Derlin-1 ameliorates nonalcoholic hepatic steatosis by promoting ubiquitylation and degradation of FABP1

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Research Article

Keywords: Fatty acid-binding protein 1, Derlin-1, Nonalcoholic fatty liver disease, Ubiquitination degradation

Posted Date: May 8th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2870906/v1

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Abstract

Background

The functions of liver fatty acid binding protein (FABP1) in the regulation of nonalcoholic fatty liver disease (NAFLD) have been demonstrated. However, it is not fully understood how FABP1 expression is dynamically regulated in metabolic disorders. Previous studies have reported that ubiquitin proteasome-mediated degradation of FABP1 is involved, but the mechanism remains unknown.

Methods

Dysregulated expression of hepatic FABP1 and Derlin-1 was observed in NAFLD patients. We performed mice hepatic tissue co-immunoprecipitation (IP)-based mass spectrum (MS) assays. Derlin-1 interacts with FABP1 and modifies its ubiquitin status, as confirmed by co-IP. The role of Derlin-1 in lipid deposition was tested using adenovirus-mediated overexpression in C57 mice, Derlin-1 overexpression (Derlin-1-OE), or Derlin-1 knockdown (Derlin-1-KO) HepG2 cells.

Results

As a subunit of the endoplasmic reticulum-associated degradation complex (ERAD), Derlin-1 is negatively associated with NAFLD patients and interacts with and ubiquitinates FABP1. Derlin-1 suppresses FABP1 protein levels and inhibits lipid deposition through a FABP1-dependent pathway. Additionally, Trim25, an E3 ubiquitin ligase present in the endoplasmic reticulum (ER), is recruited to promote Derlin-1-related polyubiquitylation of FABP1, thereby creating a ubiquitin-associated network for FABP1. Overexpression of Derlin-1 ameliorates hepatic steatosis in both C57 mice and HepG2 cells, and contributes to attenuated weight gain, lower liver weight, and visceral fat mass.

Conclusions

FABP1, a master enzyme that maintains fatty acid metabolism, undergoes degradation by Derlin-1 through ubiquitin modification. The activation of Derlin-1 in vivo may represent a potential therapeutic strategy for NAFLD.

Trial registration:

Clinical Trials. gov ID: NCT02118376.

Background

Nonalcoholic fatty liver disease (NAFLD) encompasses a spectrum of liver pathologies, ranging from benign steatosis to lobular inflammation, with variable degrees of fibrosis leading to cirrhosis and hepatocellular carcinoma [1]. NAFLD affects up to 30% of the global population [2], and with the progressive obesity, diabetes mellitus, hypertension, and dyslipidemia epidemics. However, the molecular
mechanism underlying NAFLD initiation and progression remains elusive. Although numerous studies have aimed to unravel the etiology of NAFLD, little pharmacotherapy has been legally approved [3]. Thus, clarifying the mechanisms underlying NAFLD progression is needed to develop therapeutic strategies.

Typically, initial symptoms of NAFLD are extensive liver lipid deposition or hepatic steatosis, characterized by disturbed fatty acid metabolic homeostasis [4]. Fatty acid binding protein 1 (FABP1) is a soluble 14-kDa protein found abundantly in the cytoplasm of hepatocytes and, to a lesser extent, in the nucleus and outer mitochondrial membrane [5]; it participates in the intracellular storage and transport of fatty acids and their acyl-CoA esters, and influences the metabolic utilization and compartmentalization of long-chain fatty acids. Hepatic steatosis is closely associated with the functions of FABP1 [6–8]. Adult-onset fatty liver disease is reduced in FABP1−/− mice, whereas hepatocyte-specific FABP1 knockout mice are protected from diet-induced hepatic steatosis and dietary fibrosis [9]. FABP1 also interacts with lipid metabolism-related genes to regulate lipid uptake and metabolism [10]. FABP1 may be responsible for transporting peroxisome proliferator-activated receptor (PPAR)-α and PPARg into the nucleus, thereby promoting fatty acid oxidation and accelerating fatty acid uptake [10–13]. The intracellular concentrations of FABP1 directly correlate with the activities of PPARa and PPARg. Also, PPAR agonists enhance the transcriptional rate of FABP1 and increase FABP1 mRNA and protein levels. Therefore, abnormal expression of hepatic FABP1 may contribute to NAFLD progression. It has recently been found that 4-Hydroxynonenal adduction can result in FABP1 destabilization, targeting it for ubiquitination degradation [14]. However, FABP1 regulation during NAFLD development remains unclear, particularly at the post-translational level.

Endoplasmic reticulum-associated degradation (ERAD) [15], involving proteasome-mediated degradation of a specific protein through a crucial ERAD E3 ligase, is a vital post-translational modification of cellular proteins. Numerous studies have shown that Derlin-1 plays a pivotal role in recognizing misfolded proteins by promoting retro-translocation channel formation in the endoplasmic reticulum (ER) membrane as an E3 ubiquitin ligase modulator and interacts with modified target proteins in the cytosol via the ubiquitin-proteasome degradation pathway [16–18]. Furthermore, we previously reported that Derlin-1 can bind to E3 ubiquitin ligase, promoting ubiquitination and degradation of the epithelial Na+ channel[19]. The ubiquitin-proteasome degradation system is an essential pathway for maintaining the dynamic equilibrium of target proteins in vivo. However, no E3 ubiquitin ligase and binding mediators have been identified for FABP1, especially in nonalcoholic hepatic steatosis.

In the current study, we identified Derlin-1 as a novel negative regulator of FABP1 and lipid metabolism in the liver. Additionally, Trim25, an E3 ubiquitin ligase in the ER, was recruited to promote Derlin-1-related polyubiquitination of FABP1, thereby creating a ubiquitin-associated network for FABP1. Overexpression of Derlin-1 ameliorated hepatic steatosis in both C57 mice and HepG2 cells, which also contributed to attenuated weight gain, lower liver weight, and visceral fat mass in mice. These results revealed that Derlin-1-mediated degradation of FABP1 is crucial for lipid metabolism in fatty liver.

Methods
Participant blood samples

We took samples from 25 individuals in the NAFLD group based on the diagnostic criteria of China from the Endocrine and Metabolism Department of Shanghai Tenth People's Hospital. Individuals were recruited in this study based on the exclusion of patients diagnosed with other known chronic liver diseases, including chronic hepatitis B or C, autoimmune hepatitis, or who were excessive consumers of alcohol (> 140 g/week for men or > 70 g/week for women). Patients who had used any medication or alternative treatment that could affect liver steatosis or fibrosis and glucolipid metabolism within six months of this study were also excluded. Additionally, 25 normal controls matched for age, sex, and body mass index (BMI) were recruited from the Physical Examination Center of the Shanghai Tenth People's Hospital. For each participant, measured height and body weight and assessed alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-transaminase, (γ-GT), total cholesterol (TC), triglyceride (TG), high-density lipoprotein (HDL), and low-density lipoprotein (LDL) levels. Serum FABP1 levels were measured using enzyme-linked immunosorbent assays (ELISA; Abcam, #ab218261). Every serum sample was replicated twice, and the average was used for analysis.

Human liver samples

From January 2021 to June 2022, five individuals with NAFLD and morbid obesity who underwent laparoscopic sleeve gastrectomy (LSG) at the Endocrine and Metabolism Department of Shanghai Tenth People's Hospital were enrolled in this study. We examined liver biopsy samples from patients who may have had a biopsy before or during the LSG to stage and grade steatosis and steatohepatitis. Three adjacent normal tissues located 5 cm from benign lesions were resected as controls, and hepatitis, cirrhosis, drug-induced liver injury, and additional influencing factors were excluded. All individuals signed an informed consent form for the use of clinical specimens, and all procedures adhered to the principles of the Declaration of Helsinki.

Reagents, plasmid constructs, and antibodies

Antibodies against FABP1 were purchased from Proteintech (#13626-1-AP) and Santa Cruz Biotechnology (#sc-374537). Derlin-1 and ubiquitin (Ub) were purchased from Sigma-Aldrich (#D4443) and Cell Signaling Technology (#58395), respectively. Anti-α-tubulin, anti-b-actin, anti-HA-tag, anti-FLAG-tag, and anti-Trim25 antibodies were purchased from Proteintech (#11224-1-AP, 81115-1-RR, 51064-2-AP, #66008-4-Ig, and 12573-1-AP, respectively). Other antibodies, including IgG and secondary mouse or rabbit antibodies, were purchased from Thermo Fisher Scientific (Waltham, UK). MG132 and cycloheximide were purchased from MCE (#HY-13259 and #HY-12320, respectively). Protein A/G agarose beads were purchased from Thermo Fisher Scientific (#WF324075). FABP1 was amplified from an ultimate open reading frame clone by PCR and cloned into pcDNA3.1. Constructs of wild-type HA-tagged Derlin-1, HA-tagged Derlin-1, shRNA Derlin-1 CT, Trim25, shRNA Trim25, and shRNA Trim25 CT were designed and cloned into pcDNA3.1. Wild-type Ub and mutated Ub plasmids were amplified by PCR and cloned into pcDNA3.1. All the plasmid constructs were confirmed by DNA sequencing.
Animals

C57BL/6J (C57) mice (6–8 weeks old) were obtained from the Shanghai Laboratory Animal Center (Shanghai, China). All male mice used in our study were housed in standard cages and maintained under a 12-hour light/dark cycle at 23–26°C (Animal House, Shanghai Tenth People's Hospital of Tongji University). Water was provided ad libitum, and the relative humidity was maintained at 40 ± 5%. The study protocol was approved by the Animal Care and Use Committee of Tongji University. To establish an animal model of NAFLD and to verify FABP1 expression, mice were fed a high-fat diet (HFD), and a control group was established by feeding with a control diet (CD). In addition, we injected $2 \times 10^7$ purified viral particles per gram of body weight of control (AAV-GFP) or experimental (AAV-Derlin-1) viruses for adeno-associated virus experiments (Genechem Ltd. Shanghai, China).

Histopathological analysis, oil red staining, and immunofluorescence staining

The retrieved mouse liver tissues were fixed overnight with 4% paraformaldehyde. Paraffin-embedded and OCT-embedded liver sections were used for hematoxylin/eosin (H&E), immunohistochemistry (IHC), immunofluorescence (IF), and oil red staining (Sigma-Aldrich, #O0625), respectively. IHC and IF staining were followed by antigen retrieval and immunostaining with anti-FABP1 and Derlin-1. All images were obtained using an Olympus confocal microscope and processed using the Olympus FV1000 software.

Blood glucose and serum insulin analysis

Blood glucose levels were determined with a Gluco-meter Elite monitor (B.BRAUN, Germany), and serum insulin was measured using an insulin ELISA kit (Cusabio, #CSB-E05071m). After overnight fasting, glucose tolerance tests (GTTs) were completed by intraperitoneal injection of d-glucose (2 g/kg, Sigma, USA, #SLCK0748). Insulin tolerance tests (ITTs) were performed by i.p. injection of 1 unit/kg insulin (NovoMix 30; Bagsvaerd, Denmark) after 4 h of fasting. GTTs and ITTs were performed 15 weeks after being treated with a HFD.

Lipid analysis

Lipid metabolizing markers in the serum and hepatic tissues (TC, TG, and LDL) were measured using commercial kits (Nanjing Jiancheng, #A111-1-1, A110-1-1, and A113-1-1, respectively), according to the manufacturer’s instructions.

Co-immunoprecipitation assays and mass spectra

Protein lysates were prepared from mouse liver tissue using radioimmunoprecipitation assay buffer containing protease inhibitors for co-immunoprecipitation (CO–IP) assays, cleared by centrifugation, and the protein concentration was estimated. The lysates were washed with 100 µL of protein A/G agarose beads in 1 mL lysis buffer. The lysates were then incubated overnight with anti-FABP1 antibodies, anti-Derlin-1 antibodies, or control IgG with protein A/G agarose beads. The complexes were washed with 1 mL RIPA lysis buffer, followed by centrifugation (repeated 3–5 times), and resuspended in 2 × SDS
loading buffer. The immunoprecipitated proteins were eluted from the beads by incubation at 95°C for 5 min. The eluted proteins were detected by immunoblotting after separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

The gel pieces were dehydrated with acetonitrile and digested with trypsin for mass spectrometry-based proteomic analysis. The peptides were subjected to a nanospray source, followed by tandem mass spectrometry (MS/MS) on a Q Exactive™ Plus (Thermo) instrument coupled online to an ultra-performance liquid chromatograph. The resulting MS/MS data were processed using Proteome Discover 1.3. Liquid chromatography and tandem mass spectrometry (LC-MS/MS) analyses were performed at PTM Bio-lab (Hangzhou, Zhejiang, China).

Cell culture and transfection

Hek293t and HepG2 cells were cultured in DMEM supplemented with 10% FBS at 37 °C and 5% CO₂. First, the constructed vectors were transfected into HEK293t cells for cell transfection using Lipo3000 (Invitrogen; #L3000015). Then, 4–6 h after transfection, the medium was complemented with blood serum, and the cells were maintained in an incubator at 37 °C and 5% CO₂. Gene expression or knockdown was examined by Western blot 24–48 h after gene transfection in Hek293t cells.

HepG2 stable cell lines were also produced by lentiviral infection. The lentiviral empty vector and ORF expression vector (Derlin-1, shRAN Derlin-1, shRAN CT) were transfected into E. coli cells using Fugene HD transfection reagent (Roche) following the manufacturer's instructions. Then, two days after transfection, cell culture supernatants were collected and centrifuged. Supernatants containing lentiviral particles were used to infect HepG2 cells. After lentiviral transduction, target HepG2 cells were selected for 10–14 days in 2.5 µg/mL puromycin. The efficiency of Derlin-1 and shRNA Derlin-1 transfection was determined using immunoblot analysis.

Ubiquitination assays

Hek293t cells were transfected with FABP1, Derlin-1, HA-ubiquitin, or empty vector plasmids using Lipo3000 for 48 h. Then, 20 µM MG132 proteasome inhibitor was added to the cells; 6 h later, they were washed with ice-cold PBS (repeated two times) and cleaved in RIPA lysis buffer. The lysates were also incubated with anti-FABP1 antibodies overnight and then with protein A/G agarose beads for 4 h at 4°C. The complexes were washed with 1 mL RIPA lysis buffer, and the proteins were dissolved in 2 × SDS loading buffer from the beads. We used an anti-ubiquitin antibody to analyze the proteins released by immunoblotting.

Quantitative real-time reverse-transcriptase polymerase chain reaction

Total RNA was isolated from the liver tissues and cells using TRIzol reagent (Invitrogen, Carlsbad, CA, #15596-026). Complimentary DNA (cDNA) was synthesized by reverse transcription of RNA (0.5 mg) using a PrimeScript RT Reagent Kit (TaKaRa, Japan, #A370). Quantitative real-time polymerase chain
reaction (qRT-PCR) analyses were performed with SYBR Green Master Mix (Vazyme, #Q111-02) using a 7900HT real-time PCR system (ABI, CA, USA) to determine the expression of target genes. Expression levels were analyzed using the relative standard curve method and normalized to the housekeeping gene actin. The primer sequences for qRT-PCR were as follows. Human-Derlin-1: forwards 5'-TCGGACATCGGAGACTGGTT-3', reverse 5'-GGCAGTGATTGGCCTCCAAA-3'; human-FABP1: forwards 5'-ATGAGTTTCTCC GGAAGTACC-3', reverse 5'-ATGAGTTTCTCCCGCAAGTA-3'; human-GAPDH: forwards 5'-TTGCTGGTTG AAGTCGCAGGAG-3', reverse 5'-TGTGTCCTCGTG GATCTGA-3'; mouse-PPARa: forwards 5'-AACATCGAGT GTCGAATATGTGG-3', reverse 5'-CCGAATAGTTCGCCGAAGAA-3'; mouse-PPARb: forwards 5'-TGGCATTGTCAGCTGCCTATG-3', reverse 5'-GAGAGGTCCACAGAGCTGATT-3'; mouse-PPARd: forwards 5'-TCTCTGATGCACTGCCTATG-3', reverse 5'-ACTTGGGCTCAATGATGTCAC-3'; mouse-acyl-CoA oxidase 1 (ACOX1): forwards 5'-TAACCTTCCTCAC TCGAAGCCA-3', reverse 5'-AGTTCCATGACCCA TCTCTGTC-3'; mouse- carnitine palmitoyl-transferase 1A (CPT1A): forwards 5'-TGGCATCATCACTGGTGTGTT-3', reverse 5'-GTCTAGGGTCCGA TTGATCTTTG-3'; mouse-GAPDH: forwards 5'-AGGTCGGTGTGAACGGATTTG-3', reverse 5'-TGTAGACCATGTAGTTGAGCA-3'.

Quantification and statistical analysis

All data were analyzed using appropriate statistical methods using SPSS software (v. 20.0, IBM, USA). Normally distributed continuous data are presented as means ± standard deviations (X ± SD). A P value of < 0.05 was considered statistically significant. We determined the sample size using the Power Analysis and Sample Size software (PASS; v. 11, NCSS, Kaysville, Utah, USA) to assess the test power of the current sample size. We collected data from animal studies in a blinded manner.

Results

Patients with NAFLD have elevated serum and liver tissue FABP1 levels

We matched 25 patients with NAFLD (aged 39.82 ± 10.79 years) and 25 healthy individuals (39.27 ± 10.71 years; Con group) for age, sex, and BMI. Serum FABP1 levels in the NAFLD group were notably higher than in the Con group (21.94 ± 12.88 ng/mL vs. 10.98 ± 4.89 ng/mL, P < 0.001; Fig. 1A, Table 1). ALT, AST, γ-GT, TC, TG, and LDL also increased in the NAFLD group (24.11 ± 13.08 U/L vs. 20.32 ± 14.91 U/L, P = 0.345; 19.55 ± 6.97 U/L vs. 17.42 ± 3.58 U/L, P = 0.234; 32.17 ± 19.95 U/L vs. 23.07 ± 10.03 U/L, P = 0.047; 5.09 ± 0.80 mmol/L vs. 4.54 ± 0.86 mmol/L, P = 0.025; 1.84 ± 1.01 mmol/L vs. 1.21 ± 0.50 mmol/L, P = 0.007; 3.21 ± 0.77 mmol/L vs. 2.74 ± 0.77 mmol/L, P = 0.036), whereas HDL level decreased (1.29 ± 0.28 mmol/L vs. 1.34 ± 0.23 mmol/L, P = 0.435), in which γ-GT, TC, TG, and LDL were significantly different between the two groups (Figs. 1B–1G). Meanwhile, we found that FABP1 levels were significantly related to ALT, TC, TG, and LDL levels (r = 0.311, P = 0.048; r = 0.382, P = 0.006; r = 0.260, P = 0.039; r = 0.318, P = 0.024, Table 2). Additionally, the expression of FABP1 in the liver tissue of NAFLD patients was also increased compared with that in the Con group (Figs. 1H–1I).
Table 1
Anthropometric measurements and metabolism variables at baseline

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<th>Parameters</th>
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<td>Total (n = 50)</td>
<td>Con (n = 25)</td>
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<td>FABP1 ng/ml</td>
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<tr>
<td>BMI (kg/m²)</td>
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<td>23.79 ± 1.41</td>
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<tr>
<td>ALT (U/L)</td>
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<td>20.32 ± 14.91</td>
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<tr>
<td>AST (U/L)</td>
<td>18.60 ± 5.74</td>
<td>17.42 ± 3.58</td>
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<tr>
<td>g-GT (U/L)</td>
<td>27.62 ± 16.29</td>
<td>23.07 ± 10.03</td>
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<tr>
<td>TG (mmol/L)</td>
<td>1.52 ± 0.85</td>
<td>1.21 ± 0.50</td>
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<tr>
<td>TC (mmol/L)</td>
<td>4.82 ± 0.87</td>
<td>4.54 ± 0.86</td>
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<tr>
<td>HDL (mmol/L)</td>
<td>1.31 ± 0.25</td>
<td>1.34 ± 0.23</td>
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<tr>
<td>LDL (mmol/L)</td>
<td>2.97 ± 0.80</td>
<td>2.74 ± 0.77</td>
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Data are presented as mean ± SD or median

BMI, body mass index; CON, control group; FABP1, Liver fatty acid-binding protein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; g-GT, g-aminotransaminase; TC, total cholesterol; TG, triglyceride; HDL, high density lipoprotein; LDL, low density lipoprotein
Table 2

Association between serum FABP1 and metabolic factors

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<td>g-GT (U/L)</td>
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<tr>
<td>TC (mmol/L)</td>
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<td>TG (mmol/L)</td>
<td>0.260</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>-0.134</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>0.318</td>
</tr>
</tbody>
</table>

BMI, body mass index; FABP1, Liver fatty acid-binding protein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; g-GT, γ- transaminase; TC, total cholesterol; TG, triglyceride; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

The expression level of Derlin-1 is negatively associated with NAFLD

Mouse body and liver weight had increased observably after 16 weeks under a HFD (Fig. 2A, B), and serum FABP1 levels were significantly higher in the HFD-fed mice than in the CD group (Fig. 2C). To determine the mechanism of FABP1 upregulation, we screened for proteins interacting with endogenous FABP1 using co-IP-based LC-MS/MS in mouse liver tissue. By sorting the peptide spectra matched to the protein score and previous research [19], we concluded that Derlin-1 might be a potential binding protein for FABP1 (Fig. 2D). We found that Derlin-1 relative RNA levels were significantly lower in the HFD than in the CD group (P = 0.004; Fig. 2E). Western blot analysis of mouse liver tissue showed that FABP1 protein expression increased in the HFD group, whereas Derlin-1 levels decreased relative to the CD group (Fig. 2F). Additionally, FABP1 and Derlin-1 had combination and colocalization in the CD group mice (Fig. 2G, H). We also conducted corresponding experiments on the human liver tissues. Immunohistochemistry and Western blot analyses verified that the expression of Derlin-1 protein decreased, while that of FABP1 increased in the NAFLD group relative to the Con group (Fig. 2I, J).

Derlin-1 interacts with FABP1 and suppresses its protein level expression

*In vitro* HepG2 cells were treated with free fatty acids at two concentrations (FFAs; FFA1: 0.5 mM OA, 0.25 mM PA; FFA2: 1.0 mM OA, 0.5 mM PA) to construct a cell model of lipid deposition. We found that FABP1
expression increased, whereas Derlin-1 levels decreased after adding FFA for 24 h, but they were not affected by FFA concentration. Therefore, the FFA1 concentration was selected for subsequent cell experiments.

Next, we investigated the interaction between Derlin-1 and FABP1 using co-IP experiments. We isolated protein complexes from HepG2 cells using Derlin-1 antibodies and blotted them with anti-FABP1 antibodies. The results revealed a physical interaction between FABP1 and Derlin-1 (Fig. 3C); IPs performed with IgG as a control yielded no FABP1 signal. The binding signals of FABP1 with Derlin-1 in IPs were also confirmed using an anti-FABP1 antibody followed by Derlin-1 antibody blotting. To confirm that Derlin-1 binds to FABP1, we examined FABP1 and Derlin-1 interactions in Hek293t cells transfected with FABP1 and HA-Derlin-1. We used anti-HA antibodies to isolate the protein complexes in Hek293t cells (Fig. 3D), which suggested that these two proteins interacted specifically. Importantly, Fig. 3E illustrates the colocalization of FABP1 and Derlin-1 in HepG2 and Hek293t cells, in agreement with the biochemical data (Fig. 3C, D).

To determine whether Derlin-1 regulates FABP1 expression physiologically, we transfected Hek293t cells with or without pcDNA3.1-Derlin-1 or knockdown Derlin-1 with shRNA and control scrambled shRNA. After 48 h of transfection, FABP1 protein expression decreased markedly in response to Derlin-1 overexpression. In contrast, after Derlin-1 knockdown, FABP1 protein levels increased (Fig. 3F). We used immunofluorescence staining to examine FABP1 expression after co-transfection. These results are consistent with the western blot results (Fig. 3G). We then confirmed whether Derlin-1 affected FABP1 expression at the transcript level using qRT-PCR. No significant difference was observed in the mRNA levels between the Hek293t cells expressing Derlin-1 (Fig. 3H). According to these findings, Derlin-1 interacts with and suppresses FABP1 expression post-translationally.

Derlin-1 ubiquitinates and degrades FABP1

An inhibitor of both the synthesis and maturation of new polypeptides, cycloheximide (CHX), was used in our experiments to investigate the mechanism of Derlin-1 regulation of FABP1 with or without Derlin-1 overexpression in Hek293t cells. As shown in Fig. 4A, FABP1 disappeared much faster when Derlin-1 was overexpressed than when it was not. Furthermore, after 6 h, FABP1 was reduced to ~ 90% of the initial amount under Derlin-1 overexpression. These results show that Derlin-1 is involved in promoting the degradation of FABP1. Later, we found that FABP1 protein expression increased upon treatment with MG132, a proteasome inhibitor, indicating that FABP1 may be regulated by the ubiquitin-proteasome degradation pathway (Fig. 4B).

Transient infection with Derlin-1 resulted in a higher expression of ubiquitin in cells overexpressing Derlin-1 compared with Hek293t cells not transfected with Derlin-1 (Fig. 4C). In contrast, shRNA Derlin-1 transfection significantly reduced FABP1 ubiquitin expression in Hek293t cells (Fig. 4D). Simultaneously, HepG2 cells were stimulated with FFA and MG132; 24h later, the immunoprecipitation experiment showed that the ubiquitination level of FABP1 decreased compared to that in the group without FFA. These
findings suggest that FABP1 ubiquitination levels decrease after FFA stimulation, and Derlin-1 expression promotes the ubiquitination and degradation of FABP1 (Fig. 4E).

Trim25 triggers Derlin-1-mediated ubiquitin degradation of FABP1

Derlin-1, as a regulator of E3 ubiquitin ligase, needs to bind to specific E3 ubiquitin ligases to perform the ubiquitination degradation of FABP1. We identified the specific ubiquitin E3 ligases that assemble polyUb chains with Derlin-1 using LC-MS/MS. The CD- and HFD-fed mouse tissue lysates were immunoprecipitated with an anti-Derlin-1 antibody, and the samples were analyzed by LC-MS/MS. By co-analysis with the above IP-FABP1-MS results, ubiquitin-related proteins were screened (Fig. 5A), among which Trim25 and Trim21 were identified as potential E3 ubiquitin ligases that may jointly bind with FABP1 and Derlin-1. We also found dramatically reduced Trim25 mRNA and protein expression in HFD-fed mouse liver tissues relative to the CD group tissues, whereas Trim21 did not change significantly between the two groups (Fig. 5B, C). Co-IP experiments were also performed in HepG2 cells and CD group liver tissues to test the possibility of Trim25, Derlin-1, and FABP1 binding (Fig. 5D, E).

Immunofluorescence staining revealed plot-like colocalization of FABP1, Derlin-1, and Trim25 in the liver sections of the CD group mice. In contrast, this colocalization was reduced in HFD-fed mice (Fig. 5F). In Hek293t cells, by transfecting with pcDNA3.1-Trim25 or transfecting shRNA against Trim25, we confirmed that Trim25 is undoubtedly the E3 ligase for FABP1. As shown in Fig. 5G, pcDNA3.1-Trim25 transfection increased Trim25 protein expression by more than 2×, and shRNA Trim25 knockdown successfully reduced Trim25 protein expression in Hek293t cells. Overexpression of Trim25 reduced FABP1 levels and increased FABP1 ubiquitination levels, whereas the knockdown of Trim25 drastically suppressed the degradation of FABP1 modulated by Derlin-1 (Fig. 5G, 5H). These data indicate that Trim25, an E3 ubiquitin ligase, plays a crucial role in Derlin-1-mediated ubiquitin degradation of FABP1.

Derlin-1 overexpression attenuates liver steatosis

To further investigate the in vivo role of Derlin-1 in hepatic lipid metabolism, we injected overexpressing AAV-Derlin-1 into the tail vein of C57 mice. The results show that there was a significant upregulation in Derlin-1 14 days after tail vein injection in liver samples (Fig. 6A). The mouse livers were smaller in the AAV-Derlin-1 + HFD group compared with the AAV-GFP + HFD group (Fig. 6B). The Derlin-1 relative RNA level increased in the AAV-Derlin-1 group (Fig. 6C); however, the mRNA level of FABP1 did not differ significantly between when Derlin-1 was overexpressed versus when it was not (Fig. 6D). It was further verified that Derlin-1 did not significantly affect the FABP1 transcription level. After 13 weeks of HFD feeding, the mice in the AAV-Derlin-1 + HFD group showed significantly reduced body weight compared with those in the AAV-GFP + HFD group. At the end of 16 weeks, liver weight and visceral fat decreased in the AAV-Derlin-1 + HFD group compared to the AAV-GFP + HFD group, but there was no significant difference in dietary intake between the two groups (Fig. 6E–H). Furthermore, FABP1 expression decreased and Trim25 protein levels increased in the AAV-Derlin-1 + HFD group relative to the AAV-GFP + HFD group (Fig. 6I). Histologically, the number of lipid droplets in liver sections was significantly reduced in 80% (8/10) of the Derlin-1 overexpressing livers compared with the AAV-GFP + HFD livers (Fig. 6J).
These results suggest that the overexpression of Derlin-1 in vivo can effectively improve elevated saturated fat diet-induced hepatic steatosis.

Derlin-1 improves lipid metabolism in mice and HepG2 cells

We also measured serum lipid levels and liver lipid contents (TC, TG, LDL) of the four mouse groups. Overexpression of Derlin-1 could effectively relieve the level of hepatic steatosis in mice induced by excessive lipids. In contrast, serum TG, TC, and LDL, and liver TG and LDL levels were significantly lower in the AAV-Derlin-1 + HFD group than in the AAV-GFP + HFD group (Fig. 7A–F). Because FABP1 is a critical factor in lipid and glucose metabolism, we investigated the effects of Derlin-1 overexpression on glucose homeostasis. Derlin-1 had no significant effect on glucose metabolism in the mice (Supplementary Fig. 1). However, Derlin-1 overexpression inhibited several lipogenic genes, including PPARα, PPARδ, PPARγ, and CPT1A, although not significantly. In addition, ACOX1 expression was significantly upregulated in the AAV-Derlin-1 + HFD group compared with the AAV-GFP + HFD group (Fig. 7G).

We also constructed Derlin-1-OE and shRNA Derlin-1 (Derlin-1-KO) stable HepG2 cell lines (Fig. 7H). In these cells, FABP1 and Derlin-1 showed opposite trends (Fig. 7I). However, the levels of TG, TC, and LDL were significantly reduced in Derlin-1-OE HepG2 cells and elevated in Derlin-1-KO HepG2 cells (except for LDL; Fig. 7J, K). These experiments demonstrate that Derlin-1 overexpression can effectively ameliorate lipid metabolism.

Discussion

NAFLD results from an imbalance between the availability of lipids derived from fatty acid uptake and de novo fat production and the secretion or treatment of lipids oxidized by free fatty acids (FFA), resulting in liver steatosis [3]. NAFLD causes numerous complications, such as obesity, type 2 diabetes, and insulin resistance, and has been recognized as a major public health problem due to its sizeable global prevalence. Currently, NAFLD treatment is targeted at lipid-lowering drugs for subsequent metabolic syndromes, and antidiabetic and antioxidant pharmaceuticals, including metformin [1]. However, no specific remedy directly targets the remission of NAFLD and retards the spontaneous progression of liver steatosis. FABP1 exists in the liver at a highly elevated concentration (accounting for 5% of cytoplasmic proteins), where it participates in many biological processes [6]. Many studies [5, 20–23] have found that silencing FABP1 reduces oxidative stress, inflammation, and liver steatosis in NAFLD mice. Additionally, FABP1−/− mice can avoid the accumulation of TG in the liver induced by a HFD [24]. In the current study, FABP1 was highly expressed in the serum and liver tissue of patients with NAFLD and HFD-fed mice. Therefore, a therapeutic method that reduces the expression or function of FABP1 and thereby inhibits the accumulation of TG in the liver and improves NAFLD may be a feasible treatment plan.

Currently, the main factors affecting the expression of FABP1 include fatty acids, cholesterol, and peroxisome proliferator receptors. Mochizuki et al. [25] showed that PPARα could form a PPARα-retinol receptor α heterodimer with retinol acid receptor α, acting on the response element of the FABP1 gene
promoter and promoting the expression of FABP1. Also, Wu et al. [26] showed that the hepatitis B virus could induce steatosis by upregulating the expression of FABP1. Therefore, they proposed that FABP1 could be used as a modern treatment target for hepatitis B virus steatosis. FABP1 is also regulated by cholesterol; Kannenberg et al. [27] found a significant increase in FABP1 expression in mice fed a high-cholesterol diet when the SCP-x/SCP-2 gene is knocked out. Current research focuses on the synthesis of the FABP1 protein. However, as a critical means of affecting the expression of FABP1, the degradation pathway and its regulatory mechanisms remain unclear. A previous study reported the dynamic process of 4-Hydroxynonenal modification of FABP1 [14], suggesting that FABP1 can undergo ubiquitination degradation. However, the degradation mechanism of FABP1 has not yet been elucidated. Therefore, we studied the expression regulation of FABP1 post-translational modification-ubiquitination degradation and found that FABP1 expression is upregulated and ubiquitination levels are downregulated in HepG2 cells stimulated by FFAs. The upregulation of FABP1 expression has been suggested to be closely related to post-translational modifications.

Ubiquitin is a minor protein present in all eukaryotes [28]. Its primary function is to tag proteins for breakdown and send them to the 26S proteasome for degradation. Degradation of the ubiquitin-proteasome system (UPS) is a multi-step reaction process that connects to the substrate through E1, E2, and E3. It is the main pathway of intracellular protein degradation and contributes to the degradation of > 80% of intracellular proteins. In addition, many studies have found that ubiquitination plays a vital role in the treatment of liver disease [28–30]. Li et al. [31] found that Acly is the main enzyme that regulates adipogenesis and is degraded by Hrd1 via ubiquitin modification; they also found that activation of Hrd1 in hepatocytes may be a strategic method for NAFLD treatment. Xu et al. [32] found that the E3 ubiquitin protein ligase Trim31 attenuates NAFLD by targeting Rhbdf2 in mouse hepatocytes. Therefore, the ubiquitin–proteasome degradation pathway, a major achievement in cell biology research in recent years, has become a special target for the research and development of modern therapies. In our study, to identify relevant essential molecules that can degrade FABP1, we first identified an E3 ligase regulator, Derlin-1, which may be a different type of FABP1 post-transcriptional modulation, through IP–MS. Derlin-1 directly interacts with hepatic FABP1 to promote its ubiquitination, alleviating the pathological process of NAFLD.

Derlin-1, located on the ER membrane, is an influential regulatory molecule related to ERAD and participates in the transmembrane transport of misfolded proteins [33]. In recent years, Derlin-1 has been used as an E3 ubiquitin ligase regulator to assist the protein substrate's labeling by ubiquitination and then entering the cytoplasmic proteasome for degradation [19, 34, 35]. When Derlin-1 is missing or insufficient, misfolded proteins cannot be effectively transported to the proteasome, leading to cellular damage. Therefore, Derlin-1 plays an essential role in maintaining the balance of intracellular proteins and in preventing disease occurrence and development. Sun et al. [36] studied Derlin-1 as a mammalian ERAD substrate that plays a physiological role in the biogenesis and promotes the degradation of cystic fibrosis transmembrane conductance regulator. We have previously found that Derlin-1 promotes ubiquitination and degradation of the epithelial Na⁺ channel [19]. In this study, we found that Derlin-1 can
promote the ubiquitination degradation of FABP1. We screened the E3 ubiquitin ligases; Trim25 was recruited and found to promote Derlin-1-linked polyubiquitination of FABP1. We also used OAPA-treated HepG2 cells to mimic steatosis and found that Derlin-1 overexpression markedly prevented hepatocytes from undergoing steatosis-induced damage. Another intriguing observation is that Derlin-1 may affect the transcription level of ACOX1 in Fig. 7G, which catalyzes the first rate-limiting enzyme of the peroxisomal fatty acid β-oxidation pathway of very long-chain fatty acids; however, further experiments are needed to prove this hypothesis. To our knowledge, this is the first study to report the relationship between Derlin-1 expression and NAFLD.

Conclusions

In summary, hepatic Derlin-1 expression is negatively associated with NAFLD, and it interacts with and suppresses FABP1 via ubiquitination and degradation pathways. Furthermore, negative regulation of FABP1 by overexpression of Derlin-1 suppresses lipid metabolism in hepatocytes, significantly alleviating steatosis in C57 mice and HepG2 cells. Based on these findings, Derlin-1 may merit further exploration as a therapeutic agent for NAFLD.

Abbreviations

FABP1, liver fatty acid binding protein; NAFLD, nonalcoholic fatty liver disease; IP, immunoprecipitation; MS, mass spectrum; OE, overexpression; KO, knockdown; ERAD, endoplasmic reticulum-associated degradation complex; PPAR, peroxisome proliferator-activated receptor; BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; g-GT, g-transaminase; TC, total cholesterol; TG, triglyceride; HDL, high density lipoprotein; LDL, low density lipoprotein; ELISA, enzyme-linked immunosorbent assays; LSG, laparoscopic sleeve gastrectomy; Ub, ubiquitin; HFD, high-fat-diet; CD, control diet; H&E, hematoxylin/eosin; IHC, immunohistochemistry; IF, immunofluorescence; GTT, glucose tolerance test; ITT, insulin tolerance test; ACOX1, acyl-CoA oxidase 1; CPT1A, carnitine palmitoyltransferase 1A; FFA, free fatty acids; CHX, cycloheximide.

Declarations

Ethics approval and consent to participate

All procedures performed in the studies involving human patients were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. All individuals signed an informed consent form for the use of clinical specimens. Clinical Trials. gov ID: NCT02118376. All animal study protocols were approved by Institutional Animal Care and Use Committee at Shanghai Tenth People's Hospital. Approval number: SHDSYY-0028.

Consent for publication
Not applicable.

**Availability of data and materials**

Not applicable.

**Competing interests**

The authors declare that the research was conducted without any commercial or financial relationships that could be construed as potential conflicts of interest.

**Funding**

This work was supported by the National Natural Science Foundation of China [grant numbers 81700752, 81970677, 82170861, 82170904, and 82201867], Traditional Chinese Medicine Scientific Research Project of Shanghai Municipal Health Commission [grant number 2020_JP013], and Shanghai Tenth People's Hospital Pandeng Fund [grant numbers 2021SYPDRC059 and 2021SYPDRC050]. The funders had no role in the study design, data collection, analysis, and interpretation or in writing the manuscript.

**Author Contributions**

LB, JZ, and SQ conceived, designed, and supervised this study. XW and CZ performed the literature review and collected epidemiological and clinical data. HY, XCW, and HC drafted the manuscript. All authors read and approved the final manuscript.

**Acknowledgments**

We thank Prof. Xiubin Liang and Dongming Su of Nanjing Medical University for providing the Derlin-1 and Ubiquitin plasmids.

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Figures

Figure 1

Patients with NAFLD have elevated serum and liver tissue FABP1 levels.

(A) Serum FABP1 levels in 25 patients with and without NAFLD, *P < 0.05. (B–G) Serum liver function and lipid metabolism indicator levels in 25 patients with and without NAFLD, *P < 0.05. (H) Hematoxylin-stained sections of the human liver with and without NAFLD. (I) Immunohistochemistry for FABP1 in the liver of patients with NAFLD and patients from the control group (Con).
The expression level of Derlin-1 is negatively associated with NAFLD.

(A) Mouse weight growth curve between the CD and HFD groups (CD: Control Diet, HFD: High Fat Diet, n = 6 in each group). (B) Liver weight and (C) Serum FABP1 levels between the CD and HFD groups. (D) The top 10 candidates with the highest peptide spectra matched the protein (PSM) score in FABP1 co-IP-based LC-MS/MS analysis. (E) Derlin-1 relative mRNA levels in mouse hepatic tissues. (F) Western blot assays showing the expression level of FABP1 and Derlin-1 in mouse hepatic tissue. (G) Co-IP assay using FABP1 antibody or Derlin-1 antibody in hepatic tissues of mice from the control group (Con). (H) Immunofluorescence staining of FABP1 and Derlin-1 (scale bars=50mm, arrows indicate areas of coincident staining). (I) Immunohistochemistry for Derlin-1 in the liver of a patient with NAFLD and a patient from the control group (Con). (J) Western blot assay shows the expression level of FABP1 and Derlin-1 in human hepatic tissue.
Figure 3

**Derlin-1 interacts with FABP1 and suppresses its protein level expression.**

(A) Oil red o staining of HepG2 by treatment with free fatty acids (FFA). (B) Western blot assays showing the expression of FABP1 and Derlin-1 through different FFA concentrations. Co-IP assay in (C) HepG2 and (D) Hek293t cells. (E) Immunofluorescence staining of FABP1 and Derlin-1 in HepG2 and Hek293t cells. FABP1 was overexpressed in Hek293t cells, with and without Derlin-1 co-transfection (scale bars = 50mm), as shown by (F) western blot assays, (G) immunofluorescence staining, and (H) qRT-PCR.
Figure 4

Derlin-1 ubiquitinates and degrades FABP1.

(A) Using cycloheximide (CHX) to investigate the mechanism of Derlin-1 regulation of FABP1 with and without Derlin-1 overexpression in Hek293t cells. (B) MG132, a proteasome inhibitor, suggests that the ubiquitin-proteasome degradation pathway may regulate FABP1. (C) Western blot detected the ubiquitin
of FABP1 after transient infection with Derlin-1. (D) Western blot with shRNA Derlin-1 transfection. (E) HepG2 cells were stimulated by adding FFA and MG132; immunoprecipitation showed that the ubiquitination level of FABP1 was comparable to that of the group without FFA.

Trim25, the ubiquitin E3 ligase, triggers Derlin-1-mediated ubiquitin degradation of FABP1.
(A). Ubiquitin-linking protein co-analysis with IP-FABP1-MS and IP-Derlin-1-MS. (B) qRT-PCR and (C) western blot showing the expression of potential E3 ubiquitin ligases, Trim25 and Trim21, in HFD- and CD-fed mouse liver tissues. Co-IP experiments in (D) HepG2 cells and (E) liver tissue from the control mice to test the possibility of Trim25, Derlin-1, and FABP1 binding. (F) Plot-like colocalization of FABP1/Derlin-1/Trim25 in the liver sections of control mice and HFD-fed mice via immunofluorescence staining (scale bars = 50mm). (G) Trim25 overexpression significantly decreased the FABP1 level, and knockdown of Trim25 dramatically suppressed the degradation of FABP1 modulated by Derlin-1. (H) Overexpression of Trim25 increased FABP1 ubiquitination level, whereas knockdown of Trim25 reduced Derlin-1-mediated degradation of FABP1.

Figure 6

Derlin-1 overexpression attenuates liver steatosis.
(A) Western blot assay showing the efficacy of AAV-mediated Derlin-1 overexpression (n = 3). (B) Gross phenotypes and photomicrographs of the livers of 16-week HFD-fed mice (AAV-GFP+CD, n = 9; AAV-Derlin-1+CD, n = 9; AAV-GFP+HFD, n = 10; AAV-Derlin-1+HFD, n = 10). qRT-PCR showing both group’s mRNA levels of (C) Derlin-1 and (D) FABP1. (E) Mouse weight growth curve. (F) Liver weight/body weight (%). (G) Visceral fat mass. (H) Food intake. (I) Western blot assay showing the relative expression levels of FABP1, Derlin-1, and Trim25. (J) Representative images of H&E and oil red O staining of liver sections.

Figure 7
Figure 7

Derlin-1 improves lipid metabolism in mice and HepG2 cells.

Serum (A) TG, (B) TC, and (C) LDL levels. (D) TG, (E) TC, and (F) LDL levels in liver extracts. (G) Expression levels of key genes of lipid metabolism. (H) Western blot assay showing the efficacy of lentivirus-mediated Derlin-1 and shRNA Derlin-1 overexpression. (I) Expression of FABP1 and Derlin-1 in Derlin-1-OE and shRNA Derlin-1 (Derlin-1-KO) HepG2 cells. (J) TG, (K) TC, and (L) LDL levels in cell supernatant

Supplementary Files

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