Ablation of Deubiquitinase USP15 Ameliorates Non-Alcoholic Fatty Liver Disease and Non-Alcoholic Steatohepatitis

Kyung-Hee Chun (khchun@yuhs.ac)  Yonsei University College of Medicine  https://orcid.org/0000-0002-9867-7321
Jung-Hwan Baek  Yonsei university college of medicine
Myung Sup Kim  Yonsei University College of Medicine
Dai Hoon Han  Yonsei University College of Medicine
Yong-ho Lee  Yonsei University  https://orcid.org/0000-0002-6219-4942
Hye Ryeon Jung  Seoul National University
Eugene Yi  Seoul National University
Seung Soon Im  Kyemyung Univ
Ilseon Hwang  Keimyung University School of Medicine
Kyungeun Kim  Sungkyunkwan University School of Medicine
Joon-Yong Chung  NCI  https://orcid.org/0000-0001-5041-5982

Article

Keywords: non-alcoholic fat liver diseases, non-alcoholic steatohepatitis, deubiquitinase, USP15

Posted Date: August 25th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1826647/v1
Abstract

Non-alcoholic fatty liver disease (NAFLD) occurs due to the accumulation of fat in the liver, leading to fatal liver diseases such as non-alcoholic steatohepatitis (NASH) and cirrhosis. Elucidation of the molecular mechanisms underlying NAFLD is critical for its prevention and therapy. Here, we observed deubiquitinase USP15 expression was upregulated in mouse livers fed a high-fat diet (HFD) and liver biopsies of patients with NAFLD or NASH. USP15 interacted with lipid-accumulating proteins such as FABPs and perilipins to reduce ubiquitination and increase their protein stability. Furthermore, the severity phenotype of NAFLD induced by HFD, and NASH by the fructose/palmitate/cholesterol/trans-fat (FPC) diet was significantly ameliorated in hepatocyte-specific USP15-knockout mice. Thus, our findings reveal an unrecognized function of USP15 in the lipid accumulation of livers, which exacerbates NAFLD to NASH by overriding nutrients and inducing inflammation. Therefore, targeting USP15 can be used in the prevention and treatment of NAFLD and NASH.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is characterized by the excessive fat build-up in the liver without apparent causes such as alcohol consumption. NAFLD is the most predominant liver disease, affecting approximately 25% of the global population\(^1\)\textsuperscript{−}\textsuperscript{3}. Although the clear causes of NAFLD remain elusive, prolonged NAFLD shows a propensity to progress to non-alcoholic steatohepatitis (NASH), which is characterized by severe lipid accumulation, inflammation, and fibrosis\(^4\),\textsuperscript{5}. Moreover, NASH may eventually develop into complications such as type 2 diabetes, cirrhosis, or liver cancer\(^6\),\textsuperscript{7}. However, the only effective treatment known for NAFLD is weight loss\(^8\). Therefore, the development of targeted drugs by elucidating the molecular mechanisms underlying NAFLD is momentous.

Conjugation of ubiquitin to the substrate proteins leads to proteasomal degradation and can be reversed by the deubiquitinating enzymes\(^9\). The cellular deubiquitination system is involved in various physiological processes\(^10\). Therefore, the balance between ubiquitination and deubiquitination is strictly regulated. Importantly, dysregulation of protein homeostasis by the ubiquitin system is firmly associated with the pathogenesis of various diseases, including NAFLD and NASH\textsuperscript{11−14}. We screened the expression levels of deubiquitinases in liver tissues of NAFLD mice, and focused on USP15 (Suppl.Fig. S1). USP15 is a deubiquitinating enzyme that removes polyubiquitin chains from the target proteins. It is known to stabilize the TGF-β receptor and downstream transducer receptor-regulated SMADs, thus exhibiting an oncogenic nature\textsuperscript{15}. In addition, USP15 increases the TNF-α or IL-1β induced NF-κB activity, which results in enhanced inflammatory responses via stabilizing the TGF-β activated kinase 1 binding proteins TAB2/3\textsuperscript{16}. USP15 is also known to stabilize MDM2, which is a ubiquitin ligase that causes proteasomal degradation of the tumor suppressor, p53, and is associated with the reduced tumor cell apoptosis and antitumor T-cell response\textsuperscript{17,18}. Recently, it has been reported that USP15 regulates hepatitis C viral RNA translation and lipid droplet formation in hepatocytes\textsuperscript{19}. The extensive impact of USP15 further implicates the possible role of USP15 in the pathogenesis of NAFLD.
Here, we show that the expression of USP15 in the liver is markedly upregulated in individuals with NAFLD and NASH patients and in high-fat diet (HFD)-fed mice. Our data reveal a molecular mechanism of USP15 in the pathogenesis of NAFLD and NASH phenotype using USP15 liver specific knock-out mice.

**Materials And Methods**

**Cell culture and RNAi transfection**

The AML12 cells were kindly provided by Professor Jae-woo Kim, (Yonsei University). The human embryonic kidney 293 (HEK293) cells were purchased from the Korea Cell Line Bank (Seoul, Korea). These cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin, at 37 °C in 5% CO₂. The AML12 and HEK293 cells were transfected using lipofectamine 2000 or lipofectamine RNAiMax according to the manufacturer’s instructions²⁰ (Invitrogen, Carlsbad, CA, USA).

**RNA isolation and quantitative reverse transcription-PCR (qRT-PCR)**

Total RNA was isolated using an RNA lysis reagent (Intron Biotechnology, Korea), as previous study²¹. The complementary DNA was synthesized using the quantitative PCR master mix (TOYOBO, Osaka, Japan). qRT-PCR was performed using the SYBR Premix Ex Taq (Clontech Laboratories, Mountain View, CA, USA) on ABI instruments (Applied Biosystems, Inc., Foster City, CA, USA). The expression of genes was normalized to that of β-Actin.

**Western blot assay**

Cell lysate extractions and tissues were prepared using the radioimmunoprecipitation assay (RIPA) buffer (1% Triton X-100; 1% sodium deoxycholate; 0.1% sodium dodecyl sulfate; 150 mM NaCl; 50 mM Tris-HCl, pH 7.5; and 2 mM EDTA, pH 8.0), as described previously²²,²³. Antibodies against USP15, FABP1, FABP4, Perilipin1, Perilipin2, HA, and β-actin were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). The antibody against FLAG was obtained from Sigma-Aldrich (St. Louis, Missouri, USA). The normalization control was β-actin.

**Immunoprecipitation**

The cell lysate extraction was performed with immunoprecipitation buffer, as mentioned previously²⁴. Cell lysates were immunoprecipitated with the antibodies (FLAG, USP15, FABP1, FABP4, Perilipin1, and Perilipin2) and the protein A/G agarose bead was added to the lysates, followed by incubation. The immunoprecipitates were washed twice with washing buffer and boiled in SDS sample buffer. After centrifugation, the supernatant was analyzed with western blotting.

**Ubiquitination assay**
Ubiquitination assay was performed, as mentioned previously\textsuperscript{25}. Briefly, the cells were lysed in phosphate-buffered saline (PBS), containing 5 mM N-ethylmaleimide, (Sigma-Aldrich, St Louis, MO, USA), to hinder deubiquitination. The lysate was diluted with 0.9 ml of non-denaturing lysis buffer, followed by fragmentation of the viscous chromatin complexes by passing the lysed suspension 3–5 times through a needle attached to 1 ml syringe, and incubation on ice for 5 min. The dissociated cells were centrifuged at 13,000 rpm for 10 min at 4 °C followed by immunoprecipitation.

**Oil Red O (ORO) staining**

The fatty acid-treated AML12 cells were used as previously\textsuperscript{26}. The ORO stock solution (0.35 g/100 ml) was diluted with isopropanol to prepare a 60% ORO working solution. The dried cells were stained with the ORO working solution for 30 min and washed thrice with distilled water.

**Mouse experiments**

The USP15-floxed C57BL/6 mice were purchased from the European Mouse Mutant Archive (München, Germany). The liver-specific USP15 knockout mice (\textit{USP15}\textsuperscript{LKO}) were generated through mating USP15-floxed mice with albumin-Cre mice. Wild-type and \textit{USP15}\textsuperscript{LKO} mice were bred as heterozygotes in house and maintained on a C57BL/6 background. Heterozygous mice were bred with each other to generate wild-type and \textit{USP15}\textsuperscript{LKO} mice. The 7-week-old male wild-type and \textit{USP15}\textsuperscript{LKO} mice were fed a normal-fat diet (NFD) or an HFD (Research Diets, Inc., New Brunswick, NJ, USA) for 12 weeks. The 7-week-old male wild-type and \textit{USP15}\textsuperscript{LKO} mice were fed a fructose-palmitate-cholesterol (FPC) diet (ENVIGO, Indianapolis, IN, USA) for 16 weeks. All the mice were provided free access to food and water and kept on a 12 h light/12 h dark cycle. Body weight and food intake were measured once a week. The animal studies were approved by the Yonsei University Health System Institutional Animal Care and Use Committee (Permission number for animal experiments: 2015-0073).

**Histopathology analysis**

Mouse liver was fixed with 10% formalin and embedded in paraffin. Paraffin-embedded liver sections were stained with H&E for morphology analysis. Sirius red staining was performed using Picro Sirius Red stain kit according to the manufacturer’s instructions (Abcam, Cambridge, MA, UK). For Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, paraffin embedded sections were stained using DeadEnd\textsuperscript{TM} colorimetric TUNEL system according to the manufacturer’s instructions (Promega Corporation, Madison, WI, USA).

For Oil Red O staining, fresh mouse liver embedded in OCT solution, frozen liver sections were fixed in 10% formalin for 10 min, and washed with distilled water. Air dried sections were stained with Oil Red O solution for 15 min and washed with 60% isopropanol. Then stained sections were washed with distilled water.

**Adenoviruses**
The adenovirus expression murine USP15 was prepared using an AdenoZAP kit (O.D.260 Inc, Boise, Idaho, USA). An adenoviral vector expressing USP15 was recombined with the pZAP1.2 vector according to the manufacturer's instructions and transfected into HEK293T cells. To prepare a crude experiment viral stock from HEK293T cells, the cells were subjected to three freeze/thaw cycles and centrifuged. For adenovirus amplification, the HEK293T cells were infected with the viral stock. Large-scale purification was performed with cesium chloride gradient ultracentrifugation according to the standard protocol. For, in vivo transduction, 7-week-old male C57BL/6 mice were injected with the adenovirus via tail vein. The doses were escribed in each figure. All the mice were sacrificed seven days after injection.

**Glucose and insulin tolerance test**

For glucose tolerance test (GTT), mice were fasted for 15 hours and 1 g/kg glucose (Sigma-Aldrich, St Louis, MO, USA) was intraperitoneally injected into mice.

For insulin tolerance test (ITT), Mice were fasted for 6 hours and 1 IU/kg insulin was intraperitoneally injected into mice. Blood glucose level were measured 15. 30. 60. 90, and 120 min after injection.

**Mass spectrometry and network analysis**

For identification of USP15-interacting proteins in AML12 cells, USP15 immunoprecipitation eluates were separated by SDS-PAGE and subjected to In-gel digestion and downstream processing as described in our previous report25. Extracted peptides were suspended in 0.1% FA in water, loaded onto an EASY-Spray C18 column (75 µm×50 cm, 2 µm) and separated with a 2%–35% gradient of 0.1% FA in ACN for 65 min at a flow rate of 300 nL/min. MS spectra were recorded on a Q-Exactive hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) interfaced with a nano-ultra-HPLC system (Easy-nLC1000; Thermo Scientific). Collected MS/MS raw files were converted to mzXML files using the Trans-Proteomic Pipeline (version 4.4) and analyzed using the Sequest (version 27) algorithm in the SORCERER (Sage-N Research, Milpitas, CA, USA) platform. Protein database search was performed using the Uniprot human database (version 2016.06, 313072 entries). Full tryptic specificity and up to two missed cleavage sites were allowed. Mass tolerances for precursor ions and fragment ions were set to 10 ppm and 1 Da, respectively. Fixed modification for carbamidomethyl-cysteine (+57.0215 Da) and variable modifications for methionine oxidation (+15.9949 Da) were used. All proteins with a ProteinProphet probability of ≥ 95% with minimum two peptides and a PeptideProphet probability of ≥ 90%, peptide FDR ≤ 0.3% were identified using Scaffold (version 4.3.2; Proteome Software, Portland, OR, USA).

Network analysis of USP15-interacting proteins was performed by using the Ingenuity Pathway Analysis (IPA) software (Ingenuity System Inc, USA). Protein interaction networks functionally associated of USP15 were merged to generate a protein interaction network.

**Immunohistochemical staining**

The 62 cases of human liver biopsy were diagnosed as NAFLD (n = 18) and NASH (n = 28) according to the criteria of Kleiner et al, while 16 cases had healthy normal liver. All the procedures were conducted
according to the ethical guidelines of the Declaration of Helsinki and all individuals gave informed consent, and the study protocol was approved by the Institutional Review Board at Severance Hospital (IRB No 4–2014–0674, Seoul, South Korea). The immunohistochemistry was performed as previously\textsuperscript{27}. The immunohistochemistry was performed on 5 µm formalin-fixed paraaffin-embedded tissues. The tissue section was deparaffinized, rehydrated through graded alcohols, and subjected to heat-induced antigen retrieval using antigen retrieval buffer of pH 6.0 (for USP15 & F4/80) or pH 9.0 (for Perilipin1) (Dako, Carpinteria, CA). The endogenous peroxidase activity was quenched with 3% H\textsubscript{2}O\textsubscript{2} for 10 min. Subsequently, non-specific binding was blocked with protein block (Dako) for 20 min at room temperature. The sections were incubated with anti-USP15 rabbit polyclonal antibodies (Abcam, Cambridge, MA; cat. ab97533) at a 1:500 dilutions for 1 h, anti-Perilipin1 rabbit polyclonal antibodies (Abcam, cat. ab3526) at a 1:1000 dilution for 1 h, anti-FABP4 rabbit polyclonal antibodies (Abcam, cat. Ab13979) at a 1:2000 dilution for 0.5 h, and anti-F4/80 rabbit monoclonal antibodies (Cell Signaling, D2S9R clone, cat. #70076) at a 1:500 dilutions for 1 h. The antigen-antibody reaction was detected with Dako EnVision+ Dual Link System-HRP (Dako) and DAB+ (3, 3’-diaminobenzidine; Dako). Stained sections were lightly counterstained with hematoxylin. The negative controls, including immunoglobulin G (IgG) and omission of the primary antibody, were concurrently performed. The immunopositivity of USP15 and Perilipin1 was assessed by the proportion of positive hepatocytes (0, 0% positive; 1, ≤10%; 2, >10%).

**Quantification and statistical analysis**

We employed unpaired \( t \)-tests for comparisons between two groups. The statistical analysis was performed using Prism 5 (GraphPad, La Jolla, CA). For IHC data, the statistical analyses were performed using the PASW Statistics 18 for Windows (version 18.0; IBM SPSS Inc., Chicago, IL, USA). Crosstabs, Pearson’s \( \chi^2 \) test, and ANOVA test were used as needed. \( P \)-values were considered significant when less than 0.05.

**Results**

**USP15 is elevated in the liver tissues of mice fed an HFD and in the liver specimens of the NAFLD and NASH patients**

The mRNA expression levels of USPs were screened using the liver tissues of mice fed an HFD or NFD for 12 weeks (Suppl. Fig. S1). The levels of most USPs of 49 deubiquitinases decreased in fatty livers compared to those in normal livers. Interestingly, the mRNA expression of USP50, USP15, and USP39 increased in the fat-accumulated livers. We also re-confirmed the increment of mRNA and proteins levels (Fig. 1A and 1B) of USP15 in the liver tissues of HFD mice. IHC analysis revealed that USP15 expression was increased in the cytosol of hepatocytes in HFD mice (Fig. 1C).

The expression of USP15 was also detected in the liver tissues of NAFLD and NASH patients. The mRNA levels of USP15 were increased (Fig. 1D) and the gradient increment of USP15 proteins was detected in
the hepatocytes in the NAFLD and NASH patients (Fig. 1E). The percentage of strong expression of USP15 in patients was gradiently increased in the patient population of NAFLD and NASH (Fig. 1F). Moreover, the increase in USP15 was also parallel to the levels of AST and ALT and BMI scores in the NAFLD and NASH stages (Fig. 1G).

**USP15 directly interacts with lipid-accumulation associated proteins**

Mass spectrometry was performed to screen USP15 substrates using AML12 mouse hepatocytes (Fig. 2A). Interestingly, we determined that USP15 interacts with lipid metabolism-related proteins such as fatty acid-binding protein, FABP4 and Perilipin2. We re-confirmed the interaction between USP15 with not only FABP4 and Perilipin2 but also FABP1 and perilipin1, which are expressed in hepatocytes, by immunoprecipitation assay (Fig. 2B and 2C). We also performed an *in vitro* GST pull-down assay to detect direct interaction between USP15 with FABP4 and Perilipin1 (Fig. 2D). Furthermore, increases in FABP4 and Perilipin1 were found in steatosis and NASH stages, using the public database (GSE896320) analysis (Suppl. Fig. S2), suggesting the regulation of FABPs and perilipins by USP15 is important in NAFLD development.

Next, we determined the immunopositivity of USP15 and Perilipin1 in the liver biopsies of patients with NAFLD, NASH, and normal liver tissues. USP15 was stained in the cytoplasm of hepatocytes with a granular pattern and expressed more strongly in the hepatocytes without fat droplets. The Perilipin1 also showed cytoplasmic expression, but was concentrated around fat droplets (Fig. 2E). A parallel trend was observed for expression levels of USP15/Perilipin1 and NAFLD disease progression (Fig. 2F). Furthermore, there was a statistically significant correlation between USP15 and Perilipin1 expression in human liver tissues (Pearson's *x*² test = 7.438, *p* = 0.006, Fig. 2G). The expression of Perilipin1 had a parallel correlation with the worsening of liver function, such as the AST and ALT levels, and with the BMI score (Suppl. Fig. S3). Unfortunately, we could not detect the expression of FABP4 using immunohistochemical assays.

**USP15 increases the protein stability of FABPs and perilipins, and lipid accumulation in the hepatocytes**

Increment of USP15 increased the level of FABP4 or Perilipin1 in a dose-dependent manner (Fig. 3A). The USP15-dependent degradation was mediated by the 26S proteasome complex. MG132, the proteasome inhibitor, inhibited the protein degradation of FABP1, FABP4, Perilipin1, and Perilipin2, when USP15 was downregulated by the siRNA targeting USP15 (Fig. 3B). To determine whether the decreased protein levels of FABPs and perilipins, due to interference by USP15 expression with siRNA, resulted from the decreased protein half-life, we treated the cells with cycloheximide, a protein synthesis inhibitor. The AML12 cells transfected with siRNA targeting USP15 showed significantly reduced protein half-life of FABPs and perilipins (Fig. 3C and 3D).
Furthermore, we elucidated the function of USP15 in lipid accumulation via stabilization of these proteins. We treated the cells with oleic acid and performed ORO staining in AML12 cells. Results showed that oleic acid-treated cells accumulated a significant amount of lipids in a dose-dependent manner, whereas the cells transfected with USP15 siRNA showed reduced lipid accumulation (Fig. 3E and 3F). It was also confirmed that palmitic acid treatment induced a significant increase in the protein levels of USP15 in mouse hepatocytes (Suppl. Fig. S4).

Overexpression of wild-type of USP15 (USP15 WT) further increased the protein levels, whereas USP15 C298A, a dominant-negative form of USP15, did not result in protein increases (Fig. 3G). In line with previous data, ORO staining indicated that overexpression of USP15 WT resulted in an increase in lipid accumulation in a dose-dependent manner, whereas USP15 C298A failed to show lipid accumulation (Fig. 3H and 3I). Of note, overexpression of USP15 WT was able to accumulate significant amounts of lipids without the palmitic acid treatment. Taken together, USP15 plays a critical role in mediating the protein stability of FABPs and perilipins, which results in lipid accumulation.

**USP15 regulates the protein stability of FABPs and perilipins via deubiquitination**

We reasoned that USP15 could regulate the protein stability by affecting the ubiquitination status of FABPs and perilipins. We found that the poly-ubiquitination of FABPs and perilipins increased in response to the elimination of USP15 in AML12 cells (Fig. 4A). In addition, Ni\(^{2+}\) NTA pull-down further confirmed that USP15 is indeed the de-ubiquitination enzyme for FABP4 and Perilipin1 (Fig. 4B). Overexpression of USP15 WT in HEK293 cells caused a reduction in either FABP4 or Perilipin1 ubiquitination, whereas USP15 C298A did not reduce the ubiquitination of FABP4 and Perilipin1 (Fig. 4C). In concert with the USP15-mediated de-ubiquitination assays, the overexpression of USP15 WT significantly increased the total protein levels of FABP4 or Perilipin1, whereas USP15 C298A exhibited severe reduction in the protein levels (Fig. 4D and 4E). Together, our results indicate that the protein stability of FABPs and perilipins is regulated by USP15-mediated deubiquitination.

**Reduction of NAFLD phenotype induced by PPARγ-adenovirus in the liver-specific USP15 knockout mice**

To confirm the function of USP15 in NAFLD, we generated liver-specific USP15 knockout (USP15\(^{LKO}\)) mice (Suppl. Fig. S5A). The level of USP15 protein was decreased in the liver from USP15\(^{LKO}\) mice when compared with that of WT mice, whereas no significant difference was observed in the adipose tissues from WT or USP15\(^{LKO}\) mice (Suppl. Fig. S5B). Body weight, liver weight, liver weight to body weight ratio (Suppl. Fig. S6A), and liver phenotype (Suppl. Fig. S6B) were not different between the WT and USP15\(^{LKO}\) mice fed the NFD. In addition, there was no difference in the serum levels of glucose, triglyceride, free-fatty acid (FFA), cholesterol, AST, and ALT between the WT and USP15\(^{LKO}\) mice under the NFD (Suppl. Fig. S6C).
PPARγ upregulates the expression of genes involved in fatty acid uptake and lipid accumulation, including FABP4 and Perilipin1. Adenovirus-mediated-PPARγ overexpression induced NAFLD in mice\textsuperscript{28}. We injected PPARγ expressing-adenovirus into the WT or USP15\textsuperscript{LKO} mice via tail vein (Fig. 5A). The PPARγ mediated hepatic lipid accumulation was significantly alleviated in USP15\textsuperscript{LKO} mice compared to that in the WT mice (Fig. 5B and 5C). The increment of FABP4 and perilipin1 expression and macrophage infiltration, as a mark of inflammation, also alleviated in USP15\textsuperscript{LKO} mice (Fig. 5C). Liver weight and liver weight to body weight ratio were also reduced in USP15\textsuperscript{LKO} mice (Fig. 5D). The genes related to fatty acid accumulation, such as FABPs, perilipins, and CD36, and lipogeneses such as FASN, SCD-1, SREBP, and PPARγ, were significantly downregulated in USP15\textsuperscript{LKO} mice (Fig. 5E).

**The NAFLD phenotype induced by PPARγ-adenovirus was further increased by co-administration with USP15 overexpressing-adenovirus**

We further tested the effect of USP15 overexpression in NAFLD development. WT or C298A mutant USP15 expressing-adenoviruses were injected into the tail vein of the WT mice with or without PPARγ-adenovirus (Fig. 6A). In the absence of PPARγ-adenovirus, the fatty liver phenotype was not observed in either case of livers of mice injected with USP15-adenovirus or USP15 C298A-adenovirus (Fig. 6B and 6C), whereas the liver weight and liver to body weight ratio of mice injected with USP15 C298A adenovirus were slightly reduced (Fig. 6D). In the presence of the PPARγ-adenovirus, the hepatic steatosis effect of the PPARγ-adenovirus was dramatically enhanced by USP15 WT-adenovirus administration (Fig. 6B–D). Interestingly, the prominent hepatic steatosis effect of the PPARγ-adenovirus was less potentiated by administration of USP15 C298A-adenovirus (Fig. 6B–D), suggesting that the overexpression of USP15 WT in the liver aggravated hepatic steatosis compared to overexpression of USP15 C298A. The concentrations of triglycerides and free fatty acids were highest in liver tissues injected with both USP15 WT and PPARγ-adenovirus (Fig. 6E). It was also confirmed that administration of USP15 WT-adenovirus led to a significant increase in protein levels of FABPs and perilipins in liver tissues, whereas the increase in USP15 C298A-adenovirus infected livers was less than that of USP15 WT (Fig. 6F and G).

**HFD-induced NAFLD phenotypes were ameliorated in USP15\textsuperscript{LKO} mice with less inflammation**

After 12 weeks of HFD administration, no significant differences in body weight were observed between the WT and USP15\textsuperscript{LKO} mice (Suppl. Fig. S7A and S7B). We measured the metabolic parameters such as, food intake, drinking, oxygen consumption, energy expenditure, and physical activity, in the WT and USP15\textsuperscript{LKO} mice (Suppl. Fig. S7C and Suppl. Fig. S8). Although the energy expenditure of WT mice was slightly higher in the dark cycle (Suppl. Fig. S8), the other metabolic parameters were not different between the WT and USP15\textsuperscript{LKO} mice. The adipose tissue weight and adipose tissue weight to body weight ratio of the WT mice were similar to those of the USP15\textsuperscript{LKO} mice (Suppl. Fig. S9).
However, USP15LKO mice exhibited less hepatic steatosis (Fig. 7A), lower liver weight, and liver to body weight ratio than the WT mice (Fig. 7B). The content of triglycerides and free fatty acids in the liver was also significantly reduced in the USP15LKO mice (Fig. 7C and 7D). The ablation of USP15 in the liver led to a marked decrease in serum ALT concentration (Fig. 7E). There was significant downregulation of the expression of genes related to gluconeogenesis, fatty acid uptake, and lipogenesis in USP15LKO mice compared to WT mice (Fig. 7F). The levels of FABPs and perilipins were significantly downregulated in the liver of USP15LKO mice (Fig. 7G and 7H). We also found that USP15LKO mice had lower expression of factors involved in inflammation (Fig. 7I). Nevertheless, there were no changes in the serum concentrations of glucose, triglyceride, FFA, and cholesterol (Suppl. Fig. S10). The tolerance tests for glucose (GTT) and insulin (ITT) were performed to measure glucose homeostasis, and no difference was found in the glucose clearance and insulin sensitivity between the WT and USP15LKO mice (Suppl. Fig. S11).

**Hepatocyte deletion of USP15 suppresses liver steatosis, inflammation and fibrosis in mouse models of NASH**

As the HFD induces a mild inflammation and fibrosis, we utilized the fructose/palmitate/cholesterol/trans-fat (FPC) diet, which has been shown to induce more severe inflammation and fibrosis. After 16 weeks of FPC diet feeding, FPC-fed USP15LKO mice did not show significant differences in body weight and adipose tissue weight than the WT mice (Suppl. Fig. S12). However, similar to what was observed in HFD model, USP15LKO mice exhibited less hepatic steatosis (Fig. 8A), significantly lower liver weight and liver to body weight ratio than the WT controls (Fig. 8B). USP15LKO mice also exhibited lower contents of triglycerides and free fatty acids in the liver than the WT mice (Fig. 8C). The ablation of USP15 in the liver led to a marked reduction of serum ALT/AST concentration (Fig. 8D), whereas serum concentrations of triglyceride, free fatty acid, and total cholesterol only showed marginal differences (Suppl. Fig. S13). Notably, lipid accumulation and collagen deposition in liver were significantly down-regulated in the livers of USP15LKO mice, which were confirmed by Oil Red O staining or Sirius Red staining, respectively (Fig. 8E). Also, the expression of a profibrotic marker, alpha smooth muscle actin, or inflammation marker, F4/80, was reduced in USP15LKO mice as compared to WT controls. Of note, Smad3 is known to promote liver fibrosis by direct binding to the promoter regions of collagens upon phosphorylation. USP15LKO mice showed much less intensity of phosphorylation of SMAD3 in the nucleus (Fig. 8E). We also observed reduced FABP1 and perilipin1 expression in the livers of mice fed the FPC diet (Suppl. Fig. S14). In addition, there was significant down-regulation of gene expression levels related to inflammation and fibrosis in USP15LKO mice in response to the FPC diet (Fig. 8F and G). USP15 deficiency also led to a considerably less degree of cell death confirmed by TUNEL assay, which labels the free 3’-hydroxyl termini of genomic DNA indicating apoptotic cell death (Fig. 8H). However, tolerance tests for glucose and insulin did not show neither the enhanced glucose tolerance nor the insulin sensitivity due to hepatic USP15 depletion (Suppl. Fig. S15). Therefore, these results strongly suggested that ablation of USP15 ameliorates hepatic steatosis without significant systemic side effects.
Discussion

For understanding of the molecular mechanisms that govern the pathogenesis of chronic liver diseases, such as NAFLD and NASH, we report here the evidence supporting the regulation of protein stability in the development of them. The degree of ubiquitination and the management of protein homeostasis by the 26S proteasome play a central role in disease development and progression. In this regard, it is inferred that the regulation of protein homeostasis through deubiquitination is important in the pathogenesis of NAFLD and NASH. It is already known that several deubiquitinases are reduced in hepatic steatosis and has a protective function against hepatic steatosis\textsuperscript{11,12,14,34}. For example, the expression of USP10 is significantly reduced in NAFLD and obese mice, whereas USP10 overexpression is shown to lower the risk of metabolic dysfunction in mice\textsuperscript{12}. In addition, CYLD is known to suppress NASH reducing lipid accumulation, insulin resistance, inflammation and fibrosis in mice. However, the interaction of CYLD with E3 ligase TRIM47 results in degradation of CYLD in proportion with NASH progression\textsuperscript{11}. In this study, we screened the expression levels of deubiquitinases in liver tissues of high-fat dieted mice. Interestingly, the expression of USP15 was significantly elevated in the liver tissues of HFD mice. Consistently, the liver specimens of patients with either NAFLD or NASH showed markedly increased expression of USP15. These results suggest that increased expression levels of USP15 plays an important role in the pathogenesis of NAFLD and NASH in contrast to the reduced expression of most other deubiquitinases. In line with this, USP15 is known to mediate TGF-β signaling by stabilizing type I TGF-β receptor, leading to enhancement of TGF-β signaling\textsuperscript{15} and the NF-κB signaling pathway\textsuperscript{16}, which is a critical factor to accelerate fatal liver diseases such as NASH and cirrhosis\textsuperscript{35,36}. These results show that USP15 possesses both pro-inflammatory potential and lipid accumulation ability, suggesting that inhibition of USP15 can be a promising therapeutic approach for NAFLD/NASH.

Here, we determined the direct substrates of USP15 to regulate their stability to support NAFLD/NASH phenotype. Mass spectrometry analysis revealed that USP15 physically interacts with the proteins related to lipid metabolism, fatty acid-binding proteins (FABPs), and perilipins. FABPs are known to be involved in the uptake of long-chain fatty acids and further metabolism. Tissue-specific FABP proteins are differentially expressed. FABP1 is known to be highly expressed in the liver, whereas FABP4 is abundant in adipocytes and macrophages\textsuperscript{37}. In previous studies, FABP1 deficiency was reported to result in reduced hepatic triglyceride accumulation and inflammatory marker gene expression in mice fed the HFD\textsuperscript{38}. FABP4-deficient mice showed reduced insulin resistance with increased body weight in HFD-induced mice\textsuperscript{39}. FABP4 is speculated to be involved in lipolysis, leading to weight gain in FABP4-deficient mice. In addition, perilipins are known to associate with the surface of lipid droplets and control lipid accumulation and inflammation\textsuperscript{40}. \textit{PLIN1} is mainly expressed in adipocytes and its deficiency in HFD mice results in reduced accumulation of triglycerides in the white adipose tissues\textsuperscript{40}. Perilipin2 is a major hepatic lipid droplet-associated protein; its overexpression leads to considerable lipid droplet accumulation\textsuperscript{41}. \textit{PLIN2} knockout mice reportedly show attenuated fatty liver disease and obesity\textsuperscript{42}. In our \textit{in vivo} studies, overexpression of USP15 WT with PPARy by adenovirus was sufficient to induce stabilization of FABPs and PLINs proteins, whereas the catalytically inactive mutant form of USP15
C298A and PPARγ failed to induce protein stabilization in the livers. It may be a direct evidence that the deubiquitinating function of USP15 on FABPs and perilipins could boost NAFLD/NASH. It is also strongly supposed that FABPs and perilipins were deubiquitinated by USP15 and the enhanced stability of them synergistically contributed to increased lipid accumulation and inflammation in the development of NAFLD/NASH. 

FPC diet is used to induce features of metabolic and hepatic NASH characteristics. High sugar and palmitic acid contents are known to promote lipid synthesis and pro-inflammatory cytokines production, respectively. Also, high levels of cholesterol and trans-fat content cause further inflammation and liver damage. In this study, we believe that the absence of USP15 in hepatocytes led to reduced inflammation and fibrosis not only by lowering the expression of FABPs and perilipins, but also by suppressing TGF-β signaling pathways and cell death. Interestingly, the USP15 knockout livers of the HFD or FPC diet fed mice showed not only significantly reduced inflammatory responses and fibrosis but also hepatic triglycerides and free fatty acids. Interestingly, the USP15 KO mice fed an HFD or FPC diet did not show a significant difference in body weight when compared to the wild type mice. The fat mass measured in white adipose tissues or glucose metabolism measured with GTT or ITT also did not show any difference. These findings are indicative of the important role played by liver USP15 in the regulation of lipid accumulation and inflammatory responses in only the liver. However, we should not overlook that these phenotypes are exhibited in liver-specific deletion of USP15, not in global deletion of USP15. It could be good for less side toxic effects in whole body, but these need further study. 

Taken together, our findings have important implications that targeting deubiquitinases, a regulator of protein stability for NAFLD/NASH treatment. USP15 is involved in the protein stability of various substrates, such as FABP, perilipins, and TGF signaling pathway-associated proteins, thereby governing multiple etiologies of diseases such as lipid accumulation, inflammatory response, and fibrosis, which are important for NAFLD/NASH therapy. Thus, targeting USP15 by development specific inhibitors could represent a promising therapeutic strategy.

**Abbreviations**

NAFLD; non-alcoholic fatty liver diseases, NASH; non-alcoholic steatohepatitis, FABP; fatty acid-binding protein, PLIN; Perilipin, SMAD; sma- and mad-related protein, NFD; normal-fat diet, HFD; high-fat diet, FPC; fructose/palmitate/cholesterol/trans-fat, AST; aspartate amino- transferase, ALT; alanine amino-transferase, BMI; body mass index, FFA; free-fatty acid, GTT; glucose tolerance test, ITT; insulin tolerance test

**Declarations**

**Acknowledgements**
This study was supported by the Bio & Medical Technology Development Program of the National Research Foundation of Korea (NRF) funded by the Korean government, (NRF-2022R1A2C2007300, NRF-2022M3A9G8082639), and the NRF grant awarded by the Korean government (NRF-2019R1A2C2089237).

**Author Contributions**

JH Baek and MS Kim performed the experiments. DH Han and YH Lee prepared the patient samples, IHwang, K Kim, and JY Chung performed the pathological analysis. EC Yi and HR Jung performed the mass spectrometric analysis. SS Im performed the metabolic phenotype analysis. KH Chun designed and managed this project and prepared grants.

**Conflict of Interest**

The authors declare no competing interests.

**Ethical Approval**

The animal studies were approved by the Yonsei University Health System Institutional Animal Care and Use Committee. Human studies were conducted according to the ethical guidelines of the Declaration of Helsinki and all individuals gave informed consent, and the study protocol was approved by the Institutional Review Board at Severance Hospital.

**Data Availability Statement**

Data supporting the present study are available from the corresponding author upon reasonable request.

**References**


**Figures**
USP15 is upregulated in fatty livers. (A and B) The mRNA and protein levels of USP15 in the liver from mice fed a normal-fat diet (NFD) or a high-fat diet (HFD) for 12 weeks (n = 4/group). (C) Representative images of mouse normal and NAFLD liver tissues with low and high immunohistochemical staining for USP15. Hepatic steatosis was visualized with Hematoxylin and eosin (H&E) staining. Scale bar indicated 100 mm. (D) The level of USP15 mRNA in the livers of individuals without NAFLD (normal) or with NAFLD (n = 5/group). (E) Representative immunohistochemical staining for USP15 in formalin-fixed paraffin-embedded liver tissues. H&E staining of formalin-fixed, paraffin-embedded human liver samples diagnosed as normal, NAFLD, NASH is shown at the top of the image. Scale bar indicates 100 mm. (F) USP15 levels were significantly increased in NAFLD (p = 0.041) and NASH (p = 0.001) specimens compared to those in normal controls. (G) Positive correlation between USP15 level and serum AST concentrations, and serum ALT concentrations and BMI. *p < 0.05, **p < 0.01, ***p < 0.001 compared to NFD or normal.
**Figure 2**

**USP15 interacts with FABP1 and 4 and Perilipin1 and 2.** (A) USP15 interactome analysis. USP15 binding proteins were immunoprecipitated using a FLAG antibody and identified via mass spectrometry. (B) Interaction between endogenous USP15 and FABP, FABP4, Perilipin1, and Perilipin2. The AML12 cells were immunoprecipitated using IgG and USP15 antibodies. (C) The interaction between the exogenous USP15 and FABP4 and Perilipin1. HEK293 cells were transfected with FLAG-USP15, HA-FABP4, and HA-perilipin1 plasmids, and immunoprecipitated using FLAG antibody. (D) GST pulldown assay showing a direct interaction between USP15 and FABP4 and Perilipin1. (E) Representative immunohistochemical staining for USP15 and Perilipin1 in patients with non-alcoholic fatty liver disease. The scale bar indicated 100 µm. (F). Overexpression of Perilipin1 was observed in NAFLD ($p < 0.001$) and NASH ($p < 0.001$). (G) There was a statistically significant correlation between USP15 and Perilipin1 in non-alcoholic fatty liver specimens (Pearson’s $x^2$ test = 7.483, $p = 0.006$). ***$p < 0.001$ compared to normal.
**Figure 3**

**USP15 increases the protein stability of FABP1/4 and Perilipin1/2 and lipid accumulation in the hepatocytes.** (A) Western blot analysis showing FLAG-USP15 dose-dependency of HA-FABP4 or HA-PLIN1 in HEK293 cells with the increasing FLAG-USP15. (B) Effects of USP15 knockdown on the protein stability of FABP1/4 and Perilipin1/2. The AML12 cells were transfected with siRNA against USP15 and cells were treated with or without 20 mM MG132 for 8 h. (C) Western blots of AML12 cells treated with cycloheximide as indicated. (D) Normalized protein levels of FABP1/4 and Perilipin1/2 from (C). (E) Representative image of Oil Red O staining of AML12 cells treated with oleic acid as indicated with or without siRNA against USP15. (F) Normalization of Oil Red O staining from (E). Data are represented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 compared to scRNA. (G) Western blots of AML12 cells transfected with either USP15 WT or C298A followed by oleic acid treatments as indicated. Data are represented as mean ± SEM. *p < 0.05, **p < 0.01 compared to USP15 WT. (H) Representative image of the Oil Red O staining of AML12 cells transfected with either USP15 WT or C298A mutant. Cells were treated with palmitic acid as indicated. (I) Normalization of Oil Red O staining from (H). Data are represented as mean ± SEM. *p < 0.05, **p < 0.01 compared to empty vector.
Figure 4

**USP15 regulates the protein stability of FABP1/4 and Perilipin1/2 by deubiquitination.** (A) Western blots of de-ubiquitination assays of FABL1/4 and Perilipin1/2. The AML12 cells were transfected with or without siRNA against USP15 followed by 20 mM MG132 for 8 h. (B) Western blots of Ni²⁺ NTA pull-down show the de-ubiquitination of over-expressed FABP4 or Perilipin1 with 20 mM MG132 for 8 h. (C) Western blots of de-ubiquitination assays in AML12 cells after transfected with either USP15 WT or C298A mutant followed by 20 mM MG132 for 8 h. (D) Effects of USP15 WT or C298A mutant on the protein stability of FABP4 or Perilipin1 in a USP15 dose-dependent manner. (E) Normalization of western blots of protein levels in (D). Data are represented as mean ± SEM. *p < 0.05, **p < 0.01 compared to USP15 WT.
Liver-specific USP15 knockout mice improve hepatic steatosis in PPARγ overexpression liver. (A) Schematic representation of adenovirus injection (n = 4/group). (B) Macroscopic view of the liver from adenovirus-injected mice. (C) Slide sections of the liver from adenovirus-injected mice. Liver sections were stained with H&E. (D) Liver weight and the ratio of liver weight to the body weight of adenovirus-injected mice (n = 4/group). (E) Expression of genes involved in fatty acid uptake and lipogenesis (n = 4/group). Data in (D and E) are represented as mean ± SEM. ***p < 0.001 compared to WT AV-PPARγ.
Figure 6

Adenovirus-mediated USP15 overexpression aggravates hepatic steatosis. (A) Schematic representation of adenovirus injection (n = 4/group). (B) Macroscopic view of the liver from adenovirus-injected mice. (C) Slide sections of the liver from adenovirus-injected mice. The liver sections were stained with H&E. (D) The liver weight and the ratio of liver weight to the body weight of adenovirus-injected mice (n = 4/group). Data are represented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 compared to AV-Cont or AV-PPARγ+USP15. (E) Contents of triglycerides and free fatty acids in the liver from adenovirus-injected mice (n = 4/group). Data are represented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 compared to AV-Cont or AV-PPARγ+USP15. (F) The in vivo effects of USP15 WT or C298A mutant on FABP1/4 and Perilipin1/2 protein stability from the mice liver (n = 4/group). (G) Normalization of protein levels from (D). (H). Data are represented as mean ± SEM. *p < 0.05, **p < 0.01 compared to AV-USP15.
Liver-specific USP15 knockout mice show the amelioration of high-fat diet-induced fatty liver. Both wild-type and USP15<sup>LKO</sup> mice were fed a high-fat diet (HFD) for 12 weeks. (A) Macroscopic view of the liver from wild-type and USP15<sup>LKO</sup> mice fed an HFD. (B) Body weight, liver weight, and the ratio of liver weight to the body weight of wild-type and USP15<sup>LKO</sup> mice fed an HFD (WT: n = 10, USP15<sup>LKO</sup>: n = 9). (C) Contents of the triglyceride and free fatty acid in the liver from wild-type and USP15<sup>LKO</sup> mice fed an HFD (n = 5/group). (D) Slide sections of the liver from wild-type and USP15<sup>LKO</sup> mice fed an HFD. Liver sections were stained with H&E or Oil Red O. Scale bar indicated 100 mm. (E) Concentrations of ALT and AST in the serum from wild-type and USP15<sup>LKO</sup> mice fed an HFD (n = 4/group). (F) The expression of genes involved in gluconeogenesis, fatty acid uptake, lipogenesis, and fatty acid oxidation in the liver from the wild-type and USP15<sup>LKO</sup> mice fed an HFD (n = 4/group). (G) Protein expression of FABP1, FABP4, PLIN1, and PLIN2 in the liver from wild-type and USP15<sup>LKO</sup> mice fed an HFD. (H) Slide sections of the liver from wild-type and USP15<sup>LKO</sup> mice fed an HFD. Liver sections were immunohistochemically stained using antibodies against FABP4 and PLIN1. Scale bar indicated 100 mm. (I) The expression of factors involved in inflammation in the liver from the wild-type and USP15<sup>LKO</sup> mice fed an HFD (n = 4/group). Slide sections of the liver were stained using an antibody against F4/80. Scale bar indicated 100 mm. The mRNA expression was analyzed with quantitative RT-PCR. Data are represented as mean ± SEM. *p < 0.05, **p < 0.01 compared to WT.
Hepatocyte deletion of USP15 reduces liver steatosis, inflammation and fibrosis in FPC-fed mice. Both wild-type and USP15LKO mice were fed a fructose-palmitate-cholesterol (FPC) diet for 16 weeks. (A) Macroscopic view of the liver from wild-type and USP15LKO mice fed an FPC diet. (B) Body weight, liver weight, and the ratio of liver weight to the body weight of wild-type and USP15LKO mice fed an FPC diet (WT: n = 8, USP15LKO: n = 8). (C) Contents of the triglyceride and free fatty acid in the liver from wild-type and USP15LKO mice fed an FPC diet (n = 5/group). (D) Concentrations of ALT and AST in the serum from wild-type and USP15LKO mice fed an FPC diet (n = 5/group). (E) Slide sections of the liver from wild-type and USP15LKO mice fed an FPC diet. Liver sections were stained with H&E, Oil Red O and Sirius red. Liver sections were immunohistochemically stained using antibodies against α-SMA, F4/80 and p-SMAD3. Left panels represent 200 magnification (x200), and right panels represent 400 magnification (x 400). Scale bar indicated 100 mm. (F) The expression of factors involved in inflammation in the liver from the wild-type and USP15LKO mice fed an FPC diet (n = 4/group). The mRNA expression was analyzed with quantitative RT-PCR. (G) The expression of liver fibrosis marker in the liver from wild-type and USP15LKO mice fed an FPC diet (n = 4/group). The mRNA expression was analyzed with quantitative RT-PCR. (H) Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay of liver section from wild-type and USP15LKO mice fed an FPC diet. Scale bar indicated 100 mm. Data are represented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 compared to WT.

Supplementary Files
This is a list of supplementary files associated with this preprint. Click to download.

- SupplementalinformationEMM.docx