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Marina Serrano-Maciá, Jorge Simón, Maria González-Rellán, Mikel Azkargorta, Naroa Goikoetxea-Usandizaga, et al.. Neddylation inhibition ameliorates steatosis in NAFLD by boosting hepatic fatty acid oxidation via the DEPTOR-mTOR axis. *Molecular metabolism*, 2021, 53, pp.101275. 10.1016/j.molmet.2021.101275 . hal-03346300

HAL Id: hal-03346300

<https://hal.science/hal-03346300>

Submitted on 10 Nov 2021

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Neddylation inhibition ameliorates steatosis in NAFLD by boosting hepatic fatty acid oxidation via the DEPTOR-mTOR axis



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ABSTRACT

Objective: Neddylation is a druggable and reversible ubiquitin-like post-translational modification upregulated in many diseases, including liver fibrosis, hepatocellular carcinoma, and more recently, non-alcoholic fatty liver disease (NAFLD). Herein, we propose to address the effects of neddylation inhibition and the underlying mechanisms in pre-clinical models of NAFLD.

Methods: Hepatic neddylation measured by immunohistochemical analysis and NEDD8 serum levels measured by ELISA assay were evaluated in NAFLD clinical and pre-clinical samples. The effects of neddylation inhibition by using a pharmacological small inhibitor, MLN4924, or molecular approaches were assessed in isolated mouse hepatocytes and pre-clinical mouse models of diet-induced NAFLD, male adult C57BL/6 mice, and the AlfpCre transgenic mice infected with AAV-DIO-*shNedd8*.

Results: Neddylation inhibition reduced lipid accumulation in oleic acid-stimulated mouse primary hepatocytes and ameliorated liver steatosis, preventing lipid peroxidation and inflammation in the mouse models of diet-induced NAFLD. Under these conditions, increased Deptor levels and the concomitant repression of mTOR signaling were associated with augmented fatty acid oxidation and reduced lipid content. Moreover, *Deptor* silencing in isolated mouse hepatocytes abolished the anti-steatotic effects mediated by neddylation inhibition. Finally, serum NEDD8 levels correlated with hepatic neddylation during the disease progression in the clinical and pre-clinical models

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Received February 24, 2021 • Revision received June 9, 2021 • Accepted June 14, 2021 • Available online 18 June 2021

<https://doi.org/10.1016/j.molmet.2021.101275>

Conclusions: Overall, the upregulation of Deptor, driven by neddylation inhibition, is proposed as a novel effective target and therapeutic approach to tackle NAFLD.

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Keywords Neddylation; NAFLD; Deptor; mTOR; Fatty acid oxidation; MLN4924

Abbreviations			
NAFLD	non-alcoholic fatty liver	VLDL	very-low-density lipoprotein
MAFLD	metabolic dysfunction associated fatty liver disease	OXPHOS	oxidative phosphorylation
NASH	non-alcoholic steatohepatitis	ETC	electron transport chain
PTMs	Post-translational modifications	ROS	reactive oxygen species
NEDD8	neural precursor cell-expressed, developmentally down-regulated protein8	MCDD	methionine choline-deficient diet
HCC	Hepatocellular carcinoma	CDHFD	choline-deficient high-fat diet
mTOR	mammalian target of rapamycin	FA	fatty acid
DEPTOR	DEP-domain containing mTOR-interacting protein	OA	oleic acid
NRF2	nuclear factor erythroid 2-related factor 2	OCR	oxygen consumption rate
CRLs	Cullin-RING ubiquitin ligase	Tnf	tumor necrosis factor
FAO	fatty acid oxidation	Ccl-2	chemokine (C–C motif) ligand 2
DNL	de novo lipogenesis	Il-6	interleukin-6
		Il-1β	interleukin-1 beta
		Timp-2	tissue inhibitor of metalloproteinases 2

1. INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD), or (as recently redefined) metabolic dysfunction associated fatty liver disease (MAFLD) [1], comprises a group of conditions that all share the excessive accumulation of fat in the liver as a common feature. NAFLD ranges from simple steatosis and non-alcoholic steatohepatitis (NASH) to advanced fibrosis, eventually leading to cirrhosis and Hepatocellular Carcinoma (HCC). Patients with NAFLD have a higher risk of liver-related morbidity and mortality, along with metabolic comorbidities [2–5]. Today, NAFLD is the most common liver disorder in the Western world, as its global prevalence is estimated to be 24% [6]. Despite the alarming numbers associated with the pathology of NAFLD worldwide and the large number of emerging therapies currently undergoing clinical trials, there remains no approved pharmacotherapy for the clinical management of NAFLD. To date, ongoing studies on NAFLD therapies have been primarily designed to target four main pathways: 1) hepatic fat accumulation; 2) oxidative stress, inflammation, and apoptosis; 3) intestinal microbiomes and metabolic endotoxemia; and 4) hepatic fibrosis. Though most clinical trials have focused on monotherapies, the use of a single drug acting on multiple pathways may offer an attractive therapeutic strategy in combatting NAFLD. Post-translational modifications (PTMs) affect many biological processes. Therefore, a single pharmacological inhibitor can alter the activity, localization, or function of several proteins and simultaneously target multiple deregulated pathways.

Post-translational modifications refer to the covalent and commonly enzymatic modification of proteins after biosynthesis. PTMs are an essential mechanism needed to diversify protein functions and dynamically coordinate their signaling networks. On this basis, the study of protein expression and PTMs, along with their role in NAFLD development and progression, has attracted attention in the post-genomic era. For example, the high-throughput profiling of PTMs has the potential to provide a molecular classification of fatty liver; changes to liver protein acetylation, phosphorylation, and O–N-acetylglucosamine glycosylation were found to be altered in NAFLD states

[7]. More recently, the focus has been geared toward the mediation of PTMs by ubiquitin and ubiquitin-like proteins in NAFLD. Furthermore, several authors have shown how ubiquitin-specific peptidases and some ubiquitin ligases (E3) can regulate the development of NAFLD [8–12]. NEDD8 (neural precursor cell-expressed, developmentally down-regulated protein8) is an evolutionarily conserved 8-kD protein closely related to ubiquitin, and like ubiquitin, it can be conjugated to the specific lysine residues of target proteins in eukaryotes — a process called neddylation. Though the conjugation of NEDD8 to its targets is usually associated with the stabilization of the protein, it has also been implicated in the activity of its target proteins. Despite the conjugation of NEDD8 onto the cullin subunit of CRLs (Cullin-RING ubiquitin ligase) promoting its assembly and activating its ubiquitin ligase activity being the best-characterized NEDD8 target [13], the number of newly identified neddylation targets has been increasing in the last several years with the development of novel techniques [14,15]. Therefore, the impact of neddylation on diseases can be much more extensive. It has been recently determined that neddylation is increased in advanced liver fibrosis and HCC [16,17]. Other authors have shown that the degradation of the splicing factor SRSF3 (serine rich splicing factor 3) in a neddylation-dependent mechanism is disrupted in early metabolic liver disease and may contribute to NASH progression, cirrhosis, and ultimately HCC [18]. Finally, after treatment with a pharmacological inhibitor of neddylation, obese mice present decreased weight gain and, consequently, an improved liver phenotype [19].

Herein, we have further addressed the relevance of hepatic neddylation in NAFLD and the therapeutic efficacy of neddylation inhibition in cell models *in vitro* and mouse models of diet-induced NAFLD. We provide evidence that hepatic neddylation inhibition decreases liver steatosis by boosting fatty acid oxidation in a process partly mediated by the impaired mammalian target of rapamycin (mTOR) signaling, as regulated by DEPTOR (DEP-domain containing mTOR-interacting protein). Moreover, we have identified that the levels of NEDD8 in serum appear to correlate with NAFLD disease progression and reversal upon dietary interventions or neddylation inhibition. Overall, treating NAFLD by

targeting neddylation may be a fast and effective strategy to regulate altered signaling pathways and metabolic reactions.

2. METHODS

2.1. Human samples

The human samples used in this work were obtained after the informed consent of the patients and in accordance with the Ethical Code of the World Medical Association and Helsinki Declaration and upon approval of the Hospital Marques de Valdecilla ethics Committee. Four well-characterized cohorts of NAFLD patients, obtained from the University-Hospital Marques de Valdecilla (Santander, Spain), were used. Liver biopsies were assessed and classified by an expert pathologist. In order to study global neddylation levels in liver tissue by immunohistochemistry, two cohorts of NAFLD patients, including lean ($n = 4$) and obese patients ($n = 15$), were used. Conversely, another two cohorts of patients were analyzed to evaluate NEDD8 levels in serum ($n = 19$ -lean and $n = 24$ -obese; [Suppl. Table 1](#)). The inclusion criteria established to develop the current project were as follows: age between 18 and 75 years old, no other acute or chronic disease except for type 2 diabetes and obesity (metabolic syndrome) in accordance with the physical and biochemical analysis, and alcohol consumption should be less than 30 g/day for men and 20 g/day for women. The exclusion criteria established were viral infection or liver disease caused by drugs (drug-induced liver injury). All recruited patients were of Caucasian origin. The control groups consisted of healthy human liver and serum samples ($n = 7$ liver samples for histological study, and $n = 8$ serum samples from women and men between 40 and 70 years old). Additional protocols used are provided in the supplemental material.

The mRNA levels of all genes involved in the neddylation pathway were analyzed from liver biopsies obtained during bariatric surgery from a cohort of 125 NAFLD patients, described earlier [20]. Individuals with increased alcohol intake ($>30/20$ g/day in M/F), viral and autoimmune hepatitis, or other causes of liver disease were excluded. Informed consent was obtained from each patient, and the study protocol was approved by the Ethical Committee of the Fondazione IRCCS Ca' Granda and conformed to the ethical guidelines. Briefly, RNA was isolated from liver biopsies (miRNeasy mini-kit, Qiagen) and sequenced using a HiSeq 4000 (Illumina). Reads were aligned and counted (GRCh37, ENSEMBL v75 reference), employing STAR and RSEM packages. Count normalization was performed using DESeq2. The differential expression of the genes of interest was assessed by a two-tailed Wilcoxon Rank Sum test. Statistical analyses were conducted using R 3.5.3 software, and $p < 0.05$ was considered statistically significant.

2.2. Animal maintenance, diets, and treatment

The animal procedures were performed in accordance with the European Research Council for animal care and use and the National Institute of Health guide for care and use of Laboratory animals. The maximal authority of the Country Council of Bizkaia and the Institutional Animal Care and Use Committee of CIC bioGUNE approved the animal procedures. Adult (three-month-old) male C57BL/6J mice were acquired from Charles River Laboratories and accommodated into the AALAC-accredited CIC bioGUNE animal facilities and maintained at 21 ± 1 °C, $45 \pm 10\%$ humidity, and a 12/12 h light/dark cycle. Animal maintenance was based on ad libitum access to water and the respective diet. The control group was fed a standard chow diet with 0.3% methionine and 1.030 mg/kg of choline (Teklad Global 14% Protein Rodent Maintenance diet; Envigo 2014C). Dietary-mouse

models of NAFLD included: a high-fat diet (HFD; D12492, Research Diets), choline-deficient, high-fat diet (CDHFD; D05010402, Research Diets), and 0.1% methionine and choline-deficient diet (0.1% MCDD; A02082006i, Research Diets). A group of 5 animals, randomly divided, was treated with MLN4924 (MeDChemExpress, MCE) dissolved in a vehicle solution of 2-hydroxypropyl- β -cyclodextrin (Sigma—Aldrich) by oral gavage or subcutaneous injection at a dose of 60 mg/kg during the last two weeks of treatment in case of the animals fed 0.1% MCDD and during the last three weeks in the CDHFD model. A control group of 5 animals was treated with a vehicle solution. After 4 weeks of 0.1% MCDD and 6 weeks of CDHFD, animals were sacrificed.

In addition, a group of 10 Alfp-Cre mice was used to silence Nedd8 specifically in the liver and were randomly divided into control or silenced groups. These animals expressed Cre-recombinase open reading (ORF) under the control of albumin and α -fetoprotein, abundant in the hepatocytes [21]. They were infected with adenovirus in the tail vein, which contains a short hairpin Nedd8 RNA sequence and double floxed sequence that allows for the recombination with Cre and, therefore, the insertion of the sequence (AVV-DIO-shNedd8). The infection was performed 3 weeks before the start of the 6 weeks of CDHFD. Additional protocols used are provided in the supplemental material.

Finally, adult (three-month-old) male C57BL/6J mice were maintained during 3 weeks on a 0.1% methionine, choline-deficient, high-fat diet (CDAHFD; A06071302, Research Diets) and after this period, switched back to a standard chow diet for one additional week.

For all experiments, the liver and blood were extracted and maintained at -80 °C or formalin-fixed solution until further analysis.

2.3. Primary mouse hepatocytes and treatments

Male adult mice (C57BL/6J) were acquired from Charles River Laboratories and accommodated into the CIC bioGUNE animal facility. After liver perfusion and 3 h attachment, primary mouse hepatocyte media were changed to 0% FBS MEM (Gibco) + PSG (Gibco) overnight. The following morning, hepatocytes were incubated for 6 h with either 400 μ M oleic acid (Sigma—Aldrich) conjugated with BSA-fatty acid-free or BSA-fatty acid-free diluted in 0% MEM. Neddylation inhibition was achieved using 3 μ M of MLN4924 in DMSO, an inhibitor of the Nedd8 Activating Enzyme E1 Subunit 1 (MeDChemExpress). In some experiments, 20 μ M of Etomoxir (Sigma—Aldrich) was used during the 6 h period to inhibit fatty acid oxidation. Primary mouse hepatocytes were transfected by 12 h incubation with 100 nM Deptor siRNA (Thermo Fisher, Silencer Select siRNA DEPTOR, sequence Sense: 5'-AGACGGCGAUAAAACUCAUTT- 3' and Anti-sense: 5'-AUGAGUUUUUAGCGCCGUCUCT- 3) to evaluate the role of DEPTOR in fatty acid oxidation. Finally, Nedd8 was silenced using siNEDD8 to evaluate the physiological role of neddylation inhibition (Sigma siNEDD8 Sequence sense: 5'-CAUCUACAGUGGCAAGCAA [dT][dT]-3' and 5'-UUGCUUGCCACUGUAGAUGAG [dT][dT]-3'). The reagent used to silence DEPTOR and Nedd8 in hepatocytes was Lipofectamine 2000 (Invitrogen, Ref: 1168—019), according to the user's manual. Silencer™ Negative Control No. 1 siRNA (Thermo Fisher) was used as a negative siRNA transfection control. Additional protocols used are provided in [supplemental material](#).

2.4. Statistical analysis

Prism 8 (GraphPad Software, version 8.4.0) was used to perform statistical analyses. A one-way analysis of variance (ANOVA) followed by Tukey (comparing all pairs of columns) was used for three or more groups, while Student's t-test was used for 2 groups. Grubbs' test was performed to determine the significant outliers. A $p < 0.05$ was

considered statistically significant. Statistical parameters are reported in the figure legends.

3. RESULTS

3.1. Hepatic neddylation is augmented in clinical and pre-clinical NAFLD

Previous evidence from our laboratory has shown that NEDD8-mediated modifications are increased in patients with advanced liver fibrosis and cirrhosis and mouse models of liver fibrosis [16]. Herein, we aimed to extend our studies to assess the role of neddylation in the early stages of NAFLD. On this basis, hepatic global neddylation levels, evaluated by immunohistochemistry (IHC), were increased in liver biopsies from a cohort of well-characterized NAFLD patients, lean and obese, in comparison to age-matched healthy controls (Figure 1A, Suppl. Table 1). In-depth analyses revealed that hepatic global neddylation levels positively correlate with the NAS score (Figure 1B). Then, we evaluated the levels of hepatic neddylation in mouse models of diet-induced NAFLD, reflecting different stages of the pathology: *i*) animals on a high-fat diet (HFD) for 20 weeks, characterized by steatosis and ballooning; *ii*) animals on a choline-deficient, high-fat diet (CDHFD) for 6 weeks and characterized by mild steatosis and inflammation; and *iii*) animals on a 0.1% methionine and choline-deficient diet (0.1% MCDD) for 4 weeks and characterized by enhanced macro-steatosis and overt inflammation. Hepatic global neddylation was induced in all animal models of NAFLD (Figure 1C–E),

with higher increases corresponding to more aggressive dietary interventions. Further longitudinal hepatic characterization of steatosis, ballooning, inflammation, and fibrosis levels in the dietary mouse models of NAFLD is shown in Suppl. Fig. 1a–c. The deregulation of the neddylation pathway does not occur at the transcriptional level in the clinical setting or our animal models of diet-induced NAFLD, as no significant changes in the mRNA levels for E1, E2, and E3 or the deconjugating enzymes for the NEDD8 pathway were observed (Suppl. Fig. 2a–c).

In summary and concurrent with early evidence [19], hepatic neddylation was augmented in patients suffering from NAFLD and mouse models of diet-induced NAFLD.

3.2. Neddylation inhibition reduces lipid accumulation in NAFLD pre-clinical models

The effects of the specific small pharmacological inhibitor of neddylation targeting the NEDD8 activating enzyme (NAE1), MLN4924, ([[(1S,2S, 4R)-4-[4-[[[(1S)-2,3-dihydro-1H-inden-1-yl]amino]pyrrolo [2,3-d]pyrimidin-7-yl]-2 hydroxycyclopentyl]methyl sulfamate) (commercialized as Pevonedistat) [22], were evaluated in hepatocyte cell models of steatosis. Treatment with MLN4924 and reduced neddylation, assessed as decreased neddylated cullins in isolated mouse hepatocytes stimulated with oleic acid (OA), significantly reduced lipid content, as determined by bodipy staining (Suppl. Fig. 3a, Figure 2A). Though MLN4924 has been previously shown to induce tumor cell death, in primary mouse hepatocytes,

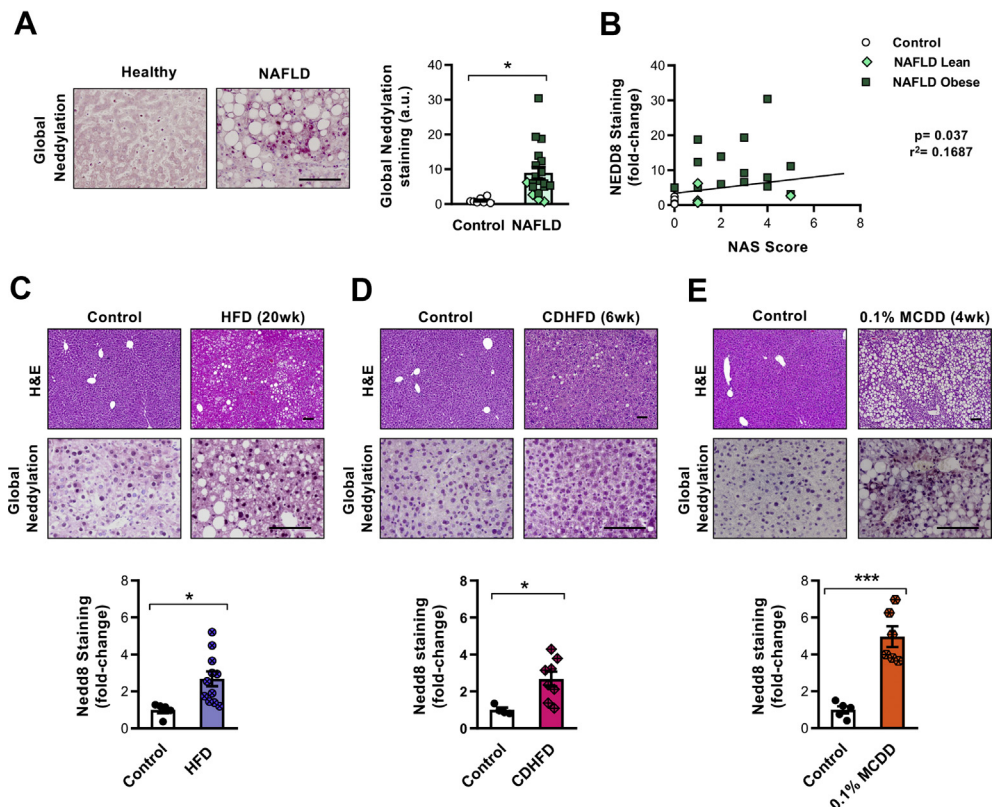


Figure 1: Neddylation is augmented in clinical and pre-clinical mouse models of non-alcoholic fatty liver disease (NAFLD). A. Hepatic neddylation levels assessed by immunohistochemistry and B. Pearson correlation and coefficient between hepatic neddylation and NAS score in NAFLD patients ($n = 19$) in comparison with a group of healthy controls ($n = 7$). Hepatic neddylation levels assessed by immunohistochemistry in healthy animals ($n = 5$) and C. animals maintained during 20 weeks on a high-fat diet (HFD) ($n = 11$), D. animals maintained during 6 weeks on a choline-deficient high-fat diet (CDHFD) ($n = 8$) and E. animals maintained during 4 weeks on a 0.1% methionine- and choline-deficient diet (0.1% MCDD) ($n = 6$). Scale bar corresponds to 100 μm . Data are shown as average \pm SEM and Student's *t*-test was used to compare groups. * $p < 0.05$ and *** $p < 0.001$ are shown.

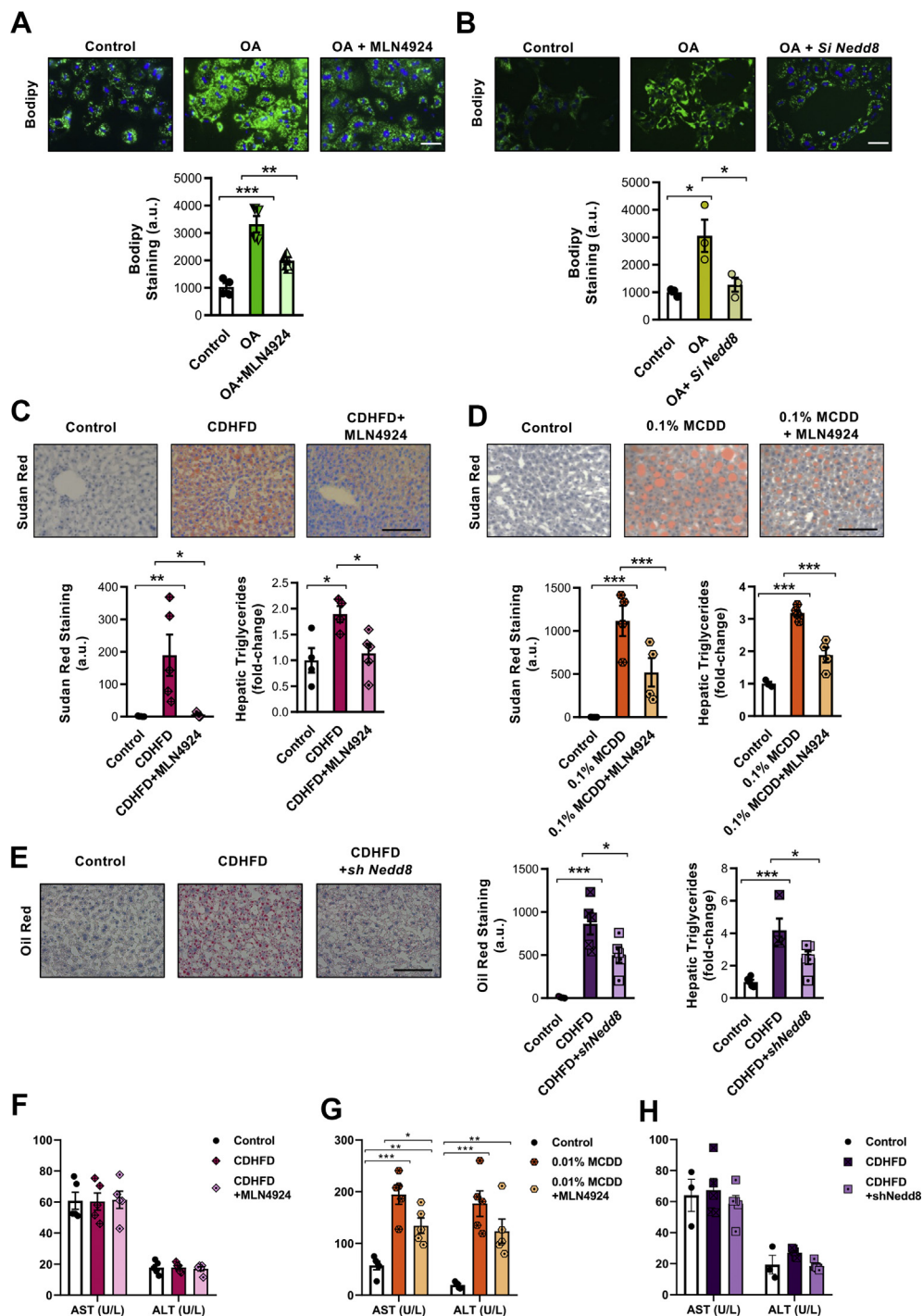


Figure 2: Neddylation inhibition reduces lipid accumulation in non-alcoholic fatty liver disease (NAFLD) pre-clinical models. **A.** and **B.** Bodipy staining in primary mouse hepatocytes isolated from wild-type mice and treated with MLN4924 3 μ M (n = 4) or siRNA against *Nedd8* (n = 3) in the presence of oleic acid (OA) during 6h. Sudan Red staining and quantification of total hepatic triglycerides in **C.** animals maintained during 6 weeks on choline-deficient and high-fat diet (CDHFD) (n = 5) and animals on CDHFD and treated with MLN4924 (CDHFD + MLN4924) (n = 5), in **D.** animals maintained during 4 weeks on 0.1% methionine- and choline-deficient diet (0.1% MCDD) (n = 4) and treated with MLN4924 (0.1%MCDD + MLN4924) (n = 4) and finally, in **E.** *Alfp-Cre/AAV-DIO-shNEDD8* mice were maintained for 6 weeks on CDHFD (n = 5). Transaminase serum levels of ALT (alanine transaminase) and AST (aspartate transaminase) in healthy animals and **F.** animals maintained during 6 weeks on CDHFD and CDHFD + MLN4924, **G.** animals maintained during 4 weeks on 0.1% MCDD and 0.1% MCDD + MLN4924, and in **H.** the hepatic silenced NEDD8 mice model (*Alfp-Cre/AAV-DIO-shNEDD8*) maintained during 6 weeks on CDHFD. Scale bar corresponds to 100 μ m. Data are shown as average \pm SEM and One-way ANOVA test was used to compare groups. *p < 0.05, **p < 0.01 and ***p < 0.001 are shown.

MLN4924 does not induce significant caspase-3 activity, an apoptosis marker (Suppl. Fig. 3b), suggesting that the observed effects in decreased lipid content do not reflect cell death. The lack of MLN4924 associated death-induced effects in steatotic hepatocytes also indicates reduced adverse effects related to MLN4924 treatment. Similar findings were obtained after *Nedd8* silencing in isolated mouse hepatocytes using molecular approaches, such as siRNA (Figure 2B; Suppl., Fig. 3c,d), suggesting that the anti-steatotic effects ascribed to MLN4924 are related to neddylation inhibition and not unspecific off-target effects.

Moreover, the effect of neddylation inhibition was evaluated in 2 mouse models of NAFLD, shown earlier to have high *Nedd8* levels (Figure 1D,E). In the CDHFD-fed mouse, animals were fed ad libitum for 3 weeks to induce mild steatosis, and afterward, MLN4924 treatment was given (60 mg/kg), once a week for other 3 weeks, totaling in 6 weeks of dietary intervention. In the 0.1% MCDD model, MLN4924 treatment (60 mg/kg) was given every 4 days, beginning after the second week of the diet, a time point characterized by steatosis and inflammation, and the experiment finished at 4 weeks of diet (Suppl. Fig. 4a). MLN4924 treatment and associated hepatic neddylation inhibition after CDHFD and 0.1% MCDD significantly decreased hepatic steatosis, quantified by Sudan red staining and biochemically measuring hepatic triglycerides (Figure 2C and D, and Suppl. Fig. 4b,c). We silenced *Nedd8*, specifically in the hepatocytes of CDHFD animals, by using *Alfp-Cre/AAV-DIO-shNedd8* to confirm further that there are no off-target effects associated with MLN4924 treatment (Suppl. Fig. 4a). Under these conditions, *Nedd8* silencing, specifically in the hepatocytes in vivo, is associated with reduced hepatic neddylation levels and liver steatosis (Figure 2E, Suppl. Fig. 4d). Supporting these data, serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were significantly reduced after neddylation inhibition, indicating diminished liver injury in the 0.1% MCDD model. In contrast, no changes were found in ALT or AST in the CDHFD model compared with control animals (Figure 2F,H). Of note, the final body weight in MLN4924-treated mice was not altered, relative to the vehicle-treated animals on NAFLD diets (CDHFD: 32.5 ± 0.9 g and CDHFD + MLN4924: 31.2 ± 1.2 g, p -value = 0.57; 0.1% MCDD: 23.7 ± 0.7 g and 0.1% MCDD + MLN4924: 24.3 ± 1.1 g, p -value = 0.63; CDHFD: 33.1 ± 0.6 g and CDHFD + *shNedd8* 32.1 ± 1.3 g, p -value = 0.45), suggesting that the anti-steatotic effects of neddylation inhibition are not related to decreased adiposity.

In summary, neddylation-specific inhibition shows anti-steatotic effects in isolated mouse hepatocytes and pre-clinical mouse models of NAFLD.

3.3. mTOR inhibition via DEPTOR accumulation plays a role in neddylation inhibition mediated anti-steatotic effects in NAFLD pre-clinical models

Recent reports have highlighted that the ATF/CREB family is involved in regulating many aspects of lipid metabolism processes, including regulating critical enzymes and regulators involved in lipogenesis, fatty acid oxidation, and lipoprotein metabolism [23]. mRNA levels of members of the ATF/CREB family, such as *Atf4* and *6* (Activating Transcription Factor 4 and 6) and *Crebh* (CAMP Responsive Element Binding Protein), show no significant regulation as a result of the MLN4924 treatment in the CD-HFD and 0.1% MCDD NAFLD mouse models (Suppl. Fig. 5a,b). Thus, we performed high-throughput proteomics Liquid Chromatography-Mass Spectrometry (LC-MS)-based analyses in animals fed CDHFD for 6 weeks and 0.1% MCDD for 4 weeks to better understand NAFLD at the proteome level. Heat map

representation shows the top 50 altered proteins after CDHFD and 0.1% MCDD (Figure 3A; Supplemental File 1). A file containing all identified proteins is provided in Supplemental File 1. Ingenuity pathway analysis (IPA) was used to identify the major canonical pathways involved in NAFLD, suggesting that eukaryotic initiation factor 2 (eIF2) signaling, which integrates a diverse array of stress-related signals to regulate both global and specific mRNA translation, and lipid metabolism, are profoundly altered. Moreover, the mTOR pathway, which plays an essential role in regulating lipid metabolism [15,24,25] is also highly altered in NAFLD (Figure 3B). In addition, proteomics LC-MS-based analysis was also performed to assess the effect of MLN4924 treatment in the dietary mouse models of NAFLD (Figure 3C; Supplemental File 1). IPA analysis aimed at identifying potential upstream regulators that explain the changes observed in the proteomics panel as a result of MLN4924 treatment was performed. The common upstream regulators identified in the two dietary models studied include MYC, HNF4A, TP53, and PDR (Figure 3D). Further studies are necessary to understand the upstream regulators involved in neddylation triggering inhibition.

mTOR signaling plays a vital role in regulating lipid metabolism, such as lipogenesis and oxidative fluxes in the liver [26]. In addition, other studies have shown that in tumor cells, DEPTOR is degraded by the SCF^{βTrCP} (Skp1-Cullin-F box proteins) E3 ubiquitin ligase, the founding member of CRLs, resulting in mTOR activation and cell proliferation [27]. Importantly, SCF^{βTrCP} E3 ubiquitin ligase is activated through NEDD8 conjugation of the conserved lysines of cullins.

Herein, we show that the neddylation inhibition in vivo in CDHFD and 0.1% MCDD induced protein DEPTOR, and as a consequence of the concomitant mTOR inhibition, the phosphorylation of S6 protein (pS6), a downstream target of mTOR, was reduced without changes in *Deptor* gene expression (Figure 4A and B). Likewise, neddylation inhibition using MLN4924 pharmacological treatment in OA-stimulated hepatocytes increased DEPTOR content (Figure 4C). Under these conditions, when silencing *Deptor* by using siRNA-based molecular approaches in primary mouse hepatocytes, MLN4924 treatment did not significantly reduce the cellular lipid content (Figure 4C–E).

Thus, mTOR inhibition via DEPTOR accumulation plays a role in the neddylation inhibition-mediated anti-steatotic effects.

3.4. Neddylation inhibition boosts fatty acid oxidation coupled with oxidative phosphorylation in NAFLD pre-clinical models

The regulation of fat homeostasis in the liver results from a balance between uptake, anabolism, catabolism, and export from the liver. Indeed, a tight regulation between fat uptake, de novo lipogenesis (DNL), fatty acid degradation/oxidation (FAO), and triglyceride-containing very-low density lipoproteins (VLDL) export occurs in the liver. Both unaltered changes in weight gain and serum triglycerides and cholesterol after MLN4924 treatment or *Nedd8* silencing in vivo in CDHFD- and 0.1% MCDD-fed animals (Suppl. Fig. 6a–c) strongly suggest that neither fat uptake nor VLDL export, respectively, are altered after neddylation inhibition. Furthermore, we know from previous evidence that DNL is reduced due to consuming methionine- and choline-deprived diets and is, therefore, not a central player in the mouse models of dietary-induced NAFLD [28,29]. mTOR signaling plays an essential role in regulating lipid metabolism, such as lipogenesis and oxidative fluxes in the liver. Indeed, pharmacologic intervention via mTORC1 inhibition increases the oxidation of endogenous fatty acids in the primary cultures of rat hepatocytes [30] and ameliorates hepatic steatosis in chronic-binge ethanol-fed mice [31]. MLN4924 treatment to isolated mouse hepatocytes stimulated by OA induces FAO activity (Figure 5A). Increased FAO under these

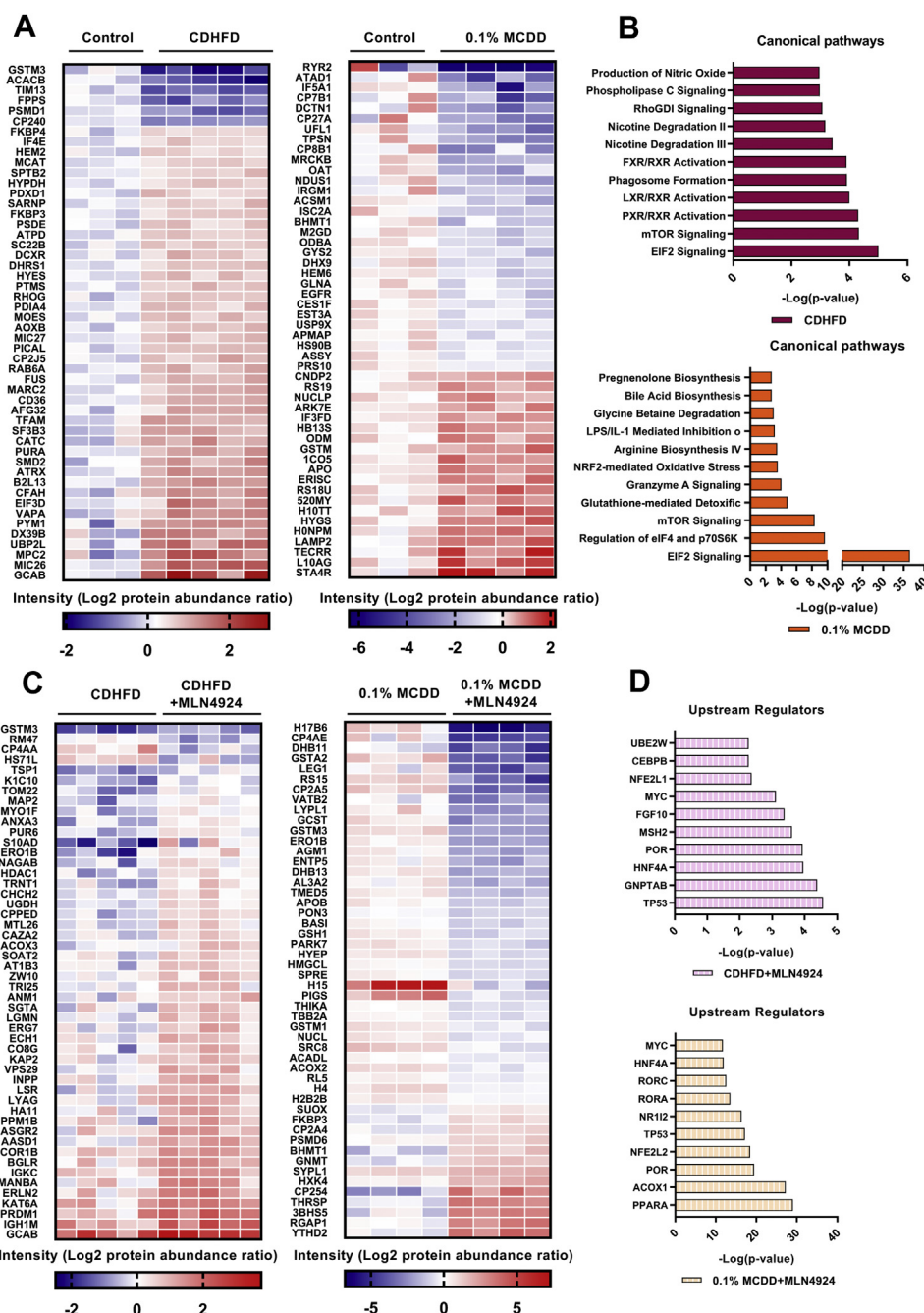


Figure 3: Liver proteomics characterization by Liquid Chromatography-Mass Spectrometry (LC-MS) in mouse models of non-alcoholic fatty liver disease (NAFLD) and treated with MLN4924. **A.** Heatmaps showing the top 50 significantly differentially expressed proteins and **B.** Ingenuity Pathway Analysis (IPA) of top canonical pathways in animals maintained during 6 weeks on choline-deficient and high-fat diet (CDHFD) as well as in animals maintained during 4 weeks on 0.1% methionine- and choline-deficient diet (0.1% MCDD). **C.** Heatmaps showing the top 50 significantly differentially expressed proteins; **D.** Ingenuity Pathway Analysis (IPA) of Upstream Regulators in animals maintained during 6 weeks on choline-deficient and high-fat diet (CDHFD) and animals on CDHFD and treated with MLN4924 (CDHFD + MLN4924), as well as in animals maintained during 4 weeks on 0.1% methionine- and choline-deficient diet (0.1% MCDD) and animals on 0.1% MCDD and treated with MLN4924 (0.1% MCDD + MLN4924).

circumstances most likely accounts for the augmented fueling of the TCA cycle by acetyl-CoA facilitating the transference of reducing equivalents from various intermediates to coenzymes and the production of NADH and FADH. These two reduced coenzymes fuel the respiratory chain. In agreement, neddylation inhibition by MLN4924 significantly induced oxidative phosphorylation (OXPHOS), an electron transport-linked phosphorylation, and ATP-linked respiration in mouse

hepatocytes, measured by Seahorse-based analysis (Figure 5B). Furthermore, in the presence of etomoxir, a well-described inhibitor of FAO, MLN4924 was unable to decrease the hepatic lipid content of mouse hepatocytes stimulated by OA (Figure 5C), further confirming that the pharmacological inhibition of neddylation, through MLN4924, may be exerting its lipid-lowering effect by increasing FAO. Likewise, FAO activity was shown to be induced in MLN4924-treated CDHFD and

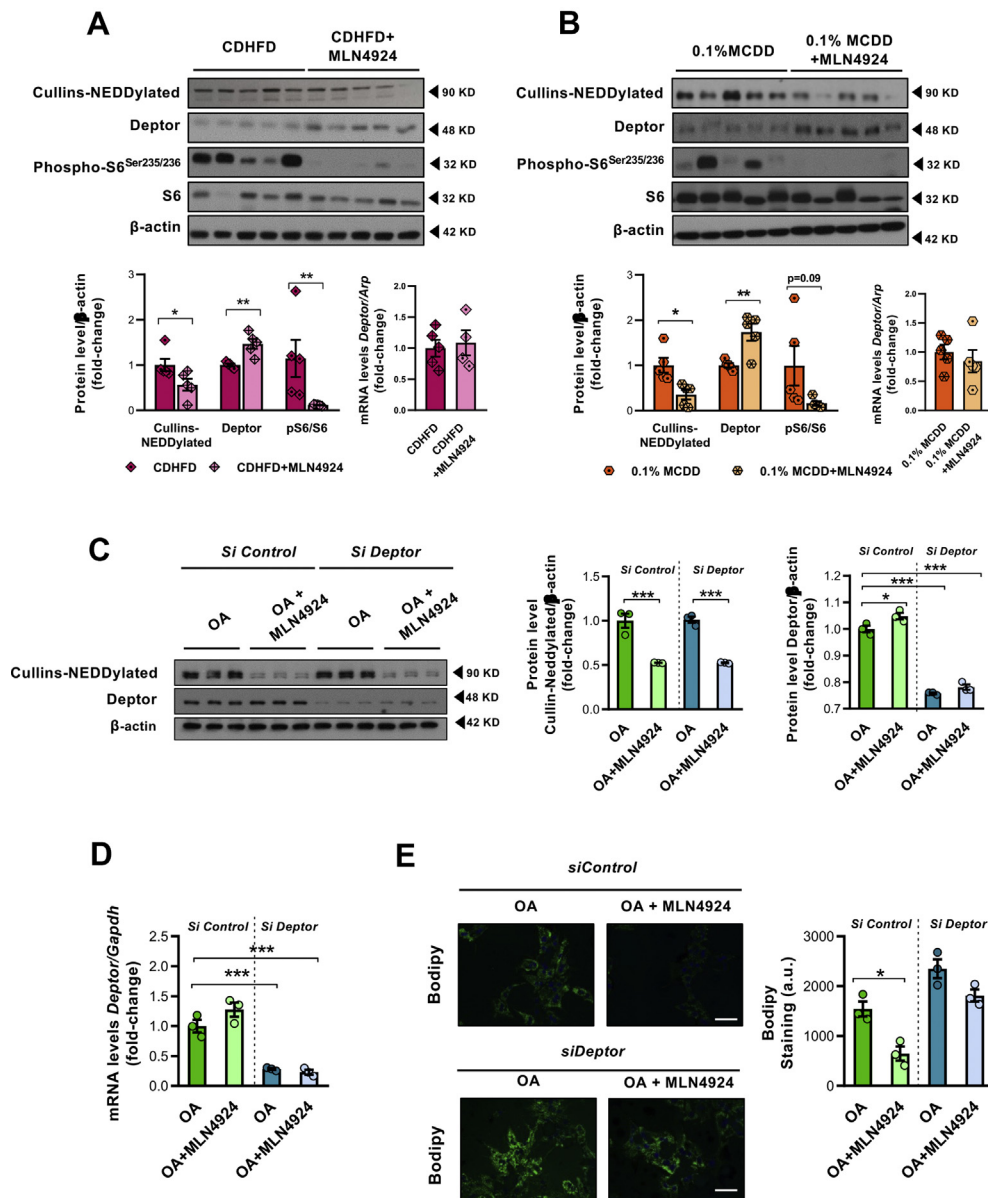


Figure 4: DEPTOR accumulation by neddylation inhibition mediates the anti-steatotic effects in pre-clinical models of non-alcoholic fatty liver disease (NAFLD). Western blot analysis of neddylated cullins, DEPTOR, phosphorylated S6 (pS6) and total S6 and beta-actin both in **A**. animals maintained during 6 weeks on choline-deficient and high-fat diet (CDHFD) ($n = 5$), and animals on CDHFD and treated with MLN4924 (CDHFD + MLN4924) ($n = 5$) as well as in **B**. animals maintained during 4 weeks on 0.1% methionine- and choline-deficient diet (0.1% MCDD) ($n = 5$) and animals on 0.1%MCDD and treated with MLN4924 (0.1% MCDD + MLN4924) ($n = 5$). **C**. Western blot of neddylated cullins, and DEPTOR ($n = 3$). **D**. mRNA analysis of DEPTOR levels ($n = 3$) and **E**. Bodipy staining of lipids in hepatocytes stimulated with OA and MLN4924 when silencing DEPTOR ($n = 3$). Data are shown as average \pm SEM. One-way ANOVA test and Student's t-test were used to compare groups. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ are shown.

0.1% MCDD-fed rodents (Figure 5D and E), whereas OXPHOS, measured with a Seahorse extracellular analyzer in isolated fresh liver mitochondria, was increased after MLN4924 treatment in CDHFD-fed rodents (Figure 5F). Overall, neddylation inhibition boosted fatty acid oxidation, coupled with oxidative phosphorylation and decreasing liver fat content.

3.5. Neddylation inhibition reduces oxidative stress, lipid peroxidation, and inflammation in NAFLD pre-clinical models

Fat accumulation in hepatocytes during NAFLD provides a potential substrate for lipid peroxidation, a process in which oxidants (e.g., free

radicals) attack lipids containing carbon–carbon double bond(s), especially polyunsaturated fatty acids (PUFAs). Malondialdehyde and, in particular, 4-hydroxy-2-nonenal (4-HNE) are the primary omega-6 fatty acid lipid peroxidation products. Herein, mouse models of diet-induced steatosis, CDHFD, and 0.1% MCDD, show an increased accumulation of dihydroethidium (DHE), a fluorescent marker of reactive oxygen species (ROS), and 4-HNE. Importantly, both DHE and 4-HNE were significantly decreased after MLN4924 treatment in vivo in the CDHFD and 0.1% MCDD-fed rodents (Figure 6A,B). Moreover, in the CDHFD and 0.1% MCDD-fed rodents, MLN4924 treatment was associated with an induced expression of NRF2 (nuclear factor

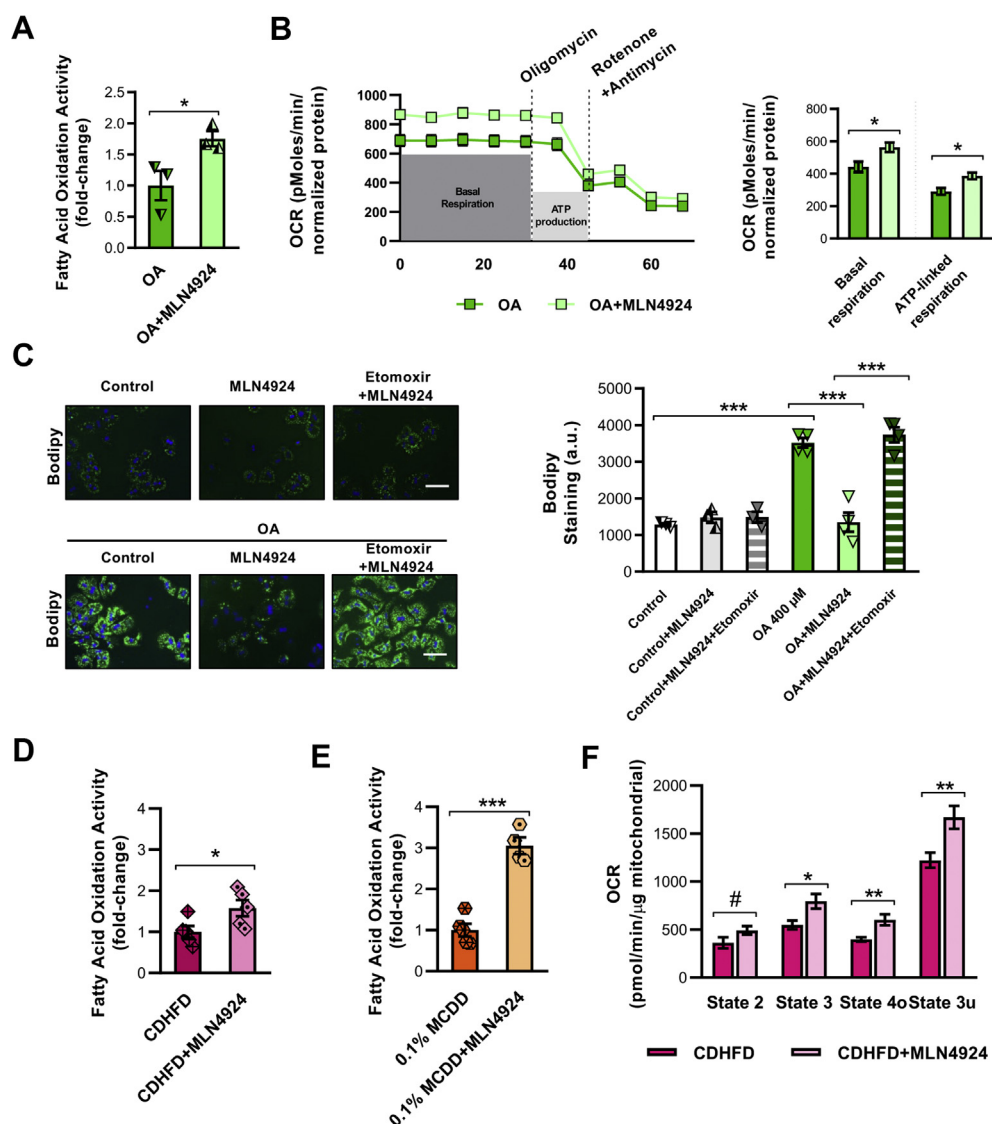


Figure 5: Neddylolation inhibition boosts fatty acid oxidation coupled with oxidative phosphorylation in non-alcoholic fatty liver disease (NAFLD) pre-clinical models. A. Fatty acid oxidation (FAO) fluxes ($n = 3$); and **B.** Oxygen consumption rate (OCR) as analyzed using a Seahorse analyzer in primary mouse hepatocytes stimulated with oleic acid (OA) during 6 h in the presence and absence of MLN4924. **C.** Immunofluorescence staining of Bodipy with or without stimulation with etomoxir, an inhibitor of FAO in primary mouse hepatocytes isolated from wild type mice and treated with MLN4924 in the presence of OA during 6h ($n = 3-4$). **D.** and **E.** Fatty acid oxidation fluxes in animal models maintained during 6 weeks on choline-deficient and high-fat diet (CDHFD), and animals on CDHFD and treated with MLN4924 (CDHFD + MLN4924) as well as in animals maintained during 4 weeks on 0.1% methionine- and choline-deficient diet (0.1% MCDD) and animals on 0.1%MCDD and treated with MLN4924 (0.1% MCDD + MLN4924) ($n = 5$ per group). **F.** OCR as analyzed using a Seahorse analyzer in isolated liver mitochondria from mice maintained during 6 weeks on CDHFD or CDHFD + MLN4924. Scale bar corresponds to 100 μm . One-way ANOVA test and Student's t-test, respectively, were used to compare groups. # $p = 0.09$, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ are shown.

erythroid 2—related factor 2; Suppl. Fig. 7a). NRF2 is a master regulator of antioxidative responses known to drive the expression of numerous cytoprotective genes involved in xenobiotic metabolism, antioxidant responses, and anti-inflammatory responses [32]. Thus, the expression of genes involved in the glutathione (GSH) metabolism was further assessed. In the CD-HFD and 0.1% MCDD MLN4924-treated groups, a tendential overexpression at the transcriptional level was observed for the subunits *GclC* and *GclM* of *Gcl* (glutamate cysteine ligase), an enzyme that catalyzes the rate-limiting step in GSH synthesis. Moreover, *Gsr* (glutathione reductase), involved in GSH reduction, and *Gsta* (glutathione S-transferase), which catalyzes the conjugation of reduced GSH to a vast number of exogenous and

endogenous hydrophobic electrophiles, was augmented after MLN4924 treatment (Suppl. Fig. 7b,c).

The oxidative stress response induced by lipid accumulation in hepatocytes may further trigger the inflammatory cytokine responses. Indeed, though the hepatic mRNA levels of proinflammatory cytokines, such as *Tnf* (tumor necrosis factor), *Ccl-2* (chemokine (C—C motif) ligand 2, and *Il-6* (interleukin-6), were increased after CDHFD, treatment with MLN4924 reduced their levels (Figure 6C). Likewise, *Tnf- α* , *Ccl-2*, and *Il-1 β* (interleukin-1 beta) were increased after 0.1% MCDD for 4 weeks and decreased after neddylolation inhibition in vivo with MLN4924. Moreover, *Timp-2* (tissue inhibitor of metalloproteinases 2), an early pro-fibrogenic gene, is increased with 0.1% MCDD and

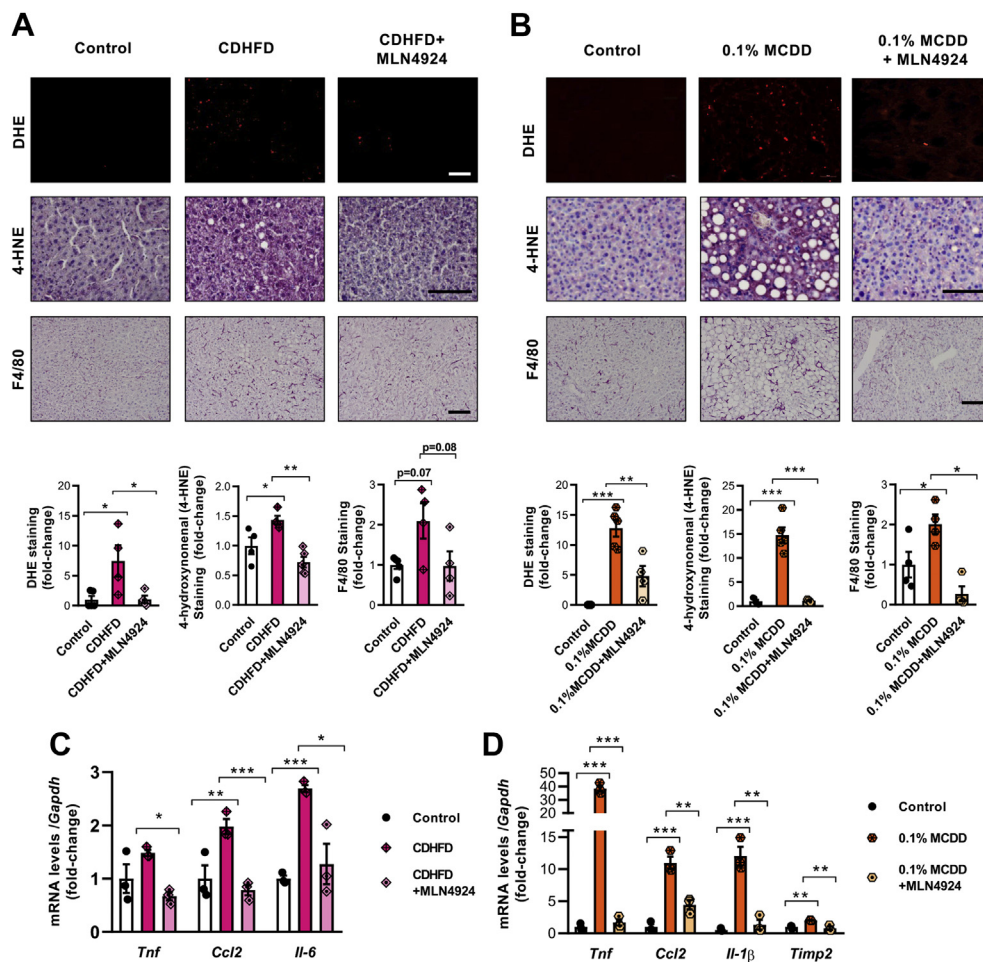


Figure 6: Neddylation inhibition reduces lipid peroxidation and inflammation in non-alcoholic fatty liver disease (NAFLD) pre-clinical mouse models. A. and B. dihydroethidium (DHE), a fluorescent marker of reactive oxygen species (ROS), 4-hydroxynonenal (4-HNE) and F4/80 immunostaining and respective quantification ($n = 4-5$); C. and D. mRNA levels of genes involved in the inflammatory and fibrotic process (*Tnf*-tumor necrosis factor, *Ccl2*- C-C motif chemokine ligand 2, *Il-6*- Interleukin-6, *Il-1β*- Interleukin-1 β, *Timp2*, tissue inhibitor of metalloproteinases 2) both in animals maintained during 6 weeks on choline-deficient and high-fat diet (CDHFD), and animals on CDHFD and treated with MLN4924 (CDHFD + MLN4924), and compared to controls on standard chow diet, as well as in animals maintained during 4 weeks on 0.1% methionine- and choline-deficient diet (0.1% MCDD) and animals on 0.1%MCDD and treated with MLN4924 (0.1% MCDD + MLN4924), and compared to controls on standard chow diet ($n = 3-5$ per group). Scale bar corresponds to 100 μm. Data are shown as average ± SEM and One-way ANOVA tests were used to compare groups. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ are shown.

reduced after MLN4924 treatment (Figure 6D). In agreement, the recruitment of the liver resident macrophages, assessed by immunostaining F4/80, a macrophage marker, is significantly reduced after neddylation inhibition in CDHFD and 0.1% MCDD-fed rodents (Figure 6A,B).

In summary, neddylation inhibition accounted for decreased oxidative stress, lipid peroxidation, and inflammation in mouse models of NAFLD.

3.6. Serum NEDD8 levels correlate with NAFLD severity and response to treatments in pre-clinical mouse models

Once hepatic neddylation was highly induced in NAFLD, we decided to evaluate NEDD8 serum levels in patients with NAFLD. Interestingly, serum NEDD8 levels, assessed by an ELISA assay, were elevated in these patients relative to healthy controls, positively correlating with the NAS score (Figure 7A and B). Similar to our findings in the clinical setting, serum levels of Nedd8 were increased in animals with a more advanced stage of NAFLD. Moreover, serum Nedd8 levels in MLN4924-

treated NAFLD rodents were reduced due to neddylation inhibition and a readout of improved liver phenotype (Figure 7C). We evaluated the Nedd8 serum levels in another diet model of NAFLD (i.e., the high-fat diet with 0.1% methionine and no added choline [CDAHFHD]) to further confirm our findings in other therapeutic approaches that do not directly target neddylation. After 3 weeks of CDAHFHD, the diet was replaced with a standard chow diet for 1 additional week. As a result of the diet reversal, the liver phenotype improved, together with lower serum transaminases and serum Nedd8 levels (Figure 7D–F).

In summary, NEDD8 serum levels correlate with disease progression, highlighting that the power of serum NEDD8 as a potential non-invasive biomarker for NAFLD should be further investigated.

4. DISCUSSION

Neddylation is a druggable and reversible PTM that has been previously shown to play an essential role in the late stages of chronic liver disease, such as advanced liver fibrosis and HCC. Under these

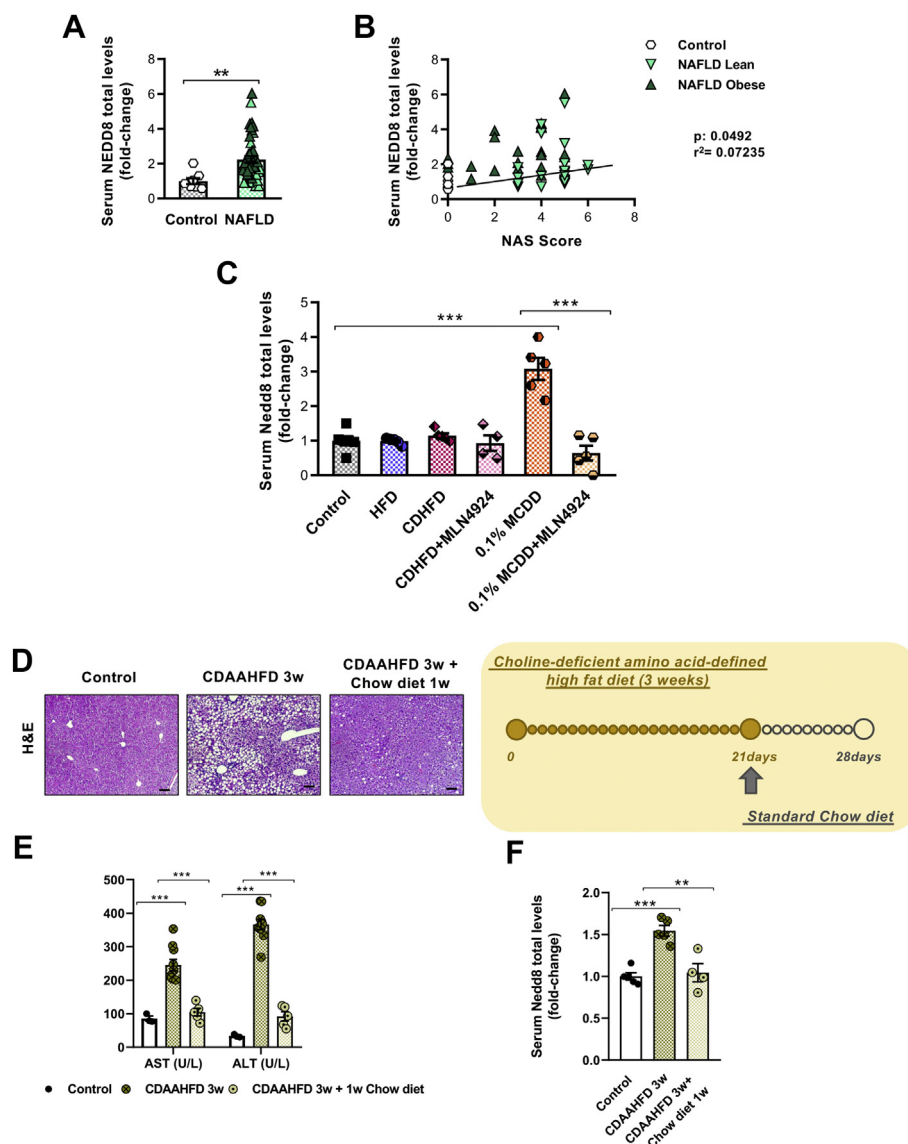


Figure 7: Serum NEDD8 levels correlate with NAFLD severity and response to treatment. **A.** Serum NEDD8 levels in NAFLD patients ($n = 46$) in comparison with a group of healthy controls ($n = 8$). **B.** Pearson correlation and coefficient between serum NEDD8 levels and NAS score. **C.** Comparison of serum Nedd8 levels between high-fat diet for 20 weeks (HFD), choline-deficient high-fat diet for 6 weeks (CDHFD), and 0.1% methionine- and choline-deficient diet for 4 weeks (0.1% MCDD) and healthy animals on a standard chow diet (control), as well as animals on CDHFD and treated with MLN4824 (CDHFD + MLN4824), and 0.1%MCDD and treated with MLN4824 (0.1% MCDD + MLN4824) ($n = 4-5$ per group). **D.** Hematoxylin and Eosin (H&E) staining; **E.** Serum transaminases (alanine aminotransferase- ALT, and aspartate aminotransferase- AST); and **F.** Serum Nedd8 levels in healthy animals (control), in a group of animals maintained during 3 weeks on a 0.1% methionine, choline-deficient, high-fat diet (3 wk CDAHFD) and in another group where this 3-week diet period was followed by one week of standard chow diet (SCD) (3 wk CDAHFD + 1wk SCD). Data are shown as average \pm SEM. One-way ANOVA test and Student's t-test, respectively, were used to compare groups. ** $p < 0.01$ and *** $p < 0.001$ are shown.

conditions, neddylation inhibition has been demonstrated to be an effective and safe therapeutic target in pre-clinical-based studies [16,17,33]. Herein, we provide strong evidence that the total levels of hepatic neddylation are increased in the liver tissue of patients with NAFLD mouse models of diet-induced NAFLD. These results concur with early findings showing increased levels of NEDD8 protein in patients with hepatic steatosis relative to healthy controls [19]. Other studies are necessary to unravel whether this regulation is dependent on other variables and associated NAFLD complications. Moreover, the triggering mechanisms of overactive neddylation pathways in NAFLD and other chronic liver diseases are not fully understood. Previous studies have shown that stress conditions, such as heat shock and

oxidative stress, may lead to an increase of the neddylation pathway in vitro [34]. Oxidative stress is a hallmark of more advanced stages of NAFLD and may be potentially involved in the neddylation upregulation in this pathology. Further studies are necessary to unravel the potential upstream regulators controlling neddylation in NAFLD.

In 2009, Soucy and colleagues described a small pharmacological compound that specifically inhibits neddylation (i.e., MLN4924/Pevonedistat) for the first time [22]. Herein, we moved beyond and used MLN4924 to treat steatosis in isolated mouse hepatocytes stimulated by OA and mouse models of diet-induced NAFLD. Neddylation inhibition with MLN4924 in CDHFD- and 0.1% MCDD-fed mice resulted in decreased hepatic lipid content without associated hepatic-related

adverse effects. In addition, we have confirmed that Nedd8 silencing and treatment with MLN4924 display similar effects in isolated mouse hepatocytes and in vivo, indicating that the anti-steatotic properties are related to neddylation modulation.

The regulation of fat homeostasis in the liver results from a balance between uptake, catabolism, and export from the liver. Here, we have found that neddylation inhibition improves liver steatosis by stimulating mitochondrial function and FAO. These results concur with recent findings from other authors reporting that neddylation is very active in mitochondria [35]. Augmenting FAO has been recently suggested as a useful therapeutic approach to reduce hepatic lipid content in NAFLD [36,37]. Others have shown that neddylation inhibition alters mitochondrial morphology in tumor proliferating cells, increasing mitochondrial fusion and inducing oxidative phosphorylation [38], concurring with our results.

Conversely, a previous report has shown that hepatic neddylation promotes FAO by maintaining electron transfer flavoproteins (ETF) in neonatal and (to a lesser extent) adult mice, preventing fasting-induced steatosis in the latter [35]. Though these results do not concur with our present evidence, the differences are most likely due to the lessened prevalence of the neddylation/ETF axis in adulthood and fasting-induced steatosis model differing from our models of diet-induced NAFLD. In addition, lipid homeostasis is regulated by many transcription factors in the liver that are altered in NAFLD [39]. For example, members of the ATF/CRBEH family have been shown to increase fatty acid oxidation [23]. Though we have not observed changes at the transcription level in the intermediates of this family, it should be considered that when we treated with MLN4924, we modify proteins post-translationally, and other regulatory mechanisms, such as cell localization and protein stability, might play a role.

Herein, we have also shown that augmented FAO is coupled with increased ETC fluxes, and therefore, ROS is not likely to be abundantly produced under these circumstances. In addition, we show that NRF2, a master regulator of cellular oxidant stress, is induced after MLN2924 treatment [40]. The MLN4924-induced expression of NRF2 corroborates early evidence showing that NRF2 is negatively regulated by KEAP1 (Kelch-like ECH-associated protein 1), a substrate adaptor protein for the Cullin3 (Cul3)-containing E3-ligase complex, which targets NRF2 for ubiquitination and degradation by the ubiquitin proteasome system (UPS) [41]. Under neddylation inhibition conditions, NRF2 can evade KEAP1-mediated degradation, translocate to the nucleus, and activate the ARE-dependent gene. In fact, Keap1/Nrf2/ARE regulates GSH levels by upregulating GSH synthetic and regenerative enzymes, including GCL, as previously observed [42]. Moreover, NRF2 also supports antioxidant and detoxification pathways by increasing the synthesis and regeneration of NADPH, a niacin-derived reducing agent. NADPH is a direct antioxidant and is used as an enzyme cofactor in many redox reactions, such as GSH reduction by glutathione reductase (an enzyme with an expression augmented after neddylation inhibition and NRF2 upregulation) [43]. Finally, glutathione S-transferases, known to have an antioxidant role, and a major determinant of the intracellular concentration of 4-HNE, is also regulated by NRF2 [44,45] and is increased after neddylation inhibition. Importantly, OXPHOS has been described to be more efficient when Nrf2 is activated [46]. Thus, reduced ROS production and the induction of an antioxidant response after neddylation inhibition in mouse models of diet-induced NAFLD may contribute to the low ROS in the presence of increased FAO fluxes.

Though the isolated accumulation of fat in the liver is considered a relatively benign hallmark of NAFLD, it is well known that steatosis may progress to NASH and, later, liver fibrosis. Indeed, prospective and

genetic data are consistent with hepatic fat driving NAFLD progression [47]. Conversely, the 'multiple hit' theory was postulated over a decade ago by Tilg and Moschen to explain the mechanisms underlying the progression of NAFLD [48]. These authors proposed that hepatic lipotoxicity and multiple events beyond the liver (e.g., adipose tissue inflammation) and gastrointestinal hits (e.g., dysbiosis) fuel the evolution of NAFLD. Over the last decade, a plethora of studies has evolved this hypothesis into an established concept [49,50]. On this basis, we have shown that neddylation inhibition in vivo not only ameliorates hepatic steatosis but also accounts for decreased lipid peroxidation. The induced oxidative stress response in NAFLD was highly associated with overwhelming inflammation. We have shown that neddylation inhibition in CDHFD- and 0.1% MCDD-fed rodents decreased inflammation markers, such as the accumulation of liver macrophages. Previously, researchers (including the present authors) have shown that neddylation inhibition can directly inhibit Kupffer cell activation [16,38]. Therefore, further studies are necessary to determine if decreased lipid inflammation induced by neddylation inhibition is a direct effect specifically targeting immune cells, an indirect consequence of decreased lipid content in hepatocytes and the concomitant non-recruitment of the liver macrophages, or a combined effect of both.

In addition, the mechanisms underlying the MLN4924-induction of FAO in NAFLD were further explored. Indeed, we provide evidence that DEPTOR and mTOR signaling are relevant in mediating MLN4924-induced FAO and decreased hepatic steatosis. DEPTOR is a naturally occurring inhibitor of mTOR that has been previously shown to be degraded by SCF E3 ubiquitin ligase, resulting in mTOR activation and cell proliferation [27]. Consistent with DEPTOR's ability to ameliorate hepatic steatosis [31], we observed that MLN4924 treatment increases the protein levels of DEPTOR by decreasing the levels of neddylated cullins and, therefore, the activity of the SCF E3 ubiquitin ligase that will otherwise promote DEPTOR ubiquitination and proteasomal degradation. Importantly, silencing DEPTOR halts MLN4924-induced lipid lowering in isolated mouse hepatocytes. Though our results indicate that intact DEPTOR signaling is essential for the MLN4924 anti-steatotic effects, additional potential targets and mechanisms should be explored and considered when assessing the MLN4924 effect in other experimental models of NAFLD.

Finally, we observed that in NAFLD, the total levels of NEDD8 in serum are proportional to hepatic neddylation levels, as hepatic and circulating NEDD8 is more elevated with disease progression, particularly in stages in which hepatic inflammation is aberrant. Furthermore, we have shown that well-established therapies for NAFLD, such as dietary interventions, decrease serum NEDD8 levels in pre-clinical mouse models. These results suggest that serum NEDD8 levels may potentially be used as a non-invasive biomarker of NAFLD progression and to precisely identify patients who could potentially benefit from a NEDD8-targeted therapy. Further studies in a larger cohort of patients are necessary to validate the usefulness of serum NEDD8 as a biomarker able to discriminate between simple steatosis and NASH.

In summary, we show that hepatic neddylation is augmented in the clinical setting and pre-clinical models of NAFLD. Importantly, neddylation inhibition decreases lipid content by increasing FAO and reduces oxidative stress, lipid peroxidation, and inflammation in mouse models of dietary NAFLD. Targeting the neddylation pathway using MLN4924 is an attractive therapeutic approach, considering that in a healthy adult liver, neddylation is almost negligible [51], and it is only upon a stress trigger, as occurs in NAFLD, liver fibrosis, and HCC, that neddylation is induced [16,17]. Therefore, it is predicted that neddylation inhibition-based therapies in NAFLD are not

associated with major adverse effects, though this prediction should be confirmed in chronic mouse models of NAFLD. Moreover, potential future clinical trials addressing the role of MLN4924 (Pevonedistat) efficacy in NAFLD can be relatively rapid, as phase I trials carried out in healthy people have already been performed for MLN4924 (a drug that is already included in phase 3 clinical trials for the treatment of acute myeloid leukemia). Importantly, serum NEDD8 levels reflect hepatic neddylation and can potentially be used as a non-invasive marker in NAFLD. Overall, we provide a novel mechanism involved in NAFLD development and open a novel therapeutic avenue to tackle NAFLD.

Author contributions to manuscript

Conception or design of the work: TCD, MLM-C.

Acquisition, analysis, or interpretation of data: MS-M, JS, MJG-R, MA, FL-O, DSU, NG-U, RR-A, SL-O, MM-G, VGJ, MB, DF-R, XB, GAB, PI, JC, EV, SCL, JMB, MAV, JJG-M, PA, UM, FE, DPX, RN, LVCV, Drafted the work: MS-M, TCD, MLM-C.

Substantively revised the work: MS-M, TCD, MLM-C, RB, DPX, JJG-M, MAV, PA, JMB, RB, UM.

ACKNOWLEDGMENTS

This work was supported by grants from Gobierno Vasco-Departamento de Salud 2013111114 (to M.L.M.-C), ELKARTEK 2016, Departamento de Industria del Gobierno Vasco (to M.L.M.-C), Ministerio de Ciencia, Innovación y Universidades MICINN: SAF2017-87301-R, and RTI2018-096759-A-100 integrado en el Plan Estatal de Investigación Científica y Técnica y Innovación, cofinanciado con Fondos FEDER (to M.L.M.-C T.C.D respectively); MCIU/AEI/FEDER, UE (RTI2018-095134-B-100) (to P.A.), AECC Bizkaia (M.S-M); Asociación Española contra el Cáncer (T.C.D), Fundación Científica de la Asociación Española Contra el Cáncer (AECC Scientific Foundation) Rare Tumor Calls 2017 (to M.L.M., J.M.B., M.A.A., J.J.G.M.), La Caixa Foundation Program (to M.L.M. and J.M.B.), 2018 BBVA Foundation Grants for Scientific Research Teams (to M.L.M.-C.), Ayudas para apoyar grupos de investigación del sistema Universitario Vasco IT971-16 (P.A.). MyFirst Grant AIRC n.16888, Ricerca Finalizzata Ministero della Salute RF-2016-02364358, Ricerca corrente Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico (to LV), the European Union (EU) Programme Horizon 2020 (under grant agreement No. 777377) for the project LITMUS- "Liver Investigation: Testing Marker Utility in Steatohepatitis" (to LV), Fondazione IRCCS Ca' Granda "Liver BIBLE" PR-0391, Fondazione IRCCS Ca' Granda core COVID-19 Biobank (RC100017A) (to LV). This research was funded by the CIBERehd (EHD15PI05/2016) and "Fondo de Investigaciones Sanitarias, Instituto de Salud Carlos III", Spain (PI16/00598 and PI19/00819, co-funded by European Regional Development Fund/European Social Fund, "Investing in your future"); Spanish Ministry of Economy, Industry and Competitiveness (SAF2016-75197-R); "Junta de Castilla y León" (SA063P17); AECC Scientific Foundation (2017/2020), Spain; "Centro Internacional sobre el Envejecimiento" (OLD-HEPAMARKER, 0348_CIE_6_E), Spain; University of Salamanca Foundation, Spain (PC-TCUE18-20_051), and Fundació Marató TV3 (Ref. 201916-31), Spain. RB acknowledges BFU2017-84653-P (MINECO/FEDER, EU), SEV-2016-0644 (Severo Ochoa Excellence Program), 765445-EU (UbiCODE Program), SAF2017-90900-REDT (UBIRed Program), and IT1165-19 (Basque Country Government). Ciberhdh_ISCIII_MINECO is funded by the Instituto de Salud Carlos III. We thank MINECO for the Severo Ochoa Excellence Accreditation to CIC bioGUNE (SEV-2016-0644). Finally, we would like to acknowledge Begonia Rodríguez Iruretagoyena for the technical support provided.

CONFLICT OF INTEREST

Dr. Martínez-Chantar advises for Mitotherapeutix LLC.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.molmet.2021.101275>.

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