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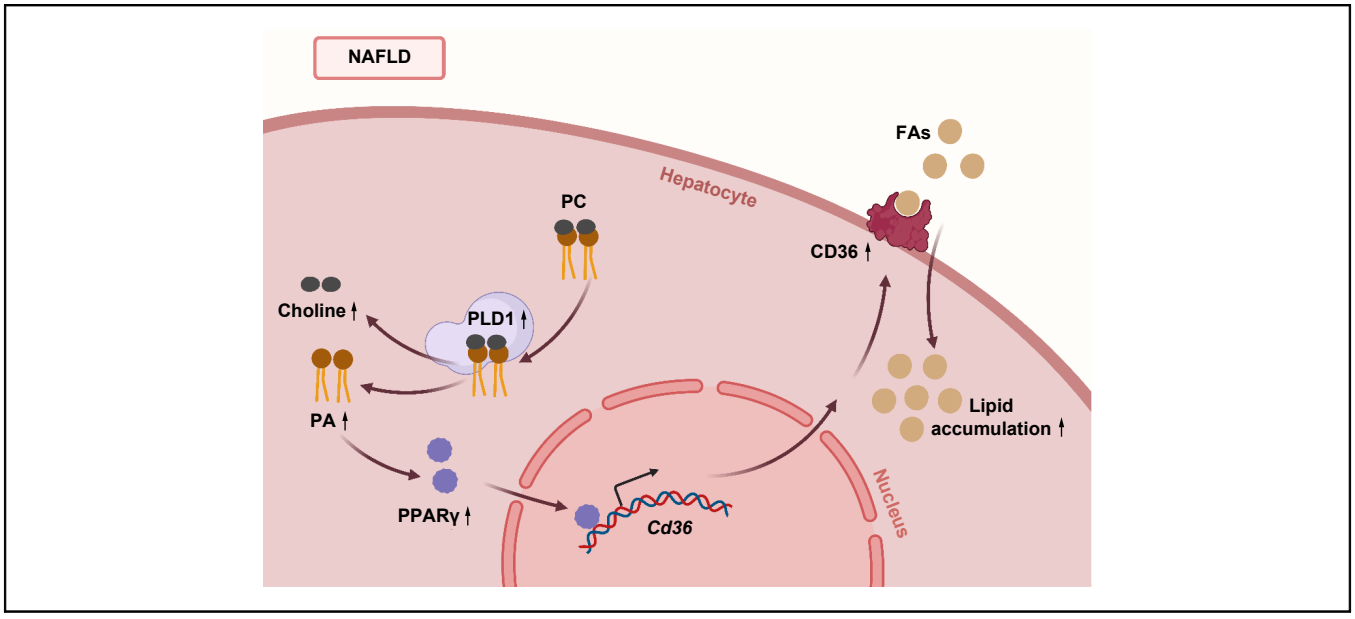
Graphical abstract



Highlights

- PLD1 is highly expressed in hepatocytes of patients with NAFLD and HFD-fed mice.
- Hepatocyte-specific deficiency of *Pld1* ameliorates hepatic steatosis.
- PLD1 promotes CD36 expression and alters lipid composition in hepatocytes.
- PA, the downstream product of PLD1, upregulates CD36 expression via PPARγ.

Impact and implications

The involvement of PLD1 in hepatocyte lipid metabolism and NAFLD has not been explicitly explored. In this study, we found that the inhibition of hepatocyte PLD1 exerted potent protective effects against HFD-induced NAFLD, which were attributable to a reduction in PPARγ/CD36 pathway-mediated lipid accumulation in hepatocytes. Targeting hepatocyte PLD1 may be a new target for the treatment of NAFLD.

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Research article

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Inhibition of phospholipase D1 ameliorates hepatocyte steatosis and non-alcoholic fatty liver disease

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Background & Aims: Phospholipase D1 (PLD1), a phosphatidylcholine-hydrolysing enzyme, is involved in cellular lipid metabolism. However, its involvement in hepatocyte lipid metabolism and consequently non-alcoholic fatty liver disease (NAFLD) has not been explicitly explored.

Methods: NAFLD was induced in hepatocyte-specific *Pld1* knockout (*Pld1*(H)-KO) and littermate *Pld1*$^{flox/flox}$ (*Pld1*-Flox) control mice feeding a high-fat diet (HFD) for 20 wk. Changes of the lipid composition in the liver were compared. Alpha mouse liver 12 (AML12) cells and mouse primary hepatocytes were incubated with oleic acid or sodium palmitate *in vitro* to explore the role of PLD1 in the development of hepatic steatosis. Hepatic PLD1 expression was evaluated in liver biopsy samples in patients with NAFLD.

Results: PLD1 expression levels were increased in the hepatocytes of patients with NAFLD and HFD-fed mice. Compared with *Pld1*-Flox mice, *Pld1*(H)-KO mice exhibited decreased plasma glucose and lipid levels as well as lipid accumulation in liver tissues after HFD feeding. Transcriptomic analysis showed that hepatocyte-specific deficiency of PLD1 decreased *Cd36* expression in steatosis liver tissues, which was confirmed at the protein and gene levels. *In vitro*, specific inhibition of PLD1 with VU0155069 or VU0359595 decreased CD36 expression and lipid accumulation in oleic acid- or sodium palmitate-treated AML12 cells or primary hepatocytes. Inhibition of hepatocyte PLD1 significantly altered lipid composition, especially phosphatidic acid and lysophosphatidic acid levels in liver tissues with hepatic steatosis. Furthermore, phosphatidic acid, the downstream product of PLD1, increased the expression levels of CD36 in AML12 cells, which was reversed by a PPARγ antagonist.

Conclusions: Hepatocyte-specific *Pld1* deficiency ameliorates lipid accumulation and NAFLD development by inhibiting the PPARγ/CD36 pathway. PLD1 may be a new target for the treatment of NAFLD.

Impact and implications: The involvement of PLD1 in hepatocyte lipid metabolism and NAFLD has not been explicitly explored. In this study, we found that the inhibition of hepatocyte PLD1 exerted potent protective effects against HFD-induced NAFLD, which were attributable to a reduction in PPARγ/CD36 pathway-mediated lipid accumulation in hepatocytes. Targeting hepatocyte PLD1 may be a new target for the treatment of NAFLD.

Introduction

Non-alcoholic fatty liver disease (NAFLD) has become the leading chronic liver disease globally; however, no effective treatment has been approved.[1] Patients with NAFLD have an increased risk for severe metabolic diseases including type 2 diabetes mellitus, insulin resistance, and dyslipidemia.[2] Increasing evidence suggests that abnormal lipid metabolism in hepatocytes is an important contributor to the initiation and progression of NAFLD.[3,4] Lipid droplet (LD) accumulation in hepatocytes is a distinctive characteristic of NAFLD, a chronic, heterogeneous liver condition that can progress to liver fibrosis and hepatocellular carcinoma.[5] This lipid accumulation may occur owing to increased *de novo* lipogenesis, increased fatty acid uptake, decreased redistribution of fatty acids to other tissues, or decreased utilisation of lipids as energy substrates.[6]

Phospholipase D (PLD) enzymes are phosphodiesterases that serve as key components of multiple signalling and metabolic pathways.[7] In mammalian cells, the PLD isoenzymes – PLD1 and

Keywords: Phospholipase D1; Hepatic steatosis; Phosphatidic acid; CD36; PPARγ.
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PLD2 – are major sources of phosphatidic acid (PA), a lipid second messenger that modulates diverse intracellular signalling.[8,9] PLD is involved in cell proliferation, inflammation, survival, redox signalling, mitochondrial function, and many pathophysiological actions; it has also been associated with neuronal ailments, cancer, thrombotic events, and infectious diseases.[7,9]

PLD activity is also linked to cell-free LD assembly.[10,11] The PLD1 isoform (but not PLD2) is present in LDs, and increased PLD1 expression promotes LD formation.[10,12] PLD1 is essential for the formation, growth, and accumulation of LDs, and its number and size are correlated with obesity and NAFLD.[5,9,13] In addition, PLD1 played a role in the development and progression of liver fibrosis in rats.[14] However, one study showed that *Pld1* systemic knockout induced NAFLD by decreasing LDs.[15] Further studies are needed to clarify the role of hepatic PLD1 in metabolic processes.

In this study, inducing hepatic steatosis in hepatocyte-specific *Pld1*-deficient mice using a high-fat diet (HFD), we provide evidence that PLD1 plays a key role in promoting hepatocyte lipid accumulation and steatosis development via the peroxisome proliferative activating receptor gamma (PPARγ)/CD36 pathway. Furthermore, PA may be the main factor involved in the activation of the PPARγ/CD36 pathway during hepatocyte lipid accumulation.

Materials and methods

Animals and experiment protocol

Pld1$^{flox/flox}$ mice were generated at the Shanghai Model Organisms Center, Inc (China). To generate hepatocyte-specific *Pld1* knockout (*Pld1*$^{flox/flox}$; Alb-Cre^{+}; *Pld1*(H)-KO) mice, *Pld1*$^{flox/flox}$ mice were crossed with *Alb*-Cre mice (003574, The Jackson Laboratory, Bar Harbor, Maine, USA). Littermate *Pld1*-Flox (*Pld1*$^{flox/flox}$; Alb-Cre^{-}) mice were used as controls. All animals were maintained in a pathogen-free, temperature-controlled environment under a 12-h light/dark cycle at Beijing Friendship Hospital, and all animal protocols were approved by the Institutional Animal Care and Ethics Committee. Male *Pld1*-Flox and *Pld1*(H)-KO mice at 8–9 wk were fed a normal control diet (NCD) or an HFD (60 kcal% fat; D12492, Research Diets, New Brunswick, New Jersey, USA).

Clinical study

The samples involved in the clinical study were collected from twelve patients diagnosed with NAFLD by liver biopsy at Beijing Friendship Hospital, Capital Medical University. Patients with NAFLD who consumed <10 g alcohol/day for women or 20 g alcohol/day for men and those with other causes of steatosis or chronic liver disease were excluded. The demographic and clinical characteristics of the patients with NAFLD are shown in Table S2. For each biopsy, a NAFLD activity score (NAS) summarising the main histological lesions was defined based on the grade of steatosis, the grade of activity (hepatocytes ballooning and lobular inflammation), and the stage of fibrosis (Table S2).[16] Healthy liver tissues were obtained from eight donors, whose livers were subsequently used for transplantation. Written informed consent was obtained from all patients before enrolment, and the study protocol was approved by the human institutional review board of the Beijing Friendship Hospital (No. 2017-P2-131-03).

Statistical analysis

Statistical analysis was performed using GraphPad Prism software (version 9.0, San Diego, California, USA), and values were expressed as mean ± SEM. Differences between two groups were compared using Student's *t* test for normal distribution and the Mann–Whitney test for abnormal distribution. One-way ANOVA with Tukey's test for normal distribution and the Kruskal–Wallis test for abnormal distribution were used for analysing differences among three or more groups. Statistical significance was set at $p < 0.05$.

For further details regarding the materials and methods used, please refer to the Supplementary CTAT Table and Supplementary Materials and methods.

Results

Hepatocyte-specific deficiency of *Pld1* ameliorated NAFLD in HFD-fed mice

After 20 wk of HFD feeding, hepatocellular PLD activity was significantly upregulated compared with that in the NCD-fed control mice (Fig. 1A). The protein and mRNA expression levels of PLD1 were markedly increased in the hepatocytes obtained from HFD-fed mice than in those obtained from NCD-fed mice (Fig. 1B–D). However, the expression level of PLD2 did not change after HFD feeding (Fig. 1B, C, and E). We further tested PLD1 expression in hepatocytes of patients with NAFLD and in those of healthy controls by immunofluorescence (Fig. 1F and G) and found the proportion of ALB^{+}PLD1^{+} area to be higher in patients with NAFLD than in healthy controls. These findings suggest that hepatocellular PLD1 is involved in the development and progression of NAFLD in both mice and humans.

To investigate the role of hepatocellular PLD1 in NAFLD development or progression, HFD was fed to *Pld1*(H)-KO and *Pld1*-Flox mice for 20 wk. The protein and mRNA expression levels of PLD1 decreased in *Pld1*-deficient hepatocytes in both HFD- and NCD-fed mice (Fig. S1A–C). PLD1 deficiency did not alter the expression of PLD2 in hepatocytes after HFD feeding (Fig. S1D–F). As shown in Fig. 2A–C, *Pld1*(H)-KO mice gained remarkably less body weight, liver size, and liver weight than age-matched *Pld1*-Flox mice, although food intake among each group was not significantly different after HFD feeding (Fig. S1G). HFD-fed mice had insulin resistance with an impaired oral glucose tolerance test (OGTT) compared with NCD-fed mice; these were improved by hepatocyte *Pld1* knockout (Fig. 2D and E). Plasma triglyceride (TG) and free fatty acid (FFA) levels did not differ between NCD- and HFD-fed mice (Fig. 2F). Plasma levels of total cholesterol (TC), LDL-cholesterol (LDL-C), glucose (GLU), alanine aminotransferase (ALT), and aspartate transaminase (AST) in mice with hepatocyte-specific deficiency of *Pld1* were significantly reduced compared with those in *Pld1*-Flox mice after HFD feeding (Fig. 2F–I). Hepatocyte-specific *Pld1* deficiency significantly decreased liver steatosis score, lobular inflammation score, and the NAS in HFD-fed *Pld1*(H)-KO mice compared with those in HFD-fed *Pld1*-Flox mice (Fig. 2J and K and Fig. S1H–J). Oil Red O staining revealed decreased lipid accumulation in the livers of *Pld1*(H)-KO mice compared with those of *Pld1*-Flox mice after HFD feeding (Fig. 2J and L). BODIPY staining showed that LDs were increased in the livers of HFD-fed mice and decreased in hepatocyte *Pld1* knockout mice (Fig. 2M and N). LD deposition was also found in a portion of Desmin^{+} hepatic stellate cells (HSCs) in both NCD- and HFD-fed mice (Fig. S1K). Furthermore, the deficiency of PLD1 could decrease liver-infiltrating CD45^{+}, F4/80^{+}, and CD3^{+} cells and the expression levels of inflammation-related genes, such as *Tnfa*, *Il1b*, *Il6*, and *Il17*, after HFD feeding (Fig. 2J, O, and P). Meanwhile, the levels of fibrosis-related genes, such as *α-SMA*, *Col1a1*, *Col3a1*,

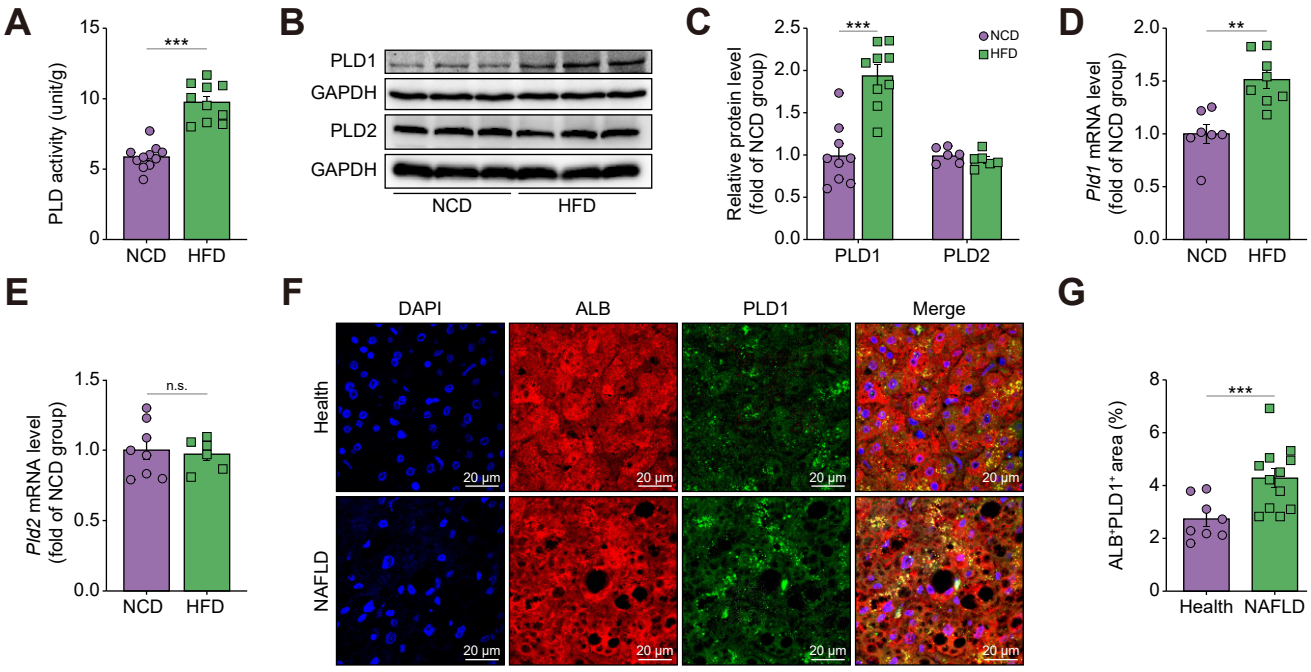

Fig. 1. PLD1 expression was increased in the hepatocytes of HFD-fed mice and patients with NAFLD. (A) PLD activity, (B) representative Western blot images showing PLD1, PLD2, and GAPDH levels, (C) quantification of PLD1 and PLD2 by densitometry and gene expression levels of (D) *Pld1* and (E) *Pld2* in the hepatocytes of *Pld1*-Flox mice after 20 wk of NCD or HFD feeding. (F) Representative immunofluorescence staining images in the liver of patients with NAFLD and healthy controls; ALB in red (546), PLD1 in green (488), and nuclei in blue (DAPI) (scale bar, 20 μm). (G) Quantification of $ALB^{+}PLD1^{+}$ area. n = 8–12 participants per group. Differences between two groups were compared using Student's *t* test for normal distribution and the Mann–Whitney test for abnormal distribution. $^{**}p$ <0.01, $^{***}p$ <0.001. ALB, albumin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HFD, high-fat diet; NAFLD, non-alcoholic fatty liver disease; NCD, normal control diet; PLD, phospholipase D; PLD1, phospholipase D1; PLD2, phospholipase D2.

and *Col6a1*, and hydroxyproline were also downregulated in the liver tissues after *Pld*1 knockout (Fig. 2Q and R).

Hepatocyte-specific deficiency of *Pld1* changed hepatic lipid metabolism in HFD-fed mice

Considering the substantial difference in liver Oil Red O and LD staining between HFD-fed *Pld1*(H)-KO and *Pld1*-Flox mice. We performed lipidomic analysis to examine lipid metabolism and lipidomic changes in the livers of *Pld1*(H)-KO and *Pld1*-Flox mice after HFD feeding. Our lipidomic data revealed 460 lipid species in the liver tissues, consisting of 181 triacylglycerols (TAGs), 49 cardiolipins, 47 phosphatidylcholines (PCs), 25 diacylglycerols (DAGs), and other lipid classes (Fig. S2A). We visualised all significantly altered lipid species using a bubble map (Fig. S2B). Using a *p* value of 0.05 as cut-off, a total of 207 species were significantly changed in the *Pld1*-deficient livers (Fig. S2B). The overall abundance of TAG and DAG, which are the most abundant lipid classes in the liver, was significantly decreased by *Pld1* deficiency (Fig. S2C). As a key enzyme regulating phospholipid metabolism, hepatocyte *Pld1* knockout had a tendency to increase the total levels of PC in the liver (Fig. S2D). The levels of PC34:0p, PC36:3p, PC32:2/0, PC34:2/1/0, PC36:4, and PC38:6 among 47 PC species were significantly increased by hepatocyte *Pld1* knockout (Fig. S2E). In addition, there were significant changes in other glycerophospholipids, including a decrease in the abundance of lysophosphatidylethanolamines and bis(monoacylglycerol)phosphate and an increase in the abundance of phosphatidylethanolamines (Fig. S2F). Sphingolipids, including sphingomyelins and ceramides, were unaffected by *Pld1* deficiency (Fig. S2G). The abundance of FFAs and cholesteryl esters was decreased by *Pld1* deficiency (Fig. S2H and I). These results suggest that the inhibition of hepatocellular PLD1 affects the composition of lipids in liver tissues.

Transcriptome sequencing analysis showed that the expression of fatty acid translocase *CD36* changed significantly in *Pld1*(H)-KO mice

To explore the impact of hepatocyte-specific *Pld1* deficiency, mRNA transcriptome sequencing was performed in the liver tissues of *Pld1*(H)-KO and *Pld1*-Flox mice after NCD or HFD feeding (Fig. S3A and B). Gene Ontology (GO) pathway analysis revealed that pathways related to lipid uptake, transport, and metabolism were upregulated and lipase activity pathways were downregulated in *Pld1*(H)-KO mice compared with those in *Pld1*-flox mice (Fig. S3C). Compared with pathways in *Pld1*-flox mice, pathways related to lipid activity were upregulated in *Pld1*(H)-KO mice and pathways related to lipid digestion, absorption, metabolism, and import cells were downregulated (Fig. 3A). Gene set expression analysis revealed that *intestinal lipid absorption* and *LD* were positively enriched in *Pld1*-Flox-HFD mice compared with those in *Pld1*-Flox-NCD mice (Fig. S3D), whereas *cellular responses to fatty acids* and *LDs* were negatively enriched in *Pld1*(H)-KO-HFD mice compared with those in *Pld1*-Flox-HFD mice (Fig. 3B). A total of 193 differentially expressed genes were obtained from the intersection of the differentially expressed genes in *Pld1*-Flox-HFD *vs.* *Pld1*-Flox-NCD mice and in *Pld1*(H)-KO-HFD *vs.* *Pld1*-Flox-HFD mice. Among these genes, four lipid metabolism-related GO pathways were enriched: the *arachidonic*

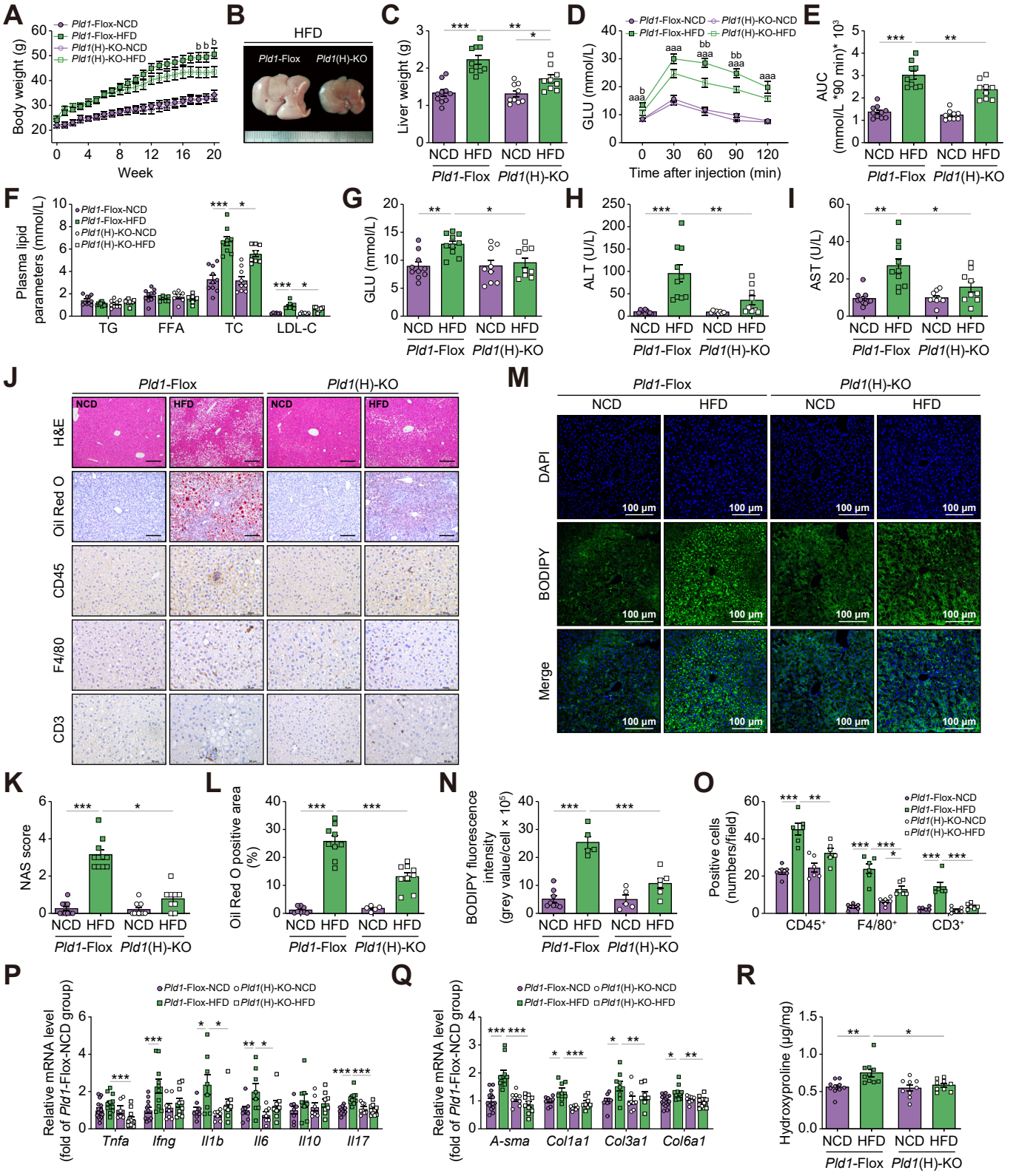

Fig. 2. Hepatocyte-specific *Pld1* deficiency alleviated NAFLD in mice. (A) Body weight was measured weekly in *Pld1*-Flox and *Pld1*(H)-KO mice after NCD or HFD feeding. (B) Representative photos of the liver, (C) liver weight, (D) OGTT, (E) AUC of the OGTT and plasma (F) TG, FFA, TC, LDL-C, (G) GLU, (H) ALT, and (I) AST levels in *Pld1*-Flox and *Pld1*(H)-KO mice after 20 wk of NCD or HFD feeding. (J) Representative images of hepatic H&E staining(scale bar, 100 μm); Oil Red O staining(scale bar, 100 μm); immunohistochemical staining of CD45, F4/80, and CD3 (scale bar, 50 μm); (K) NAS; and (L) Oil Red O positive area per total area in the liver of *Pld1*-Flox and *Pld1*(H)-KO mice after 20 wk of NCD or HFD feeding. (M) Representative immunofluorescence staining images of lipid droplet in green (BODIPY, 493/503) and nuclei in blue (DAPI) (scale bar, 100 μm); (N) BODIPY fluorescence intensity; (O) $CD45^+$, $F4/80^+$, and $CD3^+$ cell numbers per field; (P) relative mRNA levels of proinflammatory cytokine and (Q) fibrosis-related gene; and (R) hydroxyproline levels in the liver of *Pld1*-Flox and *Pld1*(H)-KO mice after

acid metabolic process, *long-chain fatty acid metabolic process*, *fatty acid metabolic process*, and *regulation of plasma lipoprotein particle levels*. Among the genes involved in these pathways, 10 were downregulated and five were upregulated in *Pld1*(H)-KO-HFD mice compared with those in *Pld1*-Flox-HFD mice. All genes were sequenced according to -$\log_{10}$(*p* value), and we found that among the genes involved in lipid metabolism, fatty acid translocase *Cd36* was in a relatively high position (Fig. 3C). To verify these observations, CD36 expression levels were measured *in vivo*. The protein and mRNA expression levels of CD36 were increased in the liver tissues of HFD-fed mice and significantly reduced by hepatocyte-specific *Pld1* deficiency (Fig. 3D–F). CD36 expression levels were also significantly higher in the liver tissues of patients with NAFLD than in those of healthy controls (Fig. 3G and H).

PLD1 inhibition and deficiency decreased the content of lipid and expression of CD36 in hepatocytes *in vitro*

To gain insight into PLD1-induced changes in lipid metabolism in hepatocytes, PLD1-specific inhibitors, VU0155069 (VU01) and VU0359595 (VU03), were used to interfere with the murine hepatocyte cell line alpha mouse liver 12 (AML12). The protein and mRNA expression levels of PLD1 and PLD activity were significantly increased after oleic acid (OA) stimulation, and the most obvious increase was observed after 500 μM OA stimulation for 48 h (Fig. 4A–D). Therefore, AML12 cells were incubated with 500 μM OA for 48 h for subsequent inhibition experiments. VU01 and VU03 reduced the proportion of $CD36^+$ cells, the mean fluorescence intensity (MFI) of CD36, and the mRNA levels of *Cd36*, which were increased by OA treatment (Fig. 4E–G). LDs and lipid accumulation were increased in the OA-treated AML12 cells and decreased by inhibition of PLD1 (Fig. 4H–K). VU01 and VU03 also decreased TG and TC levels in the OA-treated AML12 cells (Fig. 4L and M). To determine the key role of CD36 in PLD1-mediated lipid accumulation, CD36 overexpression plasmids were transfected into AML12 cells. The mRNA levels of CD36 and the proportion of $CD36^+$ cells elevated significantly after transfection and could not be reduced by VU01 (Fig. S4A and B). Oil Red O and Nile Red staining revealed increased lipid accumulation after overexpression of CD36 in OA-treated AML12 cells, which could not be decreased by inhibition of PLD1 (Fig. 4N and O and Fig. S4C and D).

In addition, primary hepatocytes from *Pld1*(H)-KO and *Pld1*-Flox mice were treated with OA to verify the role of PLD1 in lipid accumulation of hepatocytes. The expression levels of *Pld1* and CD36 increased after OA treatment in *Pld1*-Flox hepatocytes, whereas they decreased in *Pld1*-deficient hepatocytes (Fig. 4P–S). Moreover, deficiency of PLD1 reduced the elevated levels of LD, TG, and TC after OA treatment in mouse primary hepatocytes (Fig. 4T–V). These data suggested that PLD1 plays a major role in the regulation of CD36-mediated lipid accumulation in hepatocytes.

Similar results were also found in sodium palmitate (SP)-treated hepatocytes. The protein and mRNA expression levels of PLD1 were significantly increased, and PLD1 activity peaked at 48 h after stimulation with 250 μM SP (Fig. S5A–D). Therefore, AML12 cells were incubated with 250 μM SP for 48 h for subsequent experiments. The proportion of $CD36^+$ cells, the MFI of CD36, the mRNA levels of *Cd36*, LD, TG, and TC, and lipid accumulation were significantly increased in AML12 cell treated with SP and decreased after incubation with VU01 and VU03 (Fig. S5E–P). SP stimulation increased the proportion of $7AAD^-$Annexin V^+ cells and $7AAD^+$ Annexin V^+ cells, which was decreased after VU03 treatment (Fig. S5Q–S). Consistently, deficiency of PLD1 significantly decreased the levels of *Pld1* mRNA, CD36 protein and mRNA, TG, TC, and LD in SP-treated *Pld1*(H)-KO hepatocytes compared with those in SP-treated *Pld1*-Flox hepatocytes (Fig. S5T–Y).

PA (a PLD1 product) regulates CD36 expression in hepatocytes

As a key enzyme regulating phospholipid metabolism, hepatocyte *Pld1* knockout observably decreased the abundance of PA and lysophosphatidic acid (LPA) (Fig. 5A and B). The levels of PA32:2/1/0, PA34:2/1, and PA36:2/1 among 13 PA species and those of LPA16:0 and LPA18:2/1 among 5 LPA species were significantly decreased by hepatocyte *Pld1* knockout (Fig. 5C). Meanwhile, the content of PA and LPA increased in OA-treated AML12 cells and decreased by inhibition of PLD1 (Fig. 5D and E). The levels of PA32:1, PA34:2/1, PA36:2/1, and PA38:6/4/3 among 13 PA species and those of LPA16:0 and LPA18:1/0 among 3 LPA species were significantly decreased by VU01 and VU03 (Fig. 5F). SP treatment also elevated PA and LPA levels, whereas PLD1 inhibitor VU01 and VU03 downregulated PA and LPA levels, respectively (Fig. S6A and B). The levels of PA34:1, PA36:2, PA38:6/4/3, and PA40:6/5 among 13 PA species and those of LPA18:1 among 3 LPA species were significantly decreased by VU01 and VU03 (Fig. S6C).

Because PA and LPA are products of PLD1, we explored whether they regulate CD36 expression. We found that PA, but not LPA, increased the mRNA expression levels of *Cd36* in AML12 cells (Fig. 5G). Meanwhile, CD36 expression, which was reduced by the inhibition of PLD1, was normalised by PA supplementation (Fig. 5H). These results suggest that PA, catalysed by PLD1, plays a role in increasing CD36 expression in hepatocytes.

PA (a PLD1 product) impacts CD36 expression through PPARγ

To explore the specific regulatory mechanism by which PA regulates *Cd36* expression, we performed Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis on the differentially expressed genes in *Pld1*(H)-KO-HFD *vs.* *Pld1*-Flox-HFD mice and found that the PPAR signalling pathway occupied a prominent position (Fig. 6A). Among the genes that were enriched in the PPAR pathway and differentially expressed in both *Pld1*-Flox-HFD mice *vs.* *Pld1*-Flox-NCD mice and *Pld1*(H)-KO-HFD mice *vs.* *Pld1*-Flox-HFD mice, we found *Pparg* to have the same change trend as *Cd36* (Fig. 6B). Similarly, both mRNA and protein levels of PPARγ were elevated after HFD feeding and decreased by hepatocyte-specific deficiency of *Pld1* (Fig. 6C and D). In AML12 cells, inhibiting PLD1 using VU01 or VU03 decreased the mRNA expression levels of *Pparg*, which were

20 wk of NCD or HFD feeding. n = 8–10 mice per group. One-way ANOVA with Tukey's test for normal distribution and the Kruskal–Wallis test for abnormal distribution were used for analysing differences among three or more groups. $^*p$ <0.05, $^{**}p$ <0.01, $^{***}p$ <0.001. ^{aaa}p <0.001, *Pld1*-Flox-NCD mice *vs.* *Pld1*-Flox-HFD mice, ^bp <0.05, ^{bb}p < 0.01, *Pld1*(H)-KO-HFD mice *vs.* *Pld1*-Flox-HFD mice. ALT, alanine transaminase; AST, aspartate transaminase; FFA, free fatty acid; GLU, glucose; HFD, high-fat diet; LDL-C, LDL-cholesterol; NAFLD, non-alcoholic fatty liver disease; NAS, NAFLD activity score; NCD, normal control diet; OGTT, oral glucose tolerance test; PLD1, phospholipase D1; TC, total cholesterol; TG, triglyceride.

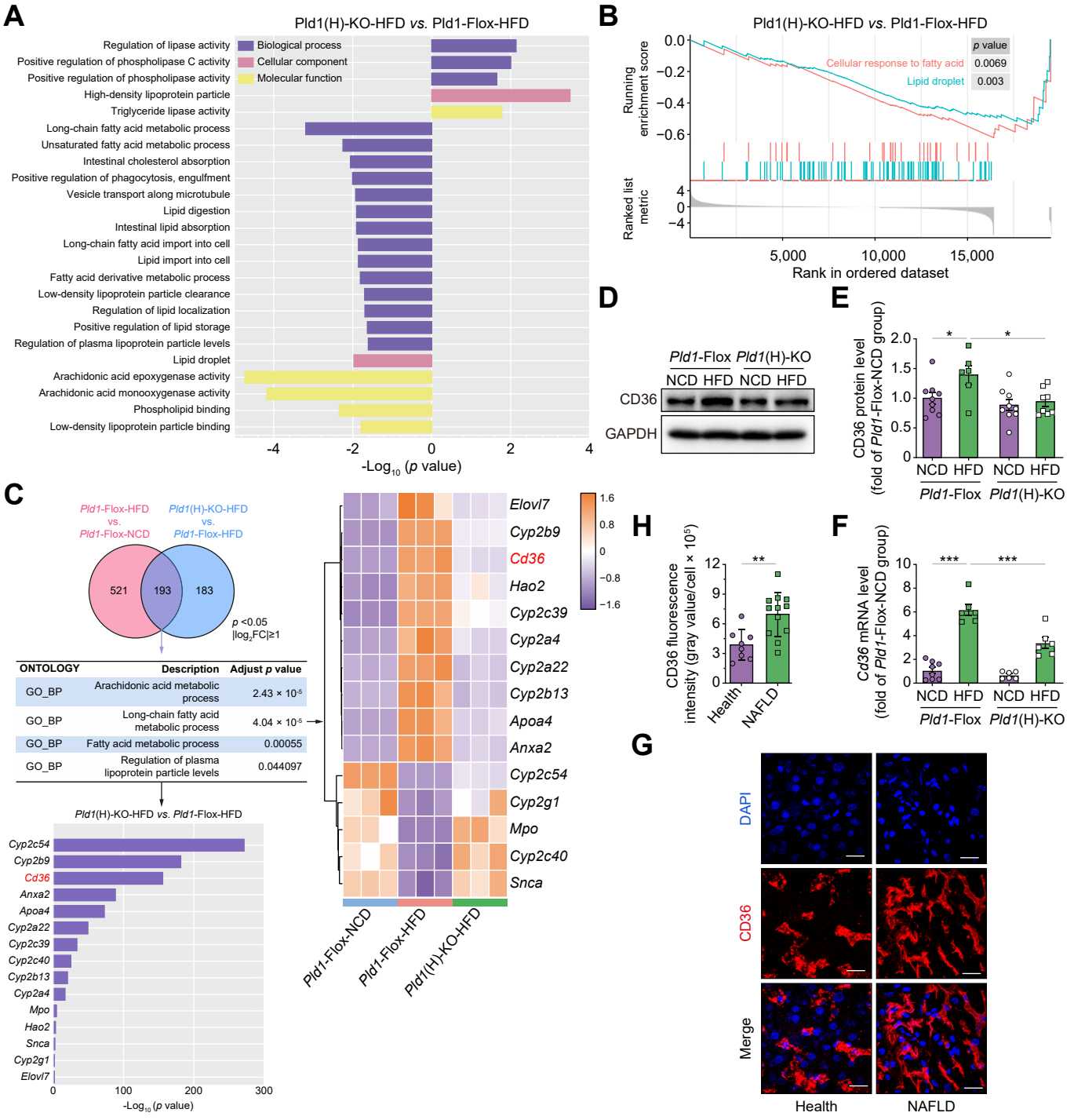

Fig. 3. Hepatocyte-specific *Pld1* deficiency induced changes in lipid and gene expression profiles. (A) Upregulated and downregulated GO pathway enriched in differentially expressed genes in *Pld1*(H)-KO-HFD mice compared with that in *Pld1*-Flox-HFD mice. (B) Negative enrichment plot for the gene set of differentially expressed genes in *Pld1*(H)-KO-HFD mice compared with that in *Pld1*-Flox-HFD mice. (C) GO enrichment analysis was performed on the genes obtained from the intersection of *Pld1*-Flox-HFD *vs.* *Pld1*-Flox-NCD mice and *Pld1*(H)-KO-HFD *vs.* *Pld1*-Flox-HFD mice (top left), and the genes involved in enriched pathways related to lipid metabolism were shown according to their expression levels and sorted according to -$\log_{10}(p)$ values (bottom left and right). (D) Representative Western blot images showing CD36 and GAPDH levels, (E) quantification of CD36 by densitometry, and (F) gene expression levels of *Cd36* in the livers of *Pld1*-Flox and *Pld1*(H)-KO mice after 20 wk of NCD or HFD feeding. (G) Representative immunofluorescence staining images of CD36 in red (647) and nuclei in blue (DAPI) (scale bar, 20 μm). (H) Quantification of CD36 by fluorescence intensity in the liver of patients with NAFLD and those of healthy controls. n = 6–12 participants per group. Differences between two groups were compared using Student's *t* test for normal distribution and the Mann–Whitney test for abnormal distribution. One-way ANOVA with Tukey's test for normal distribution and the Kruskal–Wallis test for abnormal distribution were used for analysing differences among three or more groups. *p <0.05, **p <0.01, ***p <0.001. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GO, gene ontology; HFD, high-fat diet; NAFLD, non-alcoholic fatty liver disease; NCD, normal control diet; PLD1, phospholipase D1.

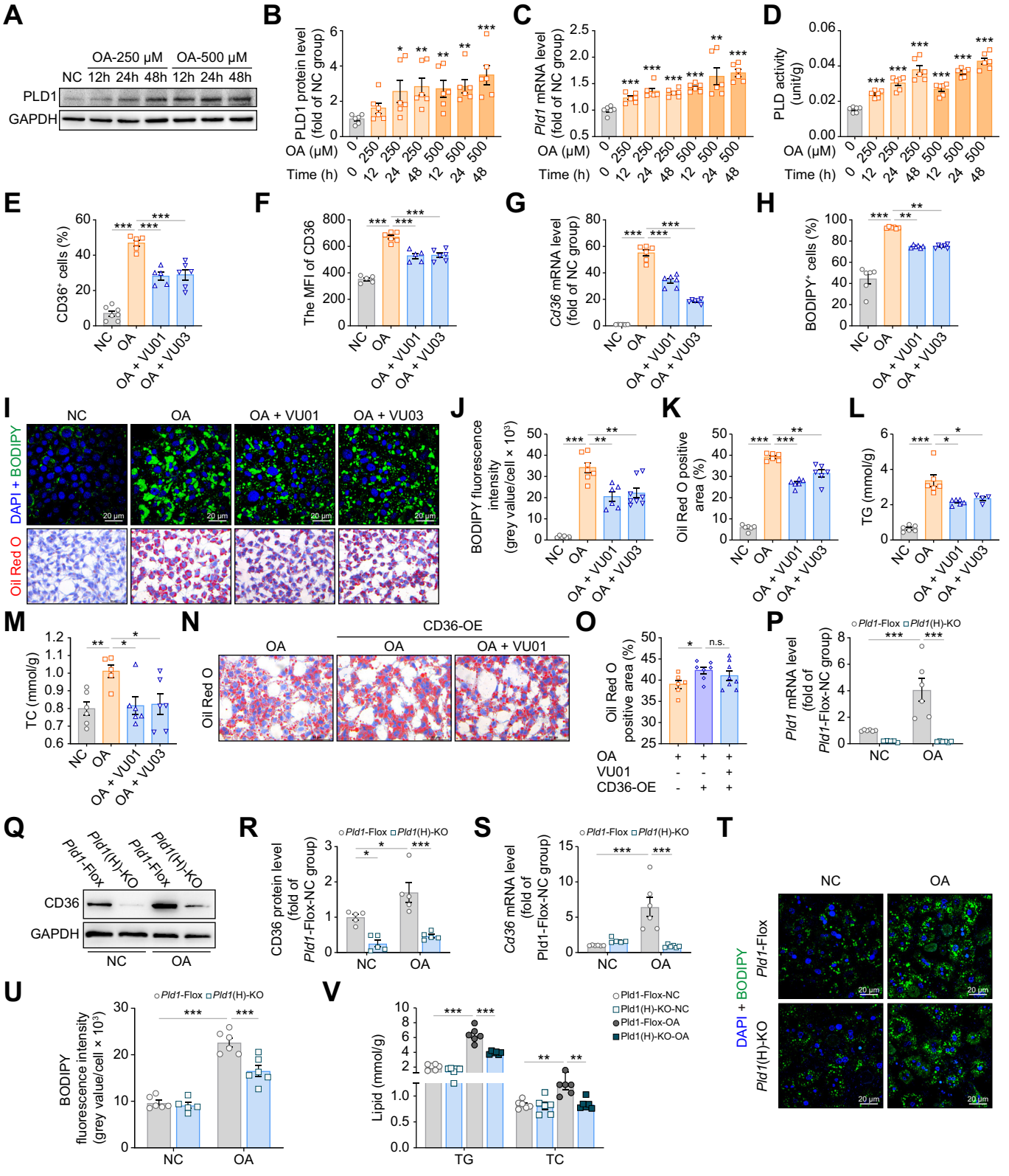

Fig. 4. Inhibition of PLD1 reduced OA-induced increase in CD36 expression and lipid levels in hepatocytes. (A) Representative Western blot images showing PLD1 and GAPDH levels, (B) quantification of PLD1 by densitometry, (C) gene expression levels of *Pld1*, and (D) PLD activity in OA-treated AML12 cells. (E) The ratio of CD36$^+$ cells, (F) the MFI of CD36, (G) the mRNA expression levels of *Cd36*, and (H) the ratio of BODIPY$^+$ cells in OA-stimulated AML12 cells with or without PLD1 inhibitor treatment (VU01 or VU03). (I) Representative staining images of lipid droplet in green (BODIPY, 493/503), nuclei in blue (DAPI), and Oil Red O. (J) BODIPY fluorescence intensity; and (K) Oil Red O positive area per total cell area, and (L) TG and (M) TC levels in the OA-stimulated AML12 cells with or without PLD1 inhibitor. (N) Representative staining images of Oil Red O and (O) Oil Red O positive area per total cell area in OA- and VU01-treated AML12 cells after CD36

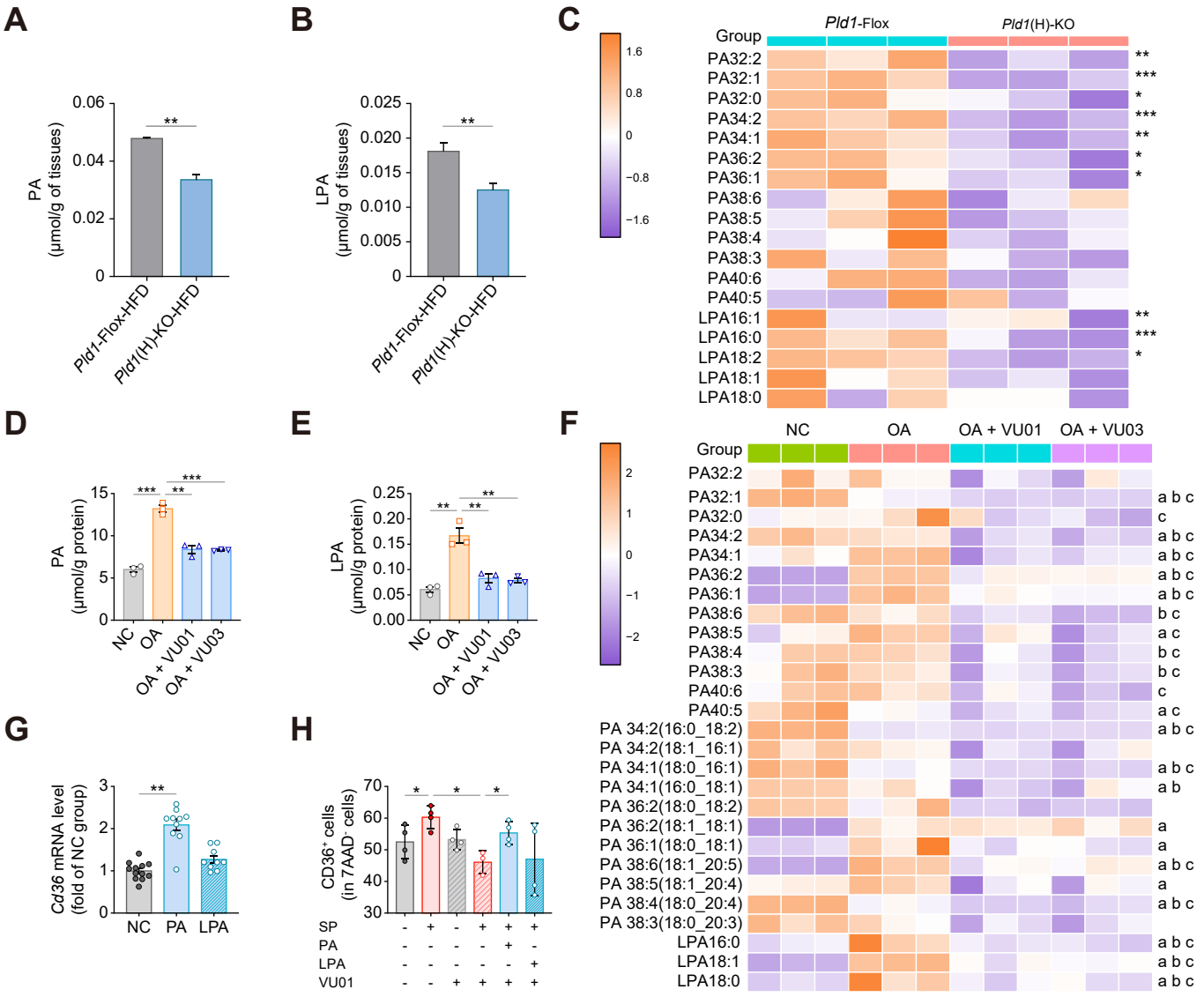

Fig. 5. PLD1 inhibition or knockdown decreased the content of PA and LPA. The changes of (A) PA and (B) LPA, and (C) heatmap of the different isoforms of PA and LPA determined by lipidomic analysis in the liver of *Pld1*-Flox and *Pld1*(H)-KO mice after 20 wk of HFD feeding. n = 3–5 mice per group. The changes of (D) PA and (E) LPA, and (F) heatmap of the different isoforms of PA and LPA determined by lipidomic analysis in the AML12 cells after OA treatment. (G) Gene expression levels of *Cd36* in PA- or LPA-treated AML12 cells. (H) The ratio of $CD36^+$ cells to $7AAD^-$ cells. n = 3–12 dishes of cells per group. Differences between two groups were compared using Student's *t* test for normal distribution and the Mann–Whitney test for abnormal distribution. One-way ANOVA with Tukey's test for normal distribution and the Kruskal–Wallis test for abnormal distribution were used for analysing differences among three or more groups. n = 3–6 dishes of cells per group. $^*p$ <0.05, $^{**}p$ <0.01, $^{***}p$ <0.001. AML12, alpha mouse liver 12; HFD, high-fat diet; LPA, lysophosphatidic acid; NC, normal control; OA, oleic acid; PA, phosphatidic acid; PLD1, phospholipase D1; VU01, VU0155069; VU03, VU0359595.

increased by OA or SP treatment (Fig. 6E and Fig. S6D). The mRNA expression levels of *Pparg* were also increased in OA-treated *Pld1*-Flox hepatocytes, whereas they were decreased in *Pld1*(H)-KO hepatocytes (Fig. 6F). PA stimulation could increase *Pparg* gene expression in AML12 cells (Fig. 6G). *Pparg* reportedly participates in the transcriptional regulation of *Cd36*, and we also

overexpression. (P) Gene expression levels of *Pld1*, (Q) representative Western blot images showing CD36 and GAPDH levels, (R) quantification of CD36 by densitometry, (S) the mRNA expression levels of *Cd36*, (T) representative immunofluorescence staining images of lipid droplet in green (BODIPY, 493/503) and nuclei in blue (DAPI), (U) BODIPY fluorescence intensity, and (V) the levels of TG and TC in OA-treated primary hepatocytes from *Pld1*-Flox and *Pld1*(H)-KO mice. n = 3–6 dishes of cells per group. Differences between two groups were compared using Student's *t* test for normal distribution and the Mann–Whitney test for abnormal distribution. One-way ANOVA with Tukey's test for normal distribution and the Kruskal–Wallis test for abnormal distribution were used for analysing differences among three or more groups. $^*p$ <0.05, $^{**}p$ <0.01, $^{***}p$ <0.001. AML12, alpha mouse liver 12; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MFI, mean fluorescence intensity; NC, normal control; OA, oleic acid; PLD1, phospholipase D1; TC, total cholesterol; TG, triglyceride; VU01, VU0155069; VU03, VU0359595.

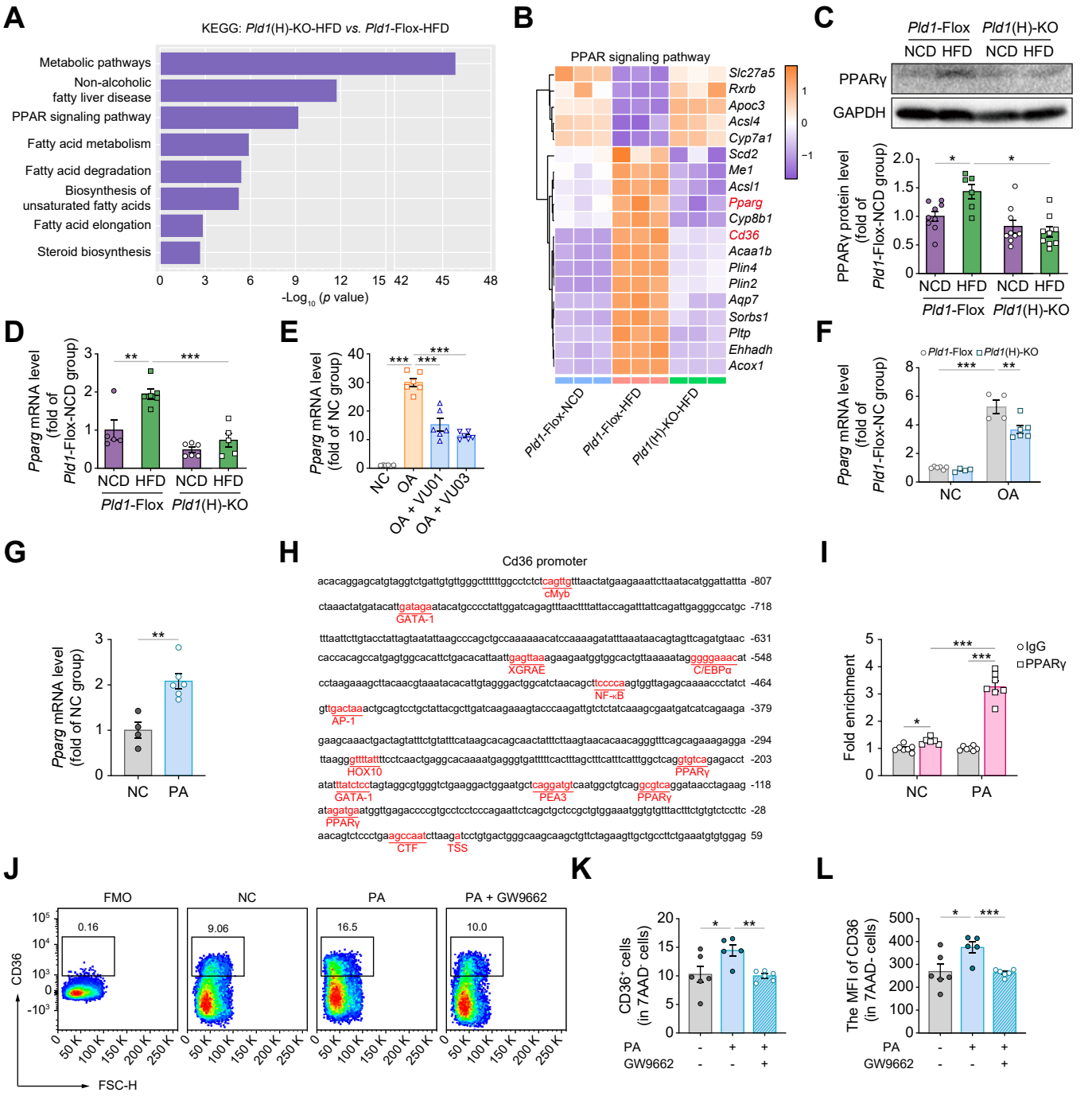

Fig. 6. PA promoted CD36 expression through PPARγ. (A) KEGG pathway enriched in differentially expressed genes in *Pld1*(H)-KO-HFD mice compared with that in *Pld1*-Flox-HFD mice. (B) Heatmap of genes related to PPAR signalling pathway according to their expression levels. (C) Representative Western blot images showing PPARγ and GAPDH levels, and quantification of PPARγ by densitometry, and (D) gene expression levels of *Pparg* in *Pld1*-Flox and *Pld1*(H)-KO mice after 20 wk of NCD or HFD feeding. (E) Gene expression levels of *Pparg* in OA-stimulated AML12 cells with or without PLD1 inhibitor treatment (VU01 or VU03). (F) Gene expression levels of *Pparg* in OA-treated primary hepatocytes from *Pld1*-Flox and *Pld1*(H)-KO mice. (G) Gene expression levels of *Pparg* in PA-treated AML12 cells. (H) Transcription factor binding site in *Cd36* promoter region. (I) Binding of PPARγ on the Cd36 promoter region was detected by CUT&Tag-qPCR experiment in AML12 cells with or without PA stimulation. (J) Representative images of flow cytometry data showing the percentages of $CD36^+$ cells to $7AAD^-$ cells, (K) the ratio of $CD36^+$ cells in $7AAD^-$ cells, and (L) MFI of $CD36^+$ cells to $7AAD^-$ cells in PA treated AML12 cells with or without GW9662 (PPARγ antagonist). n = 8–10 mice per group; n = 3–6 dishes of cells per group. Differences between two groups were compared using Student's *t* test for normal distribution and the Mann–Whitney test for abnormal distribution. One-way ANOVA with Tukey's test for normal distribution and the Kruskal–Wallis test for abnormal distribution were used for analysing differences among three or more groups. $^*p$ <0.05, $^{**}p$ <0.01, $^{***}p$ <0.001. AML12, alpha mouse liver 12; CUT&Tag, Cleavage Under Targets and Tagmentation; FMO, fluorescence minus one; FSC-H, forward scatter-height; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HFD, high-fat diet; KEGG, Kyoto Encyclopedia of Genes and Genomes; MFI, mean fluorescence intensity; NC, normal control; NCD, normal control diet; OA, oleic acid; PA, phosphatidic acid; PLD1, phospholipase D1; PPAR, peroxisome proliferative activating receptor; PPARγ, peroxisome proliferative activating receptor gamma; VU01, VU0155069; VU03, VU0359595.

predicted that there were *Pparg* transcription factor-binding sites in the promoter region of *Cd36* (Fig. 6H). Cleavage Under Targets and Tagmentation (CUT&Tag)-PCR showed the presence of CD36 promoter region fragments in the DNA segment to which PPARγ protein binds in the AML12 cells. PA significantly increased the binding of PPARγ to the CD36 promoter region compared with the normal control group (Fig. 6I). The upregulation of CD36 expression caused by PA was blocked by the PPARγ antagonist GW9662 (Fig. 6J–L).

Discussion

PLD hydrolyses phospholipids into fatty acids and lipophilic substances. Phospholipases can be divided into four categories according to their catalytic activity: A, B, C, and D. There are two main subtypes of PLD in mammals: PLD1 and PLD2.[17] PLD2 is mainly associated with viral infection and Alzheimer's disease,[8] whereas PLD1 regulates LD formation, and the enzyme activity of PLD1 is very important for the formation of LDs.[10,11]

PLD1 activity and the localised production of PA are required for LD growth and accumulation.[9] In NIH 3T3 cells, PLD1 is present in LDs and promotes their formation.[10,12] In metastatic cancer cells, PLD1 promotes LD accumulation.[18] Furthermore, PA (a PLD1 product) is converted to DAG, which is further metabolised to either TG or PC, followed by enhanced lipid accumulation in mouse embryonic fibroblasts.[19] In addition, the activation of PLD1 and formation of PA are critical for the assembly and output of very low density lipoproteins in the rat liver cell line McA-RH7777.[20,21] These results suggest that PLD1 is an important regulator of lipid metabolism in various cells.

In our study, hepatocyte *Pld1*-specific knockout significantly decreased lipid accumulation in hepatocytes via downregulating CD36 expression. However, another study showed that *Pld1* systemic knockout induced NAFLD by decreasing LD decomposition owing to decreased autophagy.[15] It was reported that PLD1 also expressed in HSCs. PLD1 could decrease type I collagen levels in HSCs via induction of autophagy.[22] Thus, systemic knockout of PLD1 might increase HSC activation and promote NAFLD development and liver fibrosis, which may partially explain the discrepancy between *Pld1* systemic and hepatocyte-specific knockout mice. Further studies are needed to clarify the role of PLD1 in different liver cells during NAFLD development.

Overexpression of fatty acid uptake systems, such as CD36 scavenger receptors, is an important cause of intracellular lipid accumulation in non-adipose tissues.[23] CD36 localisation is reportedly increased in the plasma membrane of hepatocytes in patients with non-alcoholic steatohepatitis.[24] CD36-mediated hepatic FFA uptake and chemokine ligand 2-induced inflammation jointly drive the progression of NAFLD to non-alcoholic steatohepatitis.[25] The rate of fatty acid uptake is controlled by CD36 on the cell surface, which is mainly regulated by subcellular vesicle circulation from the endosome to the plasma membrane.[26] In our study, CD36 was upregulated in *Pld1*-Flox-HFD mice and downregulated in *Pld1*(H)-KO-HFD mice, consistent with changes in the lipid accumulation phenotype and NAFLD symptoms. In AML12 cells, the expression levels of CD36 increased after OA and SP treatment and then decreased by PLD1 inhibition. These results suggest that PLD1 may increase hepatocyte lipid accumulation by promoting the CD36 expression.

Lipotoxicity is defined as a condition in which accumulation of harmful lipids can lead to organelle dysfunction, and cell injury and death, which are strongly associated with the progression from NAFLD to non-alcoholic steatohepatitis.[27] Several lines of evidence indicated that alleviating lipotoxicity was an effective strategy for preventing or improving NAFLD.[28] When in excess, DAG, lysophosphatidylcholine, sphingolipids, ceramides, FFA, and free cholesterol behave as lipotoxic lipid species.[29,30] These toxic lipids may cause cellular damage through different mechanisms including the modification of intracellular organelle function, such as the endoplasmic reticulum and mitochondria, as well as the direct activation of death receptor signalling pathways.[29] In the present study, hepatocyte-specific *Pld1* deficiency significantly decreased hepatic DAG, FFA, and cholesteryl ester, which may reduce the hepatotoxicity-induced liver inflammation and fibrosis in HFD-fed mice.

Pld1-specific deletion in hepatocytes could lower mice liver and body weight but did not affect mice food intake. PLD1, as a member of the phospholipase family, plays important roles in regulating hepatocyte lipid metabolism. Hepatocyte lipid metabolism was closely related with plasma lipid level and body weight. For example, sterol regulatory element binding protein (SREBP) cleavage-activating protein (Scap)/SREBP pathway is essential for the synthesis of fatty acids, TGs, and cholesterol in all organs. Hepatocyte-specific deletion of *Scap* in ob/ob mice could block hepatocyte fatty acid synthesis, prevent hepatic steatosis, and decrease liver weight and body weight.[31] Carbohydrate responsive element-binding protein (ChREBP) also regulates lipogenesis through transcriptional regulation of lipogenic genes, which significantly increase in ob/ob mice liver. Liver-specific inhibition of ChREBP improves hepatic steatosis by specifically decreasing lipogenic rates and also downregulates liver and bodyweight.[32] Therefore, we speculate that hepatic specific *Pld1* knockout could firstly downregulate hepatocyte lipid metabolism, then alleviate hepatocyte lipid accumulation and hepatic steatosis, and ultimately lead to liver and body weight loss.

In this study, we also found that hepatocyte-specific *Pld1* knockout in HFD-fed mice could not only decrease lipid accumulation in hepatocytes but also decrease plasma lipids and body weight. We speculate that *Pld1* knockout in hepatocytes decreases CD36 expression, lowers lipid accumulation in hepatocytes, reduces lipotoxicity, enhances hepatocyte viability and lipid metabolism functions, and finally reduces plasma lipid levels. Meanwhile, lipid mass spectrometry analysis also showed that hepatocyte-specific *Pld1* deficiency significantly decreased hepatic DAG, FFA, and cholesteryl ester, which may also reduce the hepatotoxicity-induced hepatocyte dysfunction and NAFLD in HFD-fed mice.

PPARγ plays a key role in adipogenesis and is important in a variety of cellular processes including cell cycle regulation, cell differentiation, and insulin sensitivity.[33] Furthermore, PPARγ targets adiponectin, a spacer between adipocytes, and maintains its metabolic activity and insulin sensitivity.[34] In addition, PPARγ can increase lipid uptake by affecting CD36. For instance, osteoprotegerin promotes CD36 expression by acting on the PPAR response element (PPRE) on the CD36 promoter to aggravate liver lipid accumulation in NAFLD.[35] Consistent with this previous study's finding, we found that PPARγ upregulated the expression of CD36 and led to lipid deposition.

The breakdown of PC by PLD produces PA, an important lipid-derived second messenger that is involved in vesicle transport and fusion.[9] On the one hand, the negatively charged state of PA and the local accumulation of its negative head group promote the formation of curved membranes in the lipid bilayer, which is

conducive for the formation of vesicles.[36] During nutritional deprivation, PA recruits perilipin 3 to amplify LDs.[13] It is also worth mentioning that PA can be converted to DAG, which is further metabolised to TAG, followed by enhanced lipid accumulation in adipose tissue.[19] On the other hand, LPA (a downstream product of PA) stimulates the expression of the PPRE reporter factor and endogenous PPARγ controlling gene *Cd36*, and induces monocytes lipid accumulation from oxidised LDL.[37] Similarly, in the present study, we found that PA promoted the expression of PPARγ and CD36. Therefore, the mechanism by which PA promotes PPARγ and CD36 expression requires further investigation.

In summary, we found that the expression of PLD1 was significantly increased in the steatosis livers of mice and humans with NAFLD. Hepatocyte-specific depletion of *Pld1* significantly reduced lipid accumulation in hepatocytes, both *in vivo* and *in vitro*. *Pld1*(H)-KO mice exhibited significantly lesser weight gain, lower fasting plasma lipid and GLU levels, and improved GLU tolerance, insulin sensitivity, and NAFLD. PA may be the key factor in activating the PPARγ/CD36 pathway during hepatocyte lipid accumulation.

In conclusion, the inhibition of hepatocyte PLD1 exerted potent protective effects against HFD-induced hepatic steatosis. The improved outcomes observed were attributable to a reduction in PPARγ/CD36 pathway-mediated lipid accumulation in hepatocytes. Therefore, PLD1 inhibition may be a new target for the treatment of hepatic steatosis.

Abbreviations

ALT, alanine aminotransferase; AML12, alpha mouse liver 12; AST, aspartate transaminase; ChREBP, carbohydrate responsive element-binding protein; CUT&Tag, Cleavage Under Targets and Tagmentation; DAG, diacylglycerol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GO, gene ontology; FFA, free fatty acid; FMO, fluorescence minus one; FSC-H, forward scatter-height; GLU, glucose; HFD, high fat diet; HSC, hepatic stellate cell; KEGG, Kyoto Encyclopedia of Genes and Genomes; LD, lipid droplet; LDL-C, LDL-cholesterol; LPA, lysophosphatidic acid; MFI, mean fluorescence intensity; NAFLD, non-alcoholic fatty liver diseases; NAS, NAFLD activity score; NCD, normal control diet; OA, oleic acid; OGTT, oral glucose tolerance test; PA, phosphatidic acid; PC, phosphatidylcholine; PLD, phospholipase D; PLD1, phospholipase D1; *Pld1*(H)–KO, hepatocyte-specific *Pld1* knockout; *Pld1*-Flox, $Pld1^{flox/flox}$; PLD2, phospholipase D2; PPAR, peroxisome proliferative activating receptor; PPARγ, peroxisome proliferative activating receptor gamma; PPRE, PPAR response element; SP, sodium palmitate; Scap, SREBP cleavage-activating protein; SREBP, sterol regulatory element binding protein; TAG, triacylglycerol; TC, total cholesterol; TG, triglyceride; VU01, VU0155069; VU03, VU0359595.

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Conflicts of interest

All authors declare no conflict of interest.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Participated in performing the research: HW, YZ, YP, AY, CL, SW, ZD, ML. Participated in analysing the data: HW, YZ, DZ, GS. Participated in initiating the original draft of the article: HW, YZ. Established the hypotheses: DZ, GS. Supervised the studies: DZ, GS.
Co-wrote the manuscript: DZ, GS. Participated in establishing the hypotheses: YZ.
Provided the conditional knockout mice: YZ. Participated in the review and editing of the manuscript: SW, ZZ. Participated meaningfully in the study and have seen and approved the submission of this manuscript: all authors.

Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article and/or its Supplementary Materials and methods. Any additional data are available from the corresponding authors upon reasonable request. The data reported in this work have been uploaded in the Gene Expression Omnibus (GEO) database under accession number GSE207281. The following secure token has been created to allow review of record GSE207281 while it remains in private status: qjofseiahjqztmh.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhepr.2023.100725.

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Author names in bold designate shared co-first authorship.

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