Neddylation inhibition reduces liver steatosis in MAFLD mice models by promoting hepatic fatty acid oxidation via DEPTOR-mTOR axis

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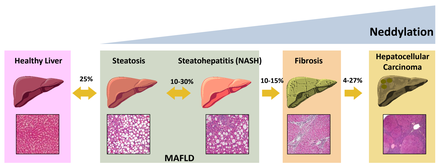
1 INTRODUCTION

Neddylation is a druggable and reversible ubiquitin-like post-translational modification upregulated in many diseases including metabolic-associated fatty liver disease (MAFLD), liver fibrosis and hepatocellular carcinoma (HCC).

MAFLD is a complex liver disease and comprehends a group of conditions being the massive accumulation of fat in the liver the main feature. Related to lipid imbalances occurring during MAFLD, mechanistic target of rapamycin (mTOR) pathway plays an essential role in lipid metabolism and pointed out as a possible trigger of the disease. In recent years, the regulation of DEP domain-containing mTOR-interacting protein (DEPTOR), a negative regulator of mTOR pathway, has been involved in the alteration of lipid homeostasis. It is known that DEPTOR is degraded by SCF (Skp1-Cullin-F box proteins) E3 ubiquitin ligase, which needs to be neddylated to be active. Therefore, we decided to evaluate the potential use of Pevonedistat (MLN4924), a neddylation inhibitor, in MAFLD therapy through regulation of mTOR signaling.

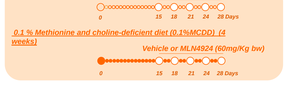


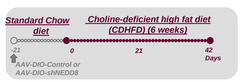
2 AIM

The present work aims to address the effects of Neddylation inhibition and the underlying mechanism in preclinical models of MAFLD.

3 METHOD

Neddylation inhibition was evaluated in mouse isolated hepatocytes. Moreover, male adult C57BL/6 mice (3-month old) fed either with 0.1% methionine and choline deficient diet (0.1%MCD diet) or with a choline-deficient high fat diet (CD-HFD) were used. After 2 weeks of 0.1%MCD diet or 3 weeks of CD-HFD, mice were treated during 2 or 3 more weeks, depending on the diet, with Pevonedistat (60mg/Kg) by oral gavage each 4 days. The effects of neddylation specific inhibition were also evaluated in male adult C57BL/6 AlfpCre mice infected with AAV-DIO-shNEED8 and maintained on CD-HFD for 6 weeks. Finally, the impact of hepatic neddylation in patients with MAFLD as well as the potential use of NEDD8 serum levels for MAFLD diagnostic purposes were evaluated.

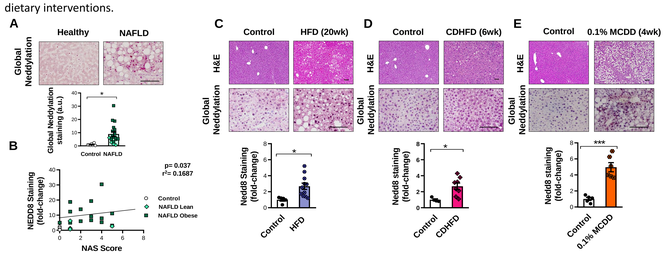




4 RESULTS

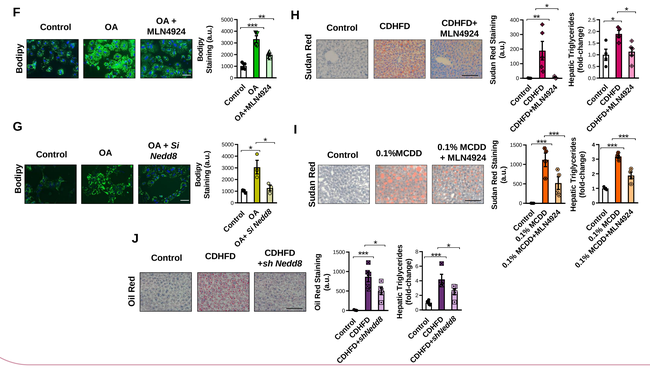
4.1 Hepatic neddylation is augmented in clinical and pre-clinical MAFLD

Hepatic global neddylation levels were increased in liver biopsies from a cohort of well-characterized MAFLD patients, both lean and obese, in comparison to age-matched healthy controls analyzed by immunohistochemistry (IHC) (**Fig. A**). In-depth analysis revealed that hepatic global neddylation levels correlate positively with the NAS score (**Fig. B**). Then, we evaluated the levels of hepatic neddylation in mouse models of diet-induced MAFLD reflecting different stages of the pathology. In all these animal models of MAFLD, hepatic global neddylation was induced (**Fig. C-E**), with higher increases corresponding to more aggressive dietary interventions.



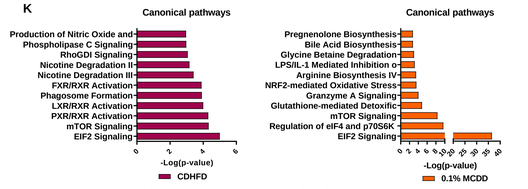
4.2 Neddylation inhibition reduces lipid accumulation in NAFLD preclinical models.

Neddylation inhibition by MLN4924 (Pevonedistat) or *Nedd8* silencing (siRNA) reduced lipid accumulation in oleic acid-stimulated mouse primary hepatocytes (**Fig. F-G**) without induce cell apoptosis. In addition, the effects of neddylation inhibition was evaluate in 2 mouse models of MAFLD. MLN4924 treatment significantly decreased hepatic steatosis, quantified both by Sudan red staining and biochemically measuring hepatic triglycerides (**Fig. H-I**). To further confirm that does not exist off-target effects associated with MLN4924 and other organs networks, *Nedd8* was specifically silenced in the hepatocytes of CDHFD animals by using Alfp-Cre/AAV-DIO-sh*Nedd8* (**Fig. J**), and the lipid contest results were similar to those treated with MLN4924.

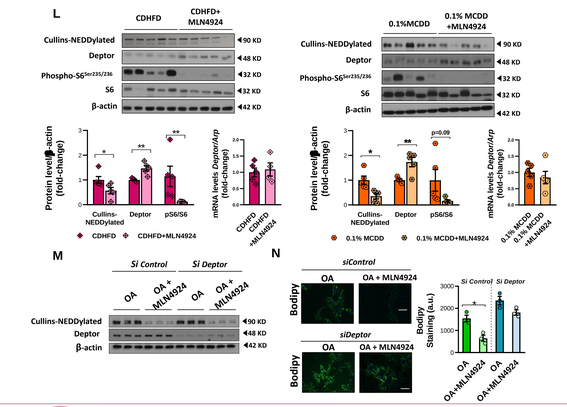


4.3 mTOR inhibition via DEPTOR accumulation plays a role in neddylation inhibition mediated anti-steatotic effects in NAFLD pre-clinical models.

To better understand MAFLD at the proteome level we performed high-throughput proteomics Liquid Chromatography-Mass Spectrometry (LC-MS)-based analyses in animals models of the disease. Ingenuity pathway analysis (IPA) identified the major canonical pathways involved in NAFLD suggesting that eukaryotic initiation factor 2 (EiF2) signaling and mTOR pathway, which plays an important role in the regulation of lipid metabolism, are highly altered in MAFLD (**Fig. K**).

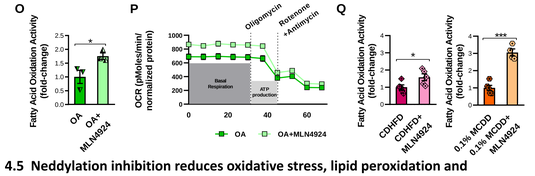


Herein, we show that neddylation inhibition *in vivo*, induced protein DEPTOR with a concomitant mTOR inhibition, the phosphorylation of S6 protein (pS6), a downstream target of mTOR, was reduced (**Fig. L**), without changes in *Deptor* gene expression. Likewise, neddylation inhibition using MLN4924 pharmacological treatment in OA-stimulated hepatocytes increased DEPTOR content (**Fig. M**). Under these conditions, when silencing *Deptor* by using siRNA-based molecular approaches in primary mouse hepatocytes, MLN4924 treatment was not able to significantly reduce the cellular lipid content (**Fig. N**). Thus, mTOR inhibition via DEPTOR accumulation plays a role in the neddylation inhibition mediated anti-steatotic effects.



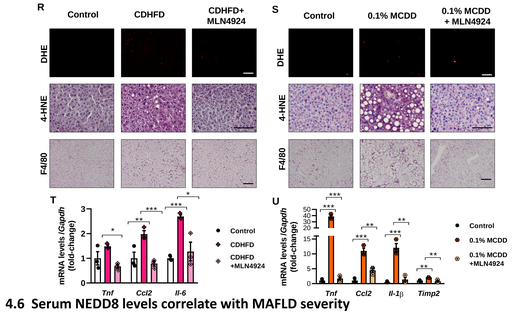
4.4 Neddylation inhibition boosts fatty acid oxidation coupled with oxidative phosphorylation in NAFLD pre-clinical models.

mTOR signaling plays an important role on regulating lipid metabolism, such as lipogenesis and oxidative fluxes in the liver. MLN4924 treatment to isolated mouse hepatocytes stimulated with OA induces FAO activity (**Fig. O**). In agreement, neddylation inhibition by MLN4924 significantly induced oxidative phosphorylation (OXPHOS), an electron transport-linked phosphorylation, as well as ATP-linked respiration in mouse hepatocytes, measured by Seahorse-based analysis (**Fig. P**). Likewise, FAO activity was also shown to be induced in MLN4924-treated CDHFD and 0.1% MCDD-fed rodents (**Fig. Q**).



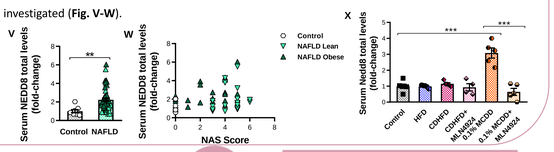
4.5 Neddylation inhibition reduces oxidative stress, lipid peroxidation and inflammation in NAFLD pre-clinical models.

Pharmacological neddylation inhibition ameliorates liver steatosis preventing lipid peroxidation, hepatic oxidative stress and inflammation in mouse models of diet induced MAFLD (**Fig. R-U**).



4.6 Serum NEDD8 levels correlate with MAFLD severity

NEDD8 serum levels correlate with disease progression, in patients and preclinical models, highlighting the power of serum NEDD8 as a potential non-invasive biomarker for MAFLD, should be further investigated (**Fig. V-W**).



5 CONCLUSIONS

Herein, we have further addressed the relevance of hepatic neddylation in MAFLD as well as the therapeutic efficacy of neddylation inhibition both *in vitro* cell models and in mouse models of diet induced MAFLD. We provide evidence that hepatic neddylation inhibition decreases liver steatosis by boosting fatty acid oxidation in a process partly mediated by impaired the mammalian target of rapamycin (mTOR) signaling as regulated by DEPTOR (DEP-domain containing mTOR-interacting protein). Moreover, we have identified for the first time that the levels of NEDD8 in serum appear to correlate with NAFLD disease progression. Overall, treating NAFLD by targeting neddylation may be a fast and effective strategy to regulate altered signaling pathways and metabolic reactions.

6 ACKNOWLEDGEMENTS

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