathways of the liver.

Discussion

Altered regulation of hepatic lipid metabolism, accompanied by chronic inflammation, is clearly linked to higher incidence of HCC in humans [13, 33]. Aberrant expression of several key genes in these pathways is associated with NAFLD, where lipid metabolic and regulatory genes are indicated, and NASH, where inflammation is thought to play a major role in driving progression to HCC [34]. Here, we show that histone reader TRIM24 plays a direct role in the regulation of liver fat metabolism and inflammatory processes. Additionally, global expression analyses of the *Trim24*$^{-/-}$ liver unveiled pathways such as the Unfolded Protein Response and EndR-Nuclear Signaling, which are closely associated with human NAFLD, inflammatory stress, apoptosis and tissue damage [35]. When *Trim24* expression is absent globally or from the liver, there is development of spontaneous NAFLD-NASH-HCC with time, despite a normal diet. This outcome is similar, in many but not all parameters, to liver-specific deletion of Pten , suggesting regulatory parallels of future interest [36]. Mouse models genetically engineered for altered expression of *Sfrp*, *Nemo*, *Pnpla3-Il48M*, *Adiponectin*, *Mttp*, *ApoB* and *ApoC3* genes exhibit this disease progression, but only when stimulated by a high fat diet [32, 34]. Our finding that TRIM24 directly regulates a subset of these, e.g. *Pnpla3*, *ApoC3*, and *Mttp*, suggests an epigenetic hierarchy. TRIM24 may be a linchpin in transcription networks that intersect to prevent NAFLD and later HCC, due to its ability to serve as either an epigenetic co-repressor or co-activator of nuclear receptors and other transcription regulators [37] [6, 7].

The phenotype of a previous *Trim24* mouse model with an exon 4 (E4) deletion, $Trim24^{dlE4/dlE4}$, suggested that the only significant function of TRIM24 is prevention of hepatic inflammation, as a co-repressor of retinoid-dependent genes and LTR-driven enhancers [7, 13]. In contrast, although specific inflammatory-associated genes were altered in liver null for *Trim24*, we did not see an early or major response of retrovirus LTR- or RAR-regulated genes associated with inflammation (Supplemental Fig. 12, 13). Rapid development of HCC in the E4-deletion $Trim24^{dlE4/dlE4}$ model lacks apparent NAFLD or NASH, and a role for TRIM24 in pathways of lipid/fatty acid/steroid metabolism, oxidation/reduction and ER stress was not apparent, as summarized in Supplemental Table 7.

Interestingly, alternative start sites of *Trim24* transcription yield native isoforms of *Trim24* lacking E4, as in the $Trim24^{dlE4/dlE4}$ mouse [13], and TRIM24 protein variants 1 and 3, which are highly similar to *BRAF* (Variant 1: 84% protein identity, Variant 3: 64% protein identity) (Supplemental Table 8). Intriguingly, chromosomal translocation and fusion of proto-oncogene *BRAF* and *TRIM24* are reported in specific leukemias [38, 39]. Comparison of the two mouse models of TRIM24 function (Supplemental Table 7) suggests that inflammation is a major driver that causes liver dysfunction to progress rapidly to HCC. However, when lipid metabolic pathways are significantly misregulated, prior to inflammation and damage, stepwise development of HCC occurs with age. These findings suggest that aggressive limitation of inflammation in human NAFLD patients may subvert or greatly delay development of HCC.

It remains unclear why aberrantly high expression of TRIM24 is associated with multiple human cancers, including HCC [11]; although, in a much smaller cohort of patients, significantly lower than normal levels of TRIM24 is reported [40]. This discontinuity may be due to TRIM24 functions in hepatic homeostasis with over- or under-expression leading to a regulatory imbalance. Additionally, not all hallmarks of NAFLD and NASH [41] are observed with loss of *Trim24*, although hepatic steatosis, inflammation and fibrosis, with hepatocyte injury and apoptosis, are present. Complete loss of *Trim24* caused a striking decrease in peripheral fat accumulation, along with elevated serum triglycerides and hepatocyte lipid accumulation, characteristics recently associated with NAFLD among nonobese patients [42]. Further study and dietary challenge of *Trim24* mouse models may offer mechanistic insights into human NAFLD, NASH and HCC, among non-obese patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

TRIM	tripartite motif protein
HCC	hepatocellular carcinoma
LoxP	Locus of Crossover in P1
Zp3	zona pellucida 3
ChIP	chromatin immunoprecipitation
NAFLD	non-alcoholic fatty liver disease
TG	triglycerides
NASH	non-alcoholic steatohepatitis
IFN	interferon
STAT	signal transducers and activators of transcription
ES	Embryonic stem
Neo	neomycin
PFA	paraformaldehyde
H&E	hematoxylin/eosin
ORO	Oil Red O
AST	aspartate aminotransferase
ALT	alanine aminotransferase
TPC	total plasma cholesterol
HDL-C	High density lipoprotein-cholesterol
STDEV	standard deviation
MEFS	mouse embryonic fibroblasts
FCA	foci of cellular alteration
HCA	hepatocellular adenoma
IHC	immunohistochemistry

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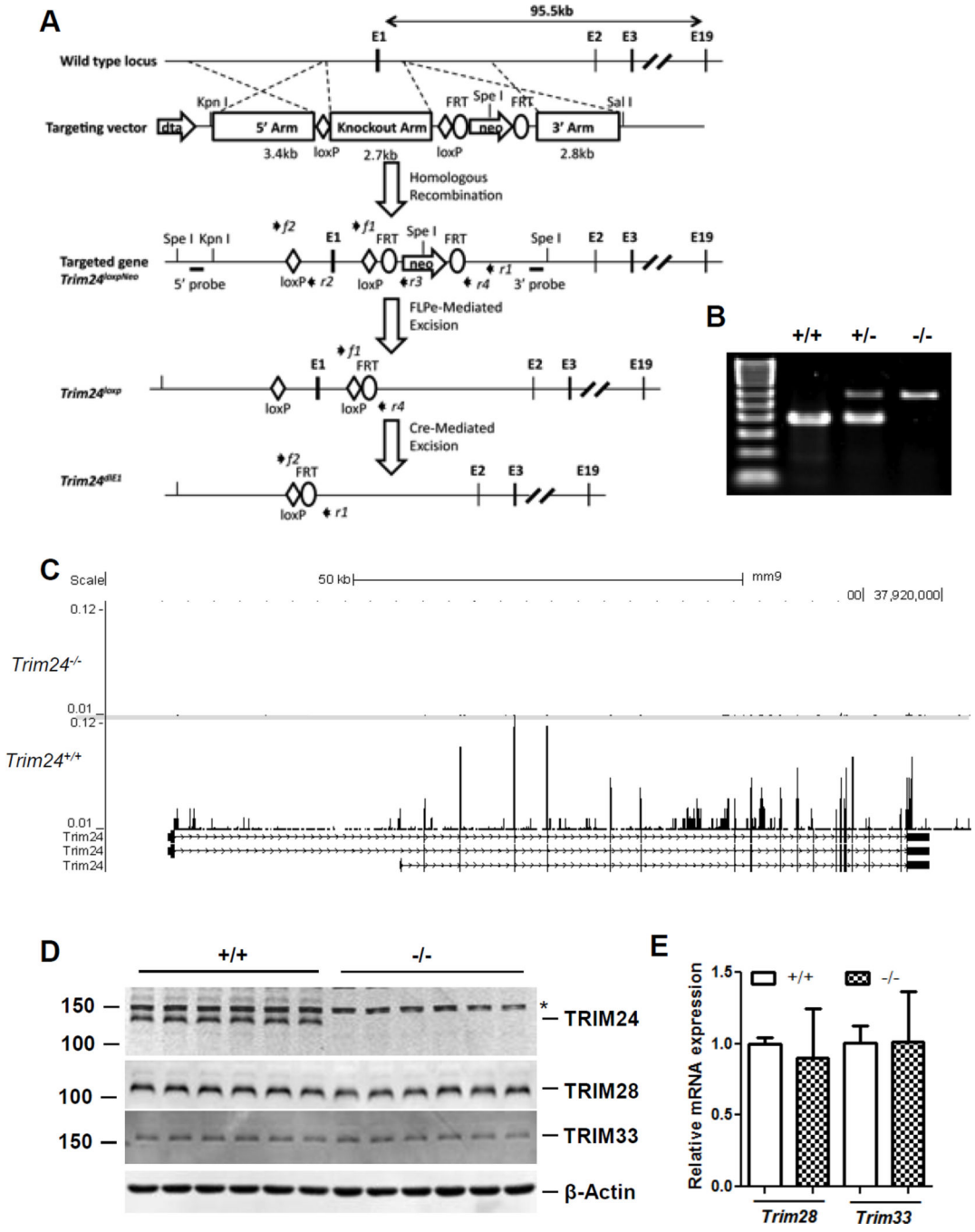

Fig. 1. Targeting of *Trim24* and expression of *Trim24* family members

(A) Murine *Trim24* gene exons (E1-E19), Southern blot -5'/ 3' probes, and primers (f1, f2, r1, r2, r3, r4) for genotyping are shown with diagrams of crosses to delete E1 and promoter. **(B)** PCR analysis confirmed deletion of *Trim24* exon 1. **(C)** Loss of *Trim24* RNA expression in *Trim24*$^{-/-}$ liver: RNA-seq of *Trim24*$^{-/-}$ and WT. **(D)** Western blot analysis of TRIM24, 28, and 33 protein expression in MEFs. β-actin - loading control and * - non-specific. **(E)** *Trim28* and *Trim33* expression in MEFs (RT-qPCR).

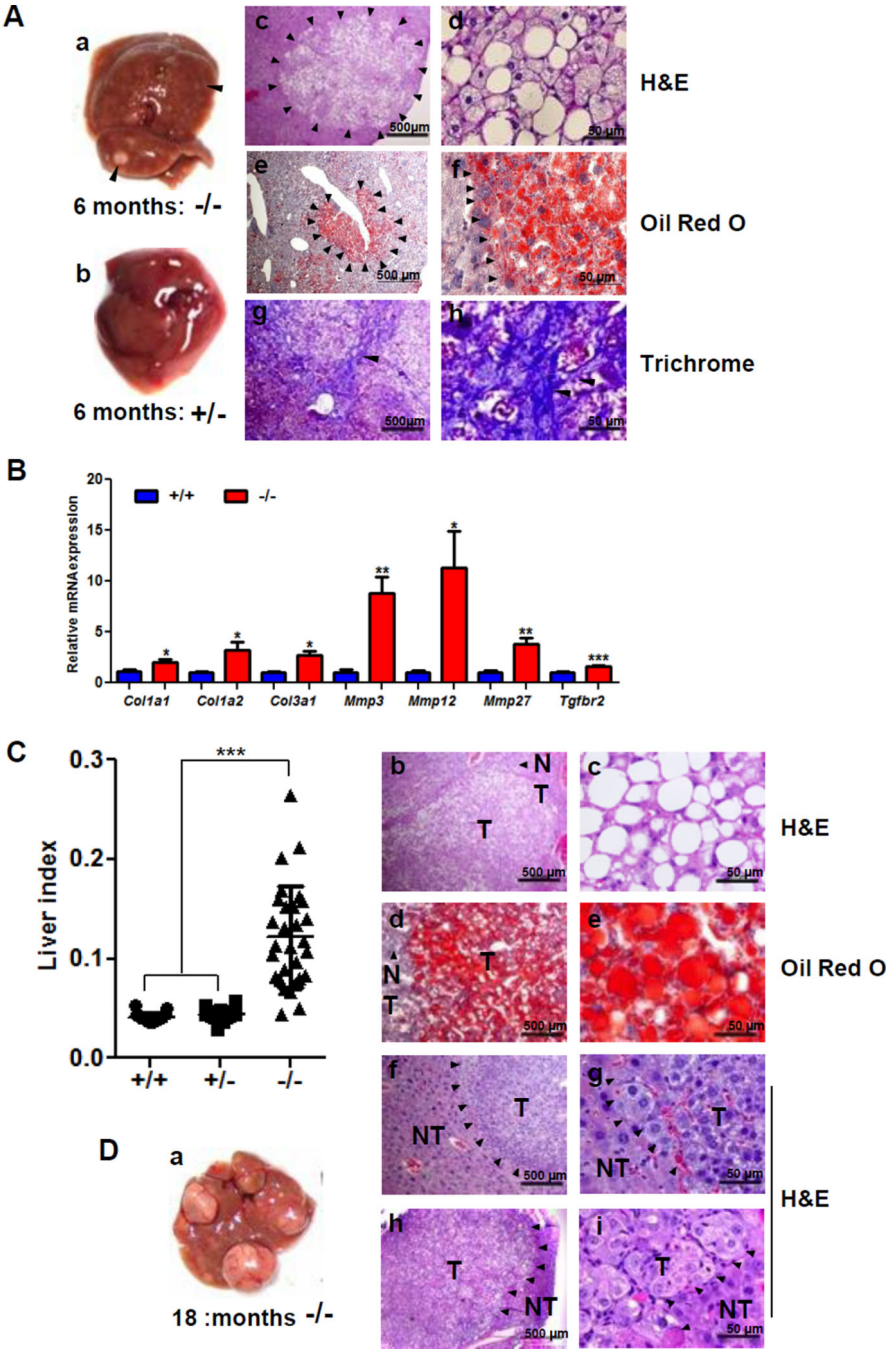

Fig. 2. Lipids and fibrosis increase in aging *Trim24*$^{-/-}$

(A) 6 month liver, lesions (arrows) **(a)** *Trim24*$^{-/-}$, **(b)** *Trim24*$^{+/-}$; **(c,d)** Vacuolated lesions with steatosis (H&E). **(e,f)** Lipid accumulation (ORO). **(g,h)** Fibrosis (arrow, Trichrome). **(B)** Fibrosis genes upregulated in 10 week *Trim24*$^{-/-}$ liver: n=6, *p<0.05; **p<0.01; ***p<0.005. **(C)** Liver index *Trim24*$^{-/-}$ (n=29), *Trim24*+/+ (n=17) and *Trim24*$^{+/-}$ (n=11). ***p<6.7E-08. Mean ± SD. **(D) (a)** 18 month *Trim24*$^{-/-}$: Multiple, large lesions (HCA) **(b, c)** Vacuolated **(d, e)** Lipid positive. **(f,g)** Invasive HCC without nodules. **(h, i)** Nodules. (Arrows = Boundary. T, tumor; NT, non-tumor). Bar: 500 µm (4X) and 50 µm (40X).

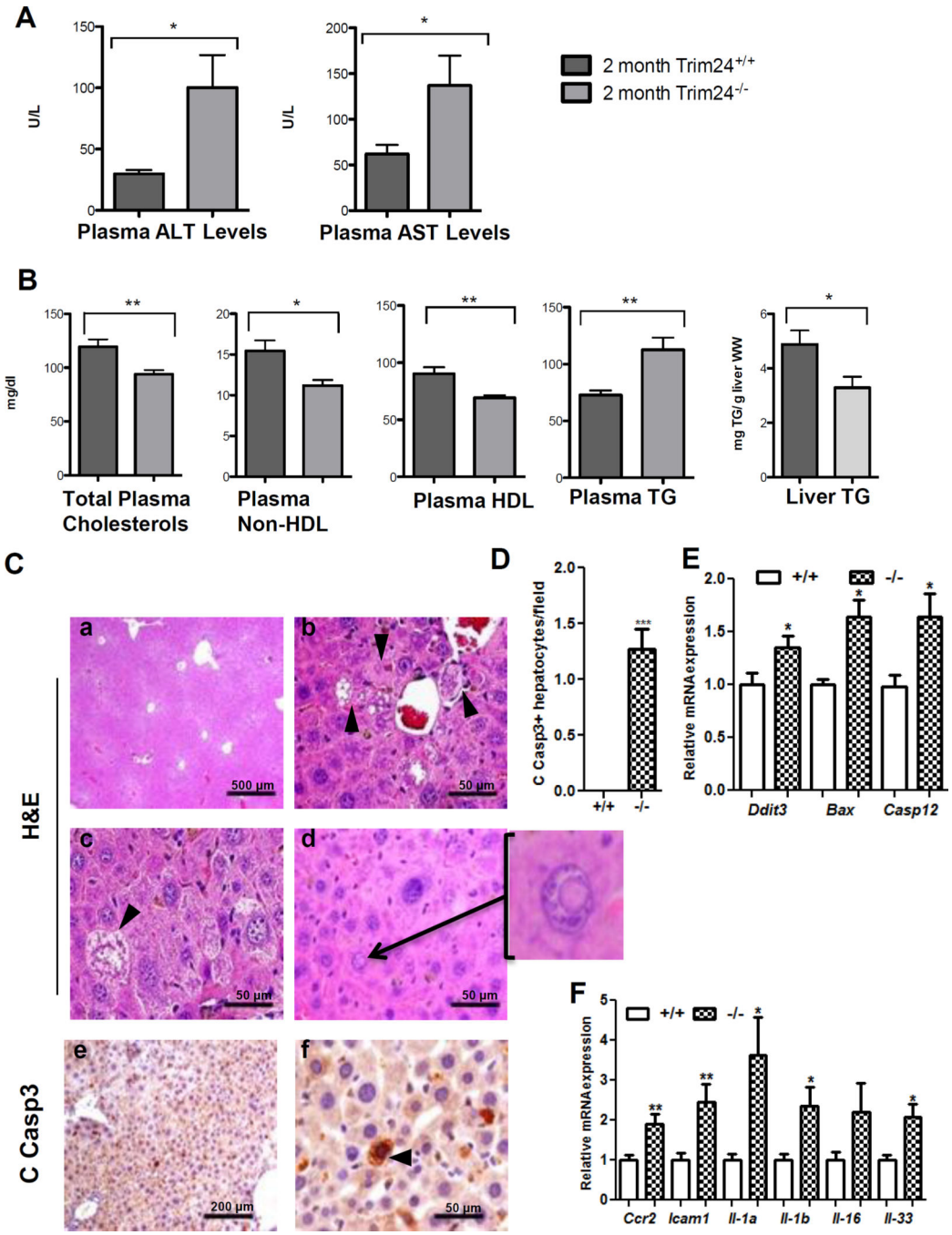

Fig. 3. Hepatocellular damage and dysfunction in 2 month *Trim24*$^{-/-}$

(A) ALT/AST: *Trim24*$^{-/-}$ (n=7), *Trim24*$^{+/+}$ (n=6). **(B)** Lipoprotein, TG: *Trim24*$^{-/-}$ (n=7), *Trim24*$^{+/+}$ (n=7; except TG n=6). Mean ± SD. * p<0.05, **p<0.01. **(C) (a–d)** Abnormal cells: *Trim24*$^{-/-}$ liver (H&E). **(b, arrows)** Damaged/vacuolated hepatocytes. **(c, arrow)** Mallory, **(d, arrow and inset)** Nuclear inclusion bodies. **(e, f, arrow)** Apoptotic hepatocytes (IHC: cleaved Caspase 3). Bar: 500 μm (4X); 200 μm (10X); 50 μm (40X). **(D)** Cleaved Caspase 3 positive hepatocytes, *Trim24*$^{-/-}$ (20X field). **(E)** Apoptosis-related genes, **(F)**

Fibrosisrelated genes, n=6 liver samples (mean ± SD),10 weeks. p value: *p<0.05; **p<0.01; ***p<0.005.

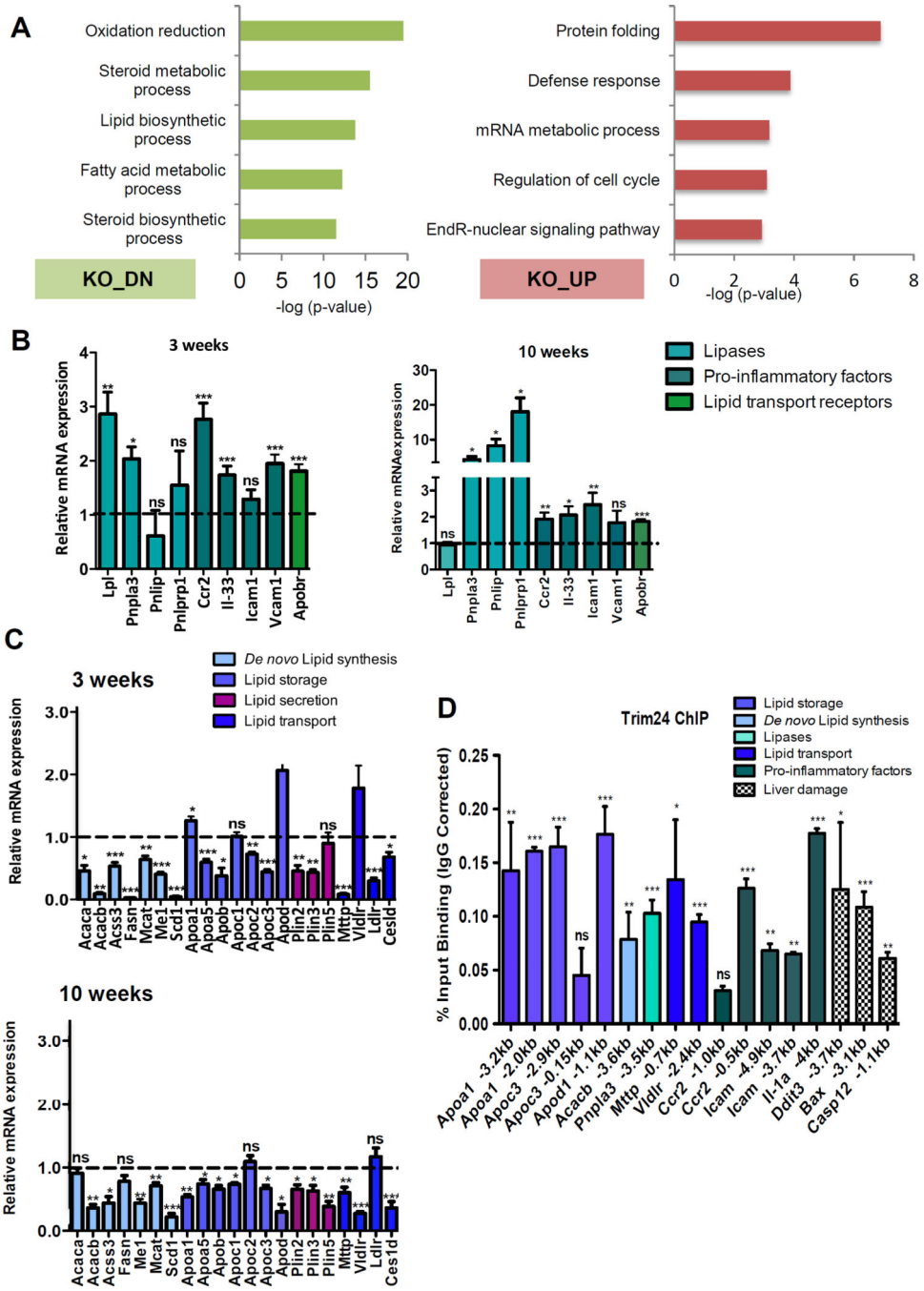

Fig. 4. TRIM24 regulates hepatic lipid metabolism, stress and inflammation genes

(A) RNA-seq, DAVID Analysis - Top 5 enriched biological functions for up-regulated and down-regulated genes in *Trim24*$^{-/-}$ versus *Trim24*$^{+/+}$ liver at 10 weeks. **(B)** TG hydrolysis (lipases), pro-inflammation, and lipid transport gene expression at 3 and 10 weeks. **(C)** *De novo* lipid synthesis, storage, secretion, and transport gene expression at 3 and 10 weeks. (B and C) qRT-PCR fold change with *Trim24*$^{+/+}$=1 (dashed line), n=3 each (mean ± SD). *p<0.05; **p<0.01; ***p<0.005. **(D)** *Trim24*$^{+/+}$ 2-months liver tissue ChIP: Significant

binding by TRIM24: *p<0.05; **p<0.01; ***p<0.001, ****p<0.0001. NS – nonspecific binding. Y-axis: TRIM24 enrichment as % input DNA.