Lysosomal-associated protein transmembrane 5 ameliorates non-alcoholic steatohepatitis through degradating CDC42

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Article

Keywords:
Abstract

Non-alcoholic steatohepatitis (NASH) has received great attention due to its extremely high incidence. Our team screened LAPTM5 associated with NASH progression through extensive bioinformatics analysis. We found protein LAPTM5 markedly decreased in response to lipotoxicity both in liver tissues from human and mouse NASH groups, and protein level of LAPTM5 was negatively correlated with NAS score. Then, we found LAPTM5 degradation was mediated through its ubiquitination modification by the E3 ubiquitin ligase NEDD4L. Hepatocyte specific depleting LAPTM5 exacerbated hepatic steatosis, inflammation and fibrosis in mouse NASH models. In contrast, LAPTM5 overexpression in hepatocyte exerted opposite effects. Mechanistically, LAPTM5 interacted with CDC42 and promoted its degradation through a lysosomal dependent manner, thus inhibited activation of the mitogen-activated protein kinase signaling pathway. Finally, adenovirus-mediated hepatic LAPTM5 overexpression ameliorated above symptoms in NASH models. These findings proved LAPTM5 as effective treatment in NASH and a potential biological marker for detecting NASH progression.

Introduction

Non-alcoholic steatohepatitis (NASH) is a pathology characterized by hepatic steatosis, hepatocyte ballooning, lobular and hepatic inflammation and interstitial fibrosis.(1, 2) As a leading cause of progression to cirrhosis and hepatocellular carcinoma (HCC), NASH accounts for one in five people with non-alcoholic fatty liver disease (NAFLD), estimated to affect approximately 25% of the world adult population according to recent reports.(3, 4) Regrettably, effective therapeutic measures to protect against the development and progression of NASH are still limited, and there is no FDA-approved pharmacological therapy available.(5, 6) The molecular targets of NASH have attracted increasing attention owing to their promising therapeutic prospects.(7)

In recent years, the role of lysosome-related regulation in disease progression has received increasing attention, it's now clear that lysosome was not just for degradating and recycling cellular waste, it's key organells in degradation, nutrient sensing, innate and adaptive immunity.(8, 9) And so far lysosome has been proved to interact with many signal pathways and regulate the progression of several kinds of diseases, such as atherosclerosis, neurodegeneration diseases, autoimmune disorders and lysosomal storage disorder etc, meanwhile the protein degradation modulated by the proteostasis system has been recognized as an attractive platform for drug targeting and it was likely to play quite meaningful role in human health.(10, 11)

The LAPTM family which consisted of LAPTM4A, LAPTM4B and LAPTM5, had been widely reported in recent years because of their specific protein transport and lysosome degradation functions which demonstrated them a special target for disease intervention. Lysosomal-associated protein transmembrane 5 (LAPTM5), belongs to the late endosomal/lysosomal transmembrane protein family, (12) and was originally identified as a regulator of protein homeostasis(13, 14) and modulator of inflammatory signaling pathways.(15) LAPTM5 improves pathological cardiac hypertrophy by regulating
the activity of the MAPK signaling pathway. According to the previous research results of our group and related literature, activation of the MAPK signaling pathway is closely related to the pathogenesis and pathological progression of NASH. We hypothesized that LAPTM5 is involved in NASH progression based on these preliminary studies.

Here, we found that the protein expression of LAPTM5 was markedly downregulated in the livers of both human NASH groups and mouse NASH models. Depleting LAPTM5 in hepatocytes significantly exacerbated hepatic steatosis, inflammation, and fibrosis in high-fat and high-cholesterol (HFHC)-diet-induced NASH mouse models, whereas LAPTM5 overexpression in hepatocytes effectively delayed and improved the above pathological changes. We further identified that LAPTM5 could directly interact with Cell Division Cycle 42 (CDC42) and overexpression of LAPTM5 promotes the lysosomal degradation of it. As a result, activation of the MAPK signaling pathway was weakened, and the inflammatory lipid toxicity response in hepatocytes was significantly ameliorated. Adenovirus-mediated therapy also ameliorated the NASH symptoms greatly. Collectively, these findings revealed a new treatment target for NASH, also new biological mechanism for regulating LAPTM5 in the progression of NASH and suggest it as a promising therapeutic target as well as biological detection marker for NASH.

Results

Hepatic LAPTM5 expression is downregulated in the pathogenesis of NASH

Although the pathophysiology of NASH is complex and multifactorial, there is no doubt that a large number of proteins which have different locations are involved in the regulation of NASH. To determine which proteins play the most critical role in the pathogenesis of NASH, we analyzed 10 clinical databases of RNA-Seq from liver samples of NASH individuals, and we found that there were three conserved proteins which located lysosome, specific granule and azurophil granule lumen all expressed in these 10 clinical databases (Fig. 1a-c). In particular, the severity of disease is most closely related to the expression of proteins localized in the lysosome (Fig. 1d). The results of 5 databases of RNA-Seq from mouse livers also confirmed this conclusion (Fig. 1e). Considering the important role of transmembrane proteins in disease progression, (21–23) 71 transmembrane proteins were identified among the above lysosomal associated proteins. High content screening analysis were employed to evaluate the role of these genes on lipid contents and the results showed that Laptm5 has the best inhibitory effect on hepatocyte lipid accumulation after PA stimulation (Fig. 1f). Then we took the liver samples of clinical patients to verify the relationship of LAPTM5 in human NASH. By analyzing multiple samples, we found the LAPTM5 protein level was markedly decreased in the NASH group compared with the not-NASH group (Fig. 1g-j). Discovered by NAS scoring system analysis, the protein expression level of LAPTM5 was negatively correlated with the NAS score, which indicated that there might be a clinical correlation between LAPTM5 and NASH (Fig. 1k-l). Interesting, we found that the protein expression of LAPTM5 was dramatically decreased in a time-dependent manner in both mouse primary hepatocytes and L02 human
hepatocytes after PA treatment (Fig. 1m-n). To further verify their effects on NASH progression, we measured LAPTM5 expression in the NAFLD liver samples. Consistent with above results, the protein levels of LAPTM5 were significantly decreased in the livers of ob/ob mice and wild type mice fed with a high-fat diet (HFD), high-fat high-cholesterol diet (HFHC) or methionine and choline-deficient diet (MCD) which were consistent with the results in human NASH samples (Fig. 1o-r).

**NEDD4L mediates protein degradation of LAPTM5 through catalyzing its K48-linked ubiquitination**

To investigate how LAPTM5 expression was decreased after metabolic stimulation, we detected the mRNA levels of LAPTM5 and found that it was comparable in *in vivo* and *in vitro* models, indicating a post-transcriptional regulation of LAPTM5 in response to metabolic stimulation (Fig. 2a-c). As it has been reported that intracellular proteins are mainly degraded through the proteasome or lysosome, MG132 and Chlq were treated in hepatocytes stimulated with palmitic acid (PA), and the results demonstrated that LAPTM5 was reduced mainly through the proteasome degradation pathway (Fig. 2d-e). IP-mass spectrometry analysis identified four E3 ubiquitin ligases, including NEDD4L, NEDD4, WWP2 and ITCH, which may participate in the degradation of LAPTM5 (Fig. 2f). CO-IP results showed that NEDD4L had the strongest interaction with LAPTM5 (Fig. 2g), and the overexpression of NEDD4L promoted the most significant degradation of LAPTM5 (Fig. 2h). Then the CO-IP and GST precipitation assays verified the interaction between LAPTM5 and NEDD4L (Fig. 2i-j). Furthermore, NEDD4L was proved can significantly enhanced the ubiquitination of LAPTM5 (Fig. 2k), and after inactivating mutations of NEDD4L, the modification was reduced (Fig. 2l). Subsequently, we performed a large-scale ubiquitination screen and found that NEDD4L predominantly promoted K48-linked ubiquitination of LAPTM5 (Fig. 2m-n). We further revealed that NEDD4L promotes LAPTM5 degradation through its E3 ligase activity (Fig. 2o).

**LAPTM5 inhibits lipid accumulation and inflammation in hepatocytes**

To further study the effect of LAPTM5 on metabolic stress, primary hepatocytes were isolated and infected with a *LAPTM5*-overexpressing adenovirus (Ad*LAPTM5*). AdGFP was served as control (Fig. 3a). LAPTM5 overexpression attenuated lipid accumulation in PAOA-treated hepatocytes compared with that in the control group, as determined by Nile red staining and triglyceride (TG) and total cholesterol (TC) concentrations detection (Fig. 3b-c). Western blot and RT-PCR analyses showed decreased expression of fatty acid absorption and synthesis markers, such as fatty acid synthase (*Fasn*), sterol regulatory element-binding protein-1c (*Srebp-1c*) and peroxisome proliferator-activated receptor gamma (*Pparγ*), but increased expression of peroxisome proliferator-activated receptor alpha (*Pparα*) in the LAPTM5 overexpression group. The expression of inflammatory response markers, including interleukin 6 (*Il6*), interleukin 1 beta (*Il1b*), tumor necrosis factor (*Tnf*), and C-C motif chemokine ligand 2 (*Ccl2*), were downregulated in Ad*LAPTM5*-PA group (Fig. 3d-e). Subsequently, hepatocytes isolated from *Laptm5* knockout (*Laptm5*-KO) mice were used for reverse verification (Fig. 3f). The deletion of LAPTM5 significantly aggravated abnormal lipid deposition in hepatocytes (Fig. 3g-h). At the same time, the
protein and mRNA levels of fatty acid synthesis and uptake and pro-inflammatory genes were aggravated in the \textit{Laptm5-KO} group compared with those in the WT group (Fig. 3i-j). Then we took mRNA from PO-stimulated WT and LAPTM5-deficient liver cells for sequencing and analysis (Fig. 3k). Clustering analysis indicated that the two groups were well-distinguished (Fig. 3l). GESA analysis and heatmaps disclosed that the pathway and genes involved in lipid metabolism, inflammation, fibrosis and apoptosis were all enriched in the \textit{Laptm5-HKO} group (Fig. 3m-o).

**Hepatocyte specific deletion of LAPTM5 exacerbates steatohepatitis**

We established hepatocyte-specific \textit{Laptm5} knockout mice (\textit{Laptm5-HKO}) (Fig. 4a-c) and subjected them to normal chow (NC) or HFD diet for 24 weeks. Compared with the NC group, the body weight, fasting blood glucose, liver weight, ratios of liver weight-to-body weight, serum TC, and liver TG, TC concentrations were all significantly increased after feeding with the HFD diet. In the meantime, these indicators in \textit{Laptm5-HKO} mice were more aggravated than those in \textit{Laptm5-Flox} mice (Fig. 4d-j). Compared with Flox mice, the larger livers and much more serious lipid deposition were displayed in livers from the \textit{Laptm5-HKO} mice feeding with the HFD diet (Fig. 4k). The expression levels of fatty acid uptake and synthesis genes (\textit{Cd36, Fasn, Scd1, Pparg}, and \textit{Srebf1}) were markedly increased (Fig. 4l-m). Immunohistochemical staining further confirmed the up-regulation of PPAR\textgamma (Fig. 4n). The concentrations of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in \textit{Laptm5-HKO} mice were much higher than those in \textit{Laptm5-Flox} mice after 24 weeks of HFD feeding (Fig. 4o).

To further investigate the influence of LAPTM5 deficiency on NASH, \textit{Laptm5-Flox} and \textit{Laptm5-HKO} mice were fed an HFHC diet. We found that the liver lipid deposition and inflammatory responses were aggravated in HKO group after 8 and 16 weeks HFHC feeding, whereas the body weight between two groups showed no significant difference (Fig. 5a-g, and Supplementary Fig. 1a-d). In addition, liver fibrosis was more severe in \textit{Laptm5-HKO} group compared with \textit{Laptm5-Flox} group after 8 and 16 weeks HFHC feeding, which was proved by PSR staining and the up-regulated expression of fibrosis-related molecules (\textit{Col1a1, Col3a1, Ctgf}, and \textit{\alpha-SMA}) (Fig. 5h-i, and Supplementary Fig. 1e). Moreover, serum ALT and AST concentrations were upregulated in \textit{Laptm5-HKO} mice, reflecting more severe liver damage in the \textit{Laptm5-HKO} group (Fig. 5j).

Considering the heterogeneity of NASH, we subsequently evaluated the function of LAPTM5 in the methionine and choline-deficient diet (MCD) induced mouse NASH model, which exhibited much more severe inflammatory cell infiltration and liver damage, the MCS diet as control.(25) Similar to the results of the HFHC-induced NASH model, LAPTM5 deficiency promotes MCD diet-induced liver lipid deposition, inflammatory response, and fibrosis (Supplementary Fig. 2a-g).

Then we also took the mRNA HFHC-induced \textit{Laptm5-HKO} and Flox mice liver tissues for sequencing and combined analysis (Fig. 5k). The two groups were well-distinguished and the same results were observed compared with cells sequence (Fig. 5l-o).
Hepatocyte-specific LAPTM5 overexpression ameliorates HFHC-induced NASH

To further verify the function of hepatic Laptm5 in NASH pathogenesis, we constructed hepatocyte-specific Laptm5 transgenic (Laptm5-HTG) mice (Fig. 6a-b) and feeding with HFHC diet to induced NASH models, the littersmates (NTG) were used as controls. Laptm5-HTG HFHC mice did not show a significant change in body weight relative to NTG HFHC mice, but the fasting blood glucose, liver weight, liver weight to body weight ratio, serum lipid and liver lipids contents decreased considerably (Fig. 6c-g). Meanwhile, lipid gathering, pro-inflammatory responses and fibrosis in Laptm5-HTG mice exhibited decreased compared with NTG mice after HFHC treatment (Fig. 6h-m). Notably, serum ALT and AST concentrations also decreased markedly in Laptm5-HTG mice (Fig. 6n).

LAPTM5 ameliorates NASH by inhibiting the activity of MAPK signaling pathway through promoting the lysosomal degradation of CDC42

To explore the molecular mechanism of LAPTM5 regulating NASH, combined the results of RNA sequencing and analysis, and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway combine analysis showed that the MAPK signaling pathway was the most significantly altered by LAPTM5 disruption (Fig. 7a). Western blot analysis confirmed that the activation of p38, JNK1/2 and ERK1/2 was inhibited by LAPTM5 overexpression, but enhanced by LAPTM5 deletion both in vivo and in vitro (Fig. 7b-e). In order to clarify how LAPTM5 regulates MAPK signaling pathway, we performed IP-mass spectrometry, and discovered that LAPTM5 interacts with the small GTP-binding protein, CDC42 (Cell Division Cycle 42) (Fig. 7f). Coincidentally, CDC42 is a major contributor to the saturated fatty acid-stimulated JNK pathway in hepatocytes. (26) CO-IP and GST assays demonstrated that LAPTM5 can interact with CDC42 (Fig. 7g-h) and the interaction was stronger under PA stimulation (Fig. 7i). CDC42 overexpression significantly aggravated the lipid deposition, inflammatory responses and activation of MAPK signaling pathway in L02 cells (Fig. 7j-m). Then, we found that LAPTM5 could inhibit the expression of CDC42 under PA stimulation (Fig. 7n-o), and the inhibition function was removed by the addition of lysosomal inhibitors Chlq (Fig. 7p-q). Immunofluorescence co-localization staining showed that CDC42 was evenly distributed in the cytoplasm, but CDC42 gradually moved closer and became granular and eventually colocalized with lysosomes in the group overexpressing LAPTM5 (Fig. 7r). Furthermore, we found that CDC42 abolished the protective effect of LAPTM5 on lipid metabolism stress (Fig. 7s-u). In general, these results indicate that LAPTM5 regulates NASH by regulating CDC42 expression.

Adenovirus-mediated hepatic LAPTM5 overexpression alleviates nonalcoholic steatohepatitis

Finally, we tested the therapeutic efficacy of targeting the LAPTM5-CDC42 axis in NASH. We injected adenovirus overexpressing LAPTM5 (AdLAPTM5) into mice which had been fed an HFHC diet for 8 weeks and continued to feed them an HFHC diet for another 4 weeks, AdGFP was used as a control
(Fig. 8a). Western blot analysis confirmed the overexpression of LAPTM5 and the downregulated expression of p-JNK1/2 and CDC42 in the livers of AdLAPTM5-injected mice (Fig. 8b). Compared to the AdGFP group, AdLAPTM5-injected mice showed a significant decrease in fasting blood glucose, liver weight, and liver weight-to-body weight ratios (Fig. 8c-d). The fatty deposition and inflammatory responses had been significantly ameliorated (Fig. 8e-i). In addition, the liver damage was also significantly reduced (Fig. 8j).

Discussion

Our results demonstrate that LAPTM5 can be ubiquitinated degradation by NEDD4L under metabolic stress. Overexpression of LAPTM5 attenuates liver steatosis, inflammatory response and fibrosis. Mechanistically, LAPTM5 can directly bind to CDC42, promotes its lysosomal degradation and then inhibit the activation of c-Jun NH2-terminal kinase signaling pathway, thus exerting its function. Hepatocyte LAPTM5 is a promising therapeutic target for NASH.

LAPTM5 is a multispanning transmembrane protein containing a ubiquitin-interacting motif (UIM) and three PY motifs that bind Nedd4-WW domains. At the same time, the NEDD4-LAPTM5 complex recruits ubiquinated GGA3 binding to the LAPTM5-UIM.(27) A previous study reported that the E3 ubiquitin ligase ITCH binds to and negatively regulates LAPTM5 through the ubiquitination pathway.(28) Our study observed that LAPTM5 was markedly decreased in NASH models, and the downregulation is via the ubiquitin-proteasome pathway. Then we found NEDD4L, a E3 ubiquitin ligases, interacting with LAPTM5 and promoted K48-linked ubiquitination of LAPTM5. Consequently, targeting the physiological regulation loop of NEDD4L-LAPTM5 may provide an effective strategy for treating NASH.

The molecular mechanisms underlying the pathogenesis of NASH are extremely complex,(29) several studies have shown that MAPK signaling pathway is involved in this process. High-fat diet can activate the expression of JNK in the livers.(30) In cellular NASH models, the saturated fatty acid palmitic acid activated PPARα, leading to c-JNK-dependent mitochondrial dysfunction and hepatocyte death.(31) P38 signaling pathway plays an important role in the inflammatory cell response and stress-induced cell apoptosis.(32) Glowacka et al. and Chen et al. proved that LAPTM5 played a significant role in modulating the activation of the MAPK signaling pathway.(33, 34) In our study, combined with bioinformatics analysis, we found that the activation of JNK1/2 and p38 was inhibited by LAPTM5 overexpression but enhanced by LAPTM5 deletion in both in vitro and in vivo NASH models.

In addition, recent studies showed that the small GTP-binding proteins CDC42 play an important role in the pathological process of NASH by modulating the activation of the MAPK signaling pathway.(35) The activation of CDC42 is required for SFA-stimulated MLK3-dependent activation of JNK in hepatocytes, and decreased expression of CDC42 can mitigate the activation of JNK.(26) Consistent with this, our study identified that LAPTM5 interacted with CDC42 and regulate its expression. Moreover, CDC42 overexpression abolished the protective effect of LAPTM5 on lipid metabolism. LAPTM5 mainly
modulates the progression of NASH via regulating the expression of CDC42 and activation of MAPK signaling pathway.

The interaction between LAPTM5 and CDC42 remains to be elucidated. LAPTM5 is localized in the lysosomal membrane and involved in many pathological and physiological processes. Kawai et al. reported that LAPTM5 promotes the lysosomal translocation and degradation of CD3ζ.(36) Ouchida et al. demonstrated that LAPTM5 interacts with BCR and promotes its lysosomal degradation in mouse B cells. (37) Additionally, lysosomal degradation pathway also plays an important role in the pathological progression of NASH. Studies have proved that TMBIM1 promoted lysosomal degradation of TLR4 and inhibited high-fat diet induced insulin resistance, hepatic steatosis and inflammation.(38) In the present study, we found that LAPTM5 could promote the lysosomal localization and degradation of CDC42.

In summary, we identified LAPTM5 as a novel suppressor of NASH that negatively regulates the p38/c-JNK pathway by promoting CDC42 lysosome degradation in hepatocytes. Adenovirus-mediated LAPTM5 therapy, as well as the significant clinical correlation between changes in LAPTM5 protein expression and NASH disease progression, these findings indicate that hepatocyte-specific LAPTM5 targeting can be an effective therapeutic strategy for NASH treatment and deserves further preclinical research. However, our study still has many limitations that needed to be resolved. For example, how does LAPTM5 carry CDC42 to the lysosome for its protein degradation, and what is the specific process? How does CDC42 regulate the activity of downstream p38 and JNK1/2 pathways? These aspects all need to be further explored. In the meantime, the correlation between LAPTM5 and NASH remains to be tested in large-scale clinical trials. Our experiment was just started in mice; maybe we need to turn our goals to larger animal models of NASH, such as nonhuman primates, before testing in the clinic.

### Methods

#### Reagents

Key reagents, antibodies and primers used in this study are listed in Supplemental Materials.

#### Generation of genetically modified mice

**Construction of liver-specific Laptm5 gene knockout mice**

*Laptm5*-floxed mice were generated using the CRISPR/Cas9 system in C57BL/6 background. Two single guide RNAs (sgRNAs) (listed in Table S1) targeting Laptm5 introns 1 and 2 were designed using an online CRISPR design tool (http://chopshop.cbu.uib.no/). sgRNA expression vectors were constructed using a pUC57-sgRNA backbone (Addgene, 51132). We also designed a donor vector that included two homologous arms, a middle coding region (CDS), and two loxP sequences in the same direction for homologous recombination repair. Next, the sgRNA and Cas9 expression vectors (Addgene, 44758) were transcribed *in vitro*, and the mixture of mRNAs obtained *in vitro* with the donor vector was injected into the zygotes of mice using a microinjection apparatus. The injected zygotes were then transplanted into
the uterus of recipient mice, and \textit{Laptm5}-flox mice were obtained by genotyping. Subsequently, founder mice were mated with C57BL/6 mice until \textit{Laptm5}-flox/flox mice were obtained and mated with liver-specific Alb-Cre transgenic mice (JAX, 003574). \textit{Laptm5}-flox/flox/Alb Cre mice (\textit{Laptm5}-HepKO), were screened. The identification primers used are listed in Table P1–P5.

\textbf{Construction of liver-specific \textit{Laptm5} transgenic mice:}

The CDS region of \textit{Laptm5} was amplified from mouse cDNA obtained by reverse transcription to construct the pALB overexpression vector. Correctly sequenced plasmids were linearized by Pvul restriction endonuclease; the recovered fragments were purified and used for microinjection, subsequently, F0 generation mice were obtained and identified. The first positive mice were mated with the wild type, and the F1 generation positive mice were selected for mating to obtain a stable genetic liver-specific \textit{Laptm5} transgenic mouse strain. The primers used are listed in Table S3.

\textbf{Statistical analysis}

All data were analyzed through corresponding statistical methods using the SPSS 21.0 software. Differences between two groups were determined using the Student's t-test. For comparisons among three or more groups, one-way ANOVA followed by the Bonferroni post hoc test (for data showing homogeneity of variance) or Tamhane T2 post hoc test (for heteroscedastic data) was applied.

When the data met a non-normal distribution, a Kruskal–Wallis nonparametric statistical test was used. Statistical significance was set at \( P < 0.05 \). All data are presented as mean \( \pm \) SD values, and the statistical methods used with the corresponding \( P \) values for the data are mentioned in each figure legend. We collected data from the animal studies in a blinded manner, and no data was excluded from the final statistical analysis.

\textbf{Data availability}

Data supporting this study are provided within the paper and supplementary files. Source data are provided with this paper.

\textbf{References}


38. Zhao, GN. et al. Tmbim1 is a multivesicular body regulator that protects against non-alcoholic fatty liver disease in mice and monkeys by targeting the lysosomal degradation of Tlr4. Nat. Med. 23, 742–752 (2017).

Declarations

Acknowledgements

We thank Dr. Hao Zhang (Union Hospital, Tongji Medical College, Huazhong University of Science and Technology), Shan-Shan Chen (Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology) for their technical assistance and mental encouragement. This work was supported by grants from The Fundamental Research Funds for the Central Universities’, (HUST No. 2021GCRC037), and the National Natural Science Foundation of China (81730015, 82170504, 81974048).

Author contributions

PY. and JX. designed the experiments. LJ., JZ., QY. and ML. performed the experiments, data analysis and wrote the manuscript. HL., XX. and PY. provided technical support. MC. provided advice and comments. PY., MC. and JX. organized and supervised the study.

Competing interests: The authors declare no competing interests.

Figures
Figure 1

Hepatic LAPTM5 expression is downregulated in the pathogenesis of NASH.

(a) The GSE derived from RNA-seq of human livers of clinical NASH patients and the health or healthy obesity. Genes categories shared between $\geq 10$ GSE data are indicated by black dots. The histogram above each plot indicates the times of activated genes categories in each category. (b) The pie chart
showed the statistical representation of the genes categories shared GSE. The integers in parentheses represent genes categories and out parentheses represent the counts of GSE shared. (c) NES dots plot of 3 conserved genes category in 10 human databases. (d) GSVA score analysis of these 3 conserved genes in the databases. (e) NES analysis of 5 databases from mouse livers. (f) Quantitative analysis of Nile red fluorescence intensity of L02 cells with 71 molecular overexpression. (g-j) Representative the LAPTM5 protein level in the human livers from NASH or Not-NASH group (n = 16-20 people/group). (k) Protein expression level of LAPTM5 with related NAS scoring system analysis (n = 16-20 people/group). (l) Immunohistochemical staining of LAPTM5 in liver sections of human in the indicated groups (n = 16-20 people/group). Scale bar, 50\(\mu\)m. (m-n) Representative the LAPTM5 protein levels in PA-stimulated primary hepatocytes (m) and L02 human hepatocytes (n), n = 3 independent experiments. (o-r) Representative the LAPTM5 protein levels in the livers from wild type and ob/ob mice (n = 4 mice/group). And LAPTM5 protein levels in the mice livers from different NASH models (n = 6 mice/group). For (g-i, o-r), Protein expression was normalized to \(\beta\)-actin levels. Data were expressed as the mean ± SD, statistical analyses were performed using a two-tailed Student’s t-test **p < 0.01.
Figure 2

NEDD4L mediates protein degradation of LAPTM5 through catalyzing its K48-linked ubiquitination.

(a) Relative Laptm5 mRNA levels in the mice livers from the indicated NASH models (left, n = 6 mice/group, middle, n = 8 mice/group, right, n = 4 mice/group). (b-c) Relative Laptm5 mRNA levels in primary hepatocytes (b) and L02 hepatocytes (c) stimulated with PA (0.5 mM) (n = 3 independent
experiments per group). (d-e) Western blot images of LAPTM5 protein levels in mouse hepatocytes (d) and L02 hepatocytes (e) treated with MG132 (50 μM), Chlq (50 μM), or DMSO (n = 3 independent experiments). (f) Procedure of identifying the E3 ubiquitin ligases interacting with LAPTM5 by analyzing IP-MS. (g) Interaction between LAPTM5 and NEDD4L, NEDD4, ITCH, WWP2 in L02 cells. (h) Western blot shows the expression of LAPTM5 after transfected with indicated plasmids. (i-j) Co-IP (i) and GST pull-down (j) shows the interaction between LAPTM5 and NEDD4L. (k) Co-IP results show the effect of NEDD4L on ubiquitination of LAPTM5 after MG132 (25 μM) treatment. (l) Co-IP assays of the ubiquitination of LAPTM5 after different treatment. (m) Ubiquitination screening of LAPTM5 by NEDD4L with the indicated types of ubiquitin. (n) Ubiquitination of LAPTM5 in L02 hepatocytes transfected with indicated plasmids. (o) LAPTM5 protein levels in L02 cells transfected with indicated plasmids. Data represent mean ± SD, *p < 0.05, **p < 0.01; n.s., not significant. (Unpaired Student t-test for two group comparison or one-way ANOVA with Holm-Sidak post hoc test for multigroup comparison).
LAPTM5 inhibits lipid accumulation and inflammation in hepatocytes.

(a) Represents LAPTM5 protein levels in mouse primary hepatocytes in the indicated groups. (b-c) Nile Red staining (b) and TG, TC contents (c) in mouse primary hepatocytes in the indicated groups. Scale bar, 25μm. (d-e) Relative mRNA (d) and protein (e) levels of markers related to fatty acid metabolism and
inflammation in the indicated groups. (f) LAPTM5 protein levels in hepatocytes isolated from *Laptm5* knockout (KO) mice or WT mice (n = 3 mice/group). (g-h) Nile Red staining (g) and TG, TC contents (h) in primary hepatocytes after indicated stimulations. Scale bar, 25μm. (i-j) Relative mRNA (n = 4 mice/group) (i) and protein (n = 3 mice/group) (j) levels of relevant markers in indicated groups. (k) Schematic images of the RNA-sequence analysis using the samples from hepatocytes. (l) Hierarchical clustering analysis of the RNA-seq data from the PA and OA stimulated primary hepatocytes isolated from WT and *Laptm5*-KO mice. (m-o) GSEA pathway enrichment analysis and Heatmaps shows the activation of pathways and gene expression of lipid metabolism and inflammatory. For (b-e, g, h, j), n = 3 independent experiments per group. PAOA, 0.5 mM/1 mM; PA, 0.5 mM. Data represent mean ± SD, *p < 0.05, **p < 0.01; n.s., not significant. (Unpaired Student t-test for two group comparison or one-way ANOVA with Holm-Sidak post hoc test for multigroup comparison).
Figure 4

**LAPTM5-HKO exacerbates HFD-induced hepatic steatosis.**

(a-b) Rudimentary diagram of the generation of Laptm5 hepatocyte-specific knockout mice Laptm5-HKO (a) and genotyping results (b). (c) LAPTM5 protein levels in the liver tissues of Laptm5-HKO and Laptm5-Flox mice (n = 3 mice/group). (d) Body weight of Laptm5-HKO and Laptm5-Flox mice after NC or HFD.
consumption for 24 weeks (n = 10-11 mice/group). (e-g) Fasting blood glucose (e), liver weight (f) and ratios of liver weight to body weight (LW/BW) (g) of Laptm5-HKO and Laptm5-Flox mice after NC or HFD consumption for 24 weeks (n = 10-11 mice/group). (h-j) Hepatic TG (h), TC (i) and serum TC (j) content of mice in the indicated groups (n = 10-11 mice/group). (k) Macroscopic and histological images of liver (left, Scale bar, 1 cm), H&E (middle) and Oil Red O (right) (Scale bar, 100μm) staining of the liver sections of mice in the indicated groups (n = 6 mice/group). (l) Relative mRNA levels of relevant markers in the livers of indicated groups (n = 4 mice/group). (m) Relative protein levels of relevant markers in the livers of indicated groups (n = 3 mice/group). (n) Immunohistochemical staining of PPARγ in liver sections mice in the indicated groups (n = 6 mice/group). Scale bar, 50μm. (o) Serum ALT and AST concentrations in mice in the indicated groups (n = 10-11 mice/group). Data represent mean ± SD, *p < 0.05, **p < 0.01; n.s., not significant. (Unpaired Student t-test for two group comparison or one-way ANOVA with Holm-Sidak post hoc test for multigroup comparison).
Figure 5

**LAPTM5-HKO exacerbates HFHC-induced NASH.**

(a-b) Fasting blood glucose (a), liver weight and LW/BW (b) of Laptm5-HKO and Laptm5-Flox mice for NC or HFHC consumptions (n = 10 mice/group). (c) hepatic TG, TC contents of Laptm5-HKO and Laptm5-Flox mice after NC or HFHC feeding for 8 or 16 weeks (n = 10 mice/group). (d) H&E (left) and Oil Red O
(right) staining in the liver sections of mice in the indicated groups (n = 6 mice/group). Scale bar, 100 µm.
(e) Relative mRNA levels of lipid metabolism related genes in the livers of mice in the indicated groups (n = 4 mice/group). (f) Immunofluorescence staining of CD11b (red) in the liver sections of mice in the indicated groups. (Nuclei, blue) (n = 4 mice/group). Scale bar, 50 µm. (g) Relative mRNA levels of pro-inflammatory cytokines in the livers of mice in the indicated groups (n = 4 mice/group). (h) PSR staining of mice liver sections in the indicated groups. (8 weeks, n = 6 mice/group, 16 weeks, n = 5-7 mice/group). Scale bars, 100 µm. (i) Relative mRNA levels of fibrosis related genes in the livers of mice in the indicated groups (n = 4 mice/group). (j) Serum ALT and AST concentrations of mice in the indicated groups (n = 10 mice/group). (k) Schematic images of the RNA-sequence analysis using the samples from mice. (l) Hierarchical clustering analysis the RNA-seq data from the mice fed the HFHC diet. (m-n) GSEA pathway enrichment analysis of pathways related to lipid metabolism, inflammation, apoptosis and fibrosis. (o) Heatmaps of the genes related to lipid metabolism, inflammatory responses and fibrosis (red, upregulated; blue, downregulated) in the indicated groups. Data represent mean ± SD, *p < 0.05, **p < 0.01; n.s., not significant. (Unpaired Student t-test for two group comparison or one-way ANOVA with Holm-Sidak post hoc test for multigroup comparison).
Figure 6

Hepatocyte-specific \textit{Laptm5} overexpression ameliorates HFHC-induced NASH.

(a) Rudimentary diagram of the generation of hepatocyte-specific \textit{Laptm5} transgenic (\textit{Laptm5-HTG}) mice. (b) Western blot images of LAPTM5 protein levels from liver tissues of \textit{Laptm5-HTG} or \textit{Laptm5-NTG} mice (n = 3 mice/group). (c-e) Body weight (c), fasting blood glucose (d), liver weight and LW/BW (e) of \textit{Laptm5-HTG} and \textit{Laptm5-NTG} mice after NC or HFHC consumptions for 16 weeks (n = 9-10 mice/group). (f-g) Serum TC (f), hepatic TG and TC contents (g) of mice in the indicated groups (n = 9-10 mice/group).
mice/group). (h) H&E (upper) and Oil Red O (lower) staining of mice liver sections from the indicated groups (n = 6-7 mice/group). Scale bar, 100μm. (i) Relative mRNA levels of lipid metabolism related genes in the livers of mice in the indicated groups (n = 6 mice/group). (j) Immunohistochemistry staining of CD11b in mice liver sections (n = 6 mice/group). Scale bar, 50μm. (k) Relative mRNA levels of pro-inflammatory genes in livers of mice in the indicated groups (n = 6 mice/group). (l) PSR staining in liver sections of mice in the indicated groups (n = 7 mice/group). Scale bar, 100μm. (m) Relative mRNA levels of profibrotic genes in livers of mice in the indicated groups (n = 6 mice/group). (n) Serum ALT and AST concentrations of mice in the indicated groups (n = 9-10 mice/group). Data represent mean ± SD, *p < 0.05, **p < 0.01; n.s., not significant. (Unpaired Student t-test for two group comparison or one-way ANOVA with Holm-Sidak post hoc test for multigroup comparison).
Figure 7

LAPTM5 ameliorates NASH by inhibiting the activity of MAPK signaling pathway through promoting the lysosomal degradation of CDC42.

(a) Combined KEGG analysis results showing the most enriched MAPK pathway. (b-c) Western blot images (b) and quantitative results (c) of phosphorylated and total protein levels of p38, JNK1/2 and
ERK1/2 in mice livers of the indicated group (n = 3 mice/group). (d-e) Western blot images showing phosphorylated and total protein levels of p38, JNK1/2 and ERK1/2 in the cells from indicated group. (f) Scheme of identifying the protein interacting with LAPTM5 by analyzing of IP-MS. (g-h) Co-IP (g) and GST pull-down (h) assays show the interaction between LAPTM5 and CDC42. (i) Co-IP assays for examining the difference of binding strength between LAPTM5 and CDC42 after the stimulation of PA. (j-k) Nile Red staining (j) and TG, TC contents (k) of L02 cells in the indicated groups. Scale bar, 20μm. (l) Relative mRNA levels of lipid metabolism and inflammation related genes. (m) Western blot results of the expression of MAPK signaling pathway, lipid metabolism and inflammation-related proteins. (n-o) Western blot results of exogenous (n) and endogenous (o) CDC42 expression after overexpression different concentrations of LAPTM5. (p) Western blot result of exogenous CDC42 expression after LAPTM5 overexpression and PA stimulation under Chlq or MG132 treatment. (q) Western blot images of exogenous CDC42 expression trend with LAPTM5 overexpression in a gradient under the treatment of Chlq simultaneously stimulated with PA. (r) Confocal microscopy images of the co-localization of LAMP1 (green) and CDC42 (red) in L02 cells in the indicated groups (Nuclei, blue). Scale bar, 8μm. (s-t) Nile Red staining (s) and TG, TC contents (t) of L02 cells overexpressed LAPTM5 and CDC42 separately or simultaneously. Scale bar, 20μm. (u) Western blot analyses of total and phosphorylation JNK1/2, and p38 in the indicated groups. For (D-E, M-Q), n = 3 independent experiments per group. PAOA, 0.5 mM/1 mM; PA, 0.5 mM; Chlq, 50 μM; MG132, 50 μM. Data represent mean ± SD, *p < 0.05, **p < 0.01; (Statistical analyses were performed using one-way ANOVA with Holm-Sidak post hoc test for multigroup comparison).
Figure 8

Adenovirus-mediated hepatic LAPTM5 overexpression alleviates HFHC-induced hepatic steatosis and inflammation.

(a) Scheme of constructing AdLAPTM5-mediated therapeutic NASH models in HFHC mice. (b) Represents the WB detection results of the proteins indicated in the figure (n = 3 mice/group). (c-d) Fasting blood glucose (c), liver weight and LW/BW (d) of mice in the indicated groups (n = 10 mice/group). (e) Hepatic TG and TC contents of mice in the indicated group (n = 10 mice/group). (f) H&E (upper) and Oil Red O (lower) staining in the liver sections (n = 5-6 mice/group). Scale bar, 100μm. (g) Relative mRNA levels of genes related to the fatty acid metabolism in the livers of mice in the indicated groups (n = 6
mice/group). (h) Immunofluorescence staining of CD11b (red) in the liver sections of HFHC-fed mice in the indicated groups (n = 5 mice/group). Scale bar, 50μm. (i) Relative mRNA levels of pro-inflammatory genes in the livers of mice in the indicated groups (n = 6 mice/group). (j) Serum ALT and AST concentrations of mice in the indicated groups (n = 10 mice/group). Data represent mean ± SD, *p < 0.05, **p < 0.01; n.s., not significant. (Unpaired Student t-test for two group comparison or one-way ANOVA with Holm-Sidak post hoc test for multigroup comparison).

**Supplementary Files**

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- LAPTM5SupplementaryNC.docx
- FigureS1.tif
- FigureS2.tif