Original article

MOLECULAR METABOLISM

A novel lncRNA *GM47544* modulates triglyceride metabolism by inducing ubiquitination-dependent protein degradation of APOC3

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ABSTRACT

Objective: Emerging evidence highlights the pivotal roles of long non-coding RNAs (lncRNAs) in lipid metabolism. Apoprotein C3 (ApoC3) is a well-established therapeutic target for hypertriglyceridemia and exhibits a strong association with cardiovascular disease. However, the exact mechanisms via which the lncRNAs control ApoC3 expression remain unclear.

Methods: We identified a novel long noncoding RNA (lncRNA), *GM47544*, within the ApoA1/C3/A4/A5 gene cluster. Subsequently, the effect of *GM47544* on intracellular triglyceride metabolism was analyzed. The diet-induced mouse models of hyperlipidemia and atherosclerosis were established to explore the effect of *GM47544* on dyslipidemia and plaque formation *in vivo*. The molecular mechanism was explored through RNA sequencing, immunoprecipitation, RNA pull-down assay, and RNA immunoprecipitation.

Results: *GM47544* was overexpressed under high-fat stimulation. *GM47544* effectively improved hepatic steatosis, reduced blood lipid levels, and alleviated atherosclerosis *in vitro* and *in vivo*. Mechanistically, *GM47544* directly bound to ApoC3 and facilitated the ubiquitination at lysine 79 in ApoC3, thereby facilitating ApoC3 degradation via the ubiquitin-proteasome pathway. Moreover, we identified *AP006216.5* as the human *GM47544* transcript, which fulfills a comparable function in human hepatocytes.

Conclusions: The identification of *GM47544* as a lncRNA modulator of ApoC3 reveals a novel mechanism of post-translational modification, with significant clinical implications for the treatment of hypertriglyceridemia and atherosclerosis.

Keywords Atherosclerotic cardiovascular disease; Hypertriglyceridemia; Ubiquitination; APOC3; Long noncoding RNA; *GM47544*

1. INTRODUCTION

Hypertriglyceridemia (HTG) is frequently observed in clinical settings as the predominant type of dyslipidemia and is linked to a variety of serious disorders, including atherosclerotic cardiovascular disease (ASCVD), nonalcoholic fatty liver disease (NAFLD), acute pancreatitis, as well as lipotoxic cardiomyopathy [1–4]. Dietary triglycerides (exogenous) are transported in the bloodstream as chylomicrons, whereas triglycerides produced by the liver (endogenous) circulate as particles with very low-density lipoprotein (VLDL) [5]. Lipoprotein lipase (LPL) activity serves as the primary mechanism for the hydrolysis of plasma triglycerides, facilitating the effective elimination of triglyceride-rich lipoproteins (TRLs) and remnants [6,7]. TRLs and remnants have been identified as significant contributors to the residual cardiovascular risk in patients receiving optimal LDL-lowering medication [8,9]. Despite the effectiveness of LDL-lowering drugs in decreasing the occurrence of cardiovascular disease, the burden of cardiovascular disease remains high owing to persistent dyslipidemia characterized by hypertriglyceridemia and low HDL levels [10,11]. Recently, APOC3 has become the most promising target for triglyceride-lowering drugs [12]. Significant advancements have been made using antibody, antisense, and RNAi methods [13,14]. Specifically, a monoclonal antibody was found to effectively eliminate lipoprotein-bound human APOC3 in mice expressing this protein, thereby enhancing the breakdown of triglyceride-rich lipoproteins *in vivo* [15]. AKCEA-APOCIII-LRx (olezarsen) is a liver-targeted antisense oligonucleotide conjugated with N-acetyl galactosamine, designed to specifically suppress the synthesis of APOC3 protein. It has

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been found to significantly reduce APOC3, triglycerides, and atherogenic lipoproteins in patients [16,17]. Furthermore, ISIS 304801, a novel antisense inhibitor targeting APOC3 synthesis, has demonstrated dose-dependent and sustained reductions of up to 40.0%～79.6% in triglyceride levels and parallel reduction in plasma APOC3 levels [18]. Given that targeting hepatic APOC3 protein and mRNA has been proven efficacious and safe in decreasing triglyceride (TG) levels, APOC3 is an attractive target for modulating lipid homeostasis [19]. However, the post-translational modification (PTM) patterns of APOC3 protein remain poorly understood.

Long noncoding RNA (lncRNA) transcript length exceeds 200 nt, most of which have no protein-coding potential [20]. LncRNAs facilitate DNA-protein interactions, sequester microRNAs and act as decoys for proteins. This allows them to modify mRNA and proteins to control the processes of methylation, phosphorylation, and ubiquitination, which affect a protein's stability [21–23]. Many functional lncRNAs driven by genome-wide association study (GWAS) loci participate in the regulation of epigenetic signatures and gene expression and, thus, susceptibility to human diseases [24,25]. GWAS has identified the APOA1/C3/A4/A5 cluster in the 11q23.3 region as one of the most powerful gene loci for regulating plasma triglyceride levels [26,27]. Genetic variants within the APOA1/C3/A4/A5 cluster exert a significant influence on plasma triglyceride levels and contribute to the risk and severity of coronary artery disease (CAD) [28]. However, the function and working mode of most lncRNAs within the APOA1/C3/A4/A5 cluster remain to be elucidated.

Here, we identified a novel lncRNA named *GM47544*, which was highly expressed in the livers of high-fat-fed mice. Subsequently, we investigated the potential involvement of *GM47544* in TG metabolism and its regulatory role in modulating the expression levels of the ApoA1/C3/A4/A5 cluster.

2. METHODS & PROTOCOLS

2.1. Gain- and loss-of-function studies

To generate *GM47544*-overexpressed AML-12 cells, *GM47544* cDNAs were obtained from Augct Biotech and inserted into the pLKO.1-TRC Cloning Vector (Addgene) and then transfected into HEK293T cells along with packaging vectors psPAX2 and pMD2.G (Addgene). We treated AML-12 cells with the collected culture media after 48 h. Subsequently, puromycin selection (5 μg/mL, Thermo Fisher Scientific) was employed to isolate the desired cell population. After confirming the overexpression efficiency by RT-qPCR, the cells were used for subsequent experiments. To knockdown *GM47544* in AML-12 cells, an Antisense oligonucleotide against *GM47544* gene (ASO-*GM47544*, RiboBio) was transfected using Lipofectamine 3000 (Thermo Fisher Scientific), and the lncRNA ASO Negative Control was used. The sequence of ASO-*GM47544* was 5′-CTTCCAACAAAAGCTCTCAT-3′.

2.2. Cell culture

HEK293T, AML-12, and THLE-3 cells were obtained from the Shanghai Life Academy of Sciences Cell Library. Primary mouse hepatocytes were isolated in accordance with previously described methods [29,30]. The cells were cultured at 37 °C in a 5% CO_2 atmosphere, using DMEM (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific) plus antibiotics.

2.3. Mice and diets

Male C57BL/6 mice, aged 8 weeks, were obtained from Beijing Vital River Laboratory and randomly divided into groups. Mice were fed a chow diet (CD, Research Diets, D10001) or a high-fat diet (HFD, D12492, research diets, Jiangsu Xietong Pharmaceutical Bio-engineering) for 16 weeks and then euthanized to examine the expression levels of lncRNAs within the ApoA1/C3/A4/A5 gene cluster in the liver.

Male C57BL/6 mice aged 8 weeks were purchased from the same laboratory and randomly grouped. Mice were fed an HFD for 3 weeks before receiving treatment with AAV8-*GM47544* (AAV-*GM47544*) or AAV8-GFP (AAV-Ctrl) recombinant adeno-associated virus (AAVs). Mice were kept on an HFD for another 16 weeks before being euthanized to analyze lipids in their serum and liver.

Apoe$^{-/-}$ mice were purchased from the Beijing Vital River Laboratory at the age of 8 weeks and randomly grouped. Mice were treated with either AAV8-*GM47544* (AAV-*GM47544*) or AAV8-GFP (AAV-Ctrl) recombinant AAVs and fed a high-cholesterol diet (HCD, MD12015, Research Diets, Biopike) for 16 weeks. The mice were euthanized, with lipids in their serum, liver, and atherosclerotic lesions.

AAVs can efficiently transduce host cells, allowing stable and sustained expression of exogenous genes over extended durations, with remarkable safety profiles and minimal immunogenicity. All the AAVs were obtained from Weizhen Biosciences (China). The mice were housed in SPF rooms with a 12 h light/12 h dark cycle. They were given unrestricted access to food and water. All experimental procedures adhered to the guidelines set by the National Institutes of Health (NIH) and received approval from the Animal Care and Use Committee of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China, IACUC Number: 3281).

2.4. Quantification of atherosclerotic lesions in the aorta

The control and *GM47544*-overexpressed *Apoe*$^{-/-}$ mice were euthanized following 16 weeks of high cholesterol diet (HCD) feeding. The entire aorta was isolated and incubated in 4% paraformaldehyde for 24 h. The aorta was opened longitudinally from its origin at the aortic root to its division at the iliac bifurcation after carefully removing the surrounding adipose tissue using a dissection microscope. The aorta was then treated with 60% isopropanol for 10 min and stained with Oil Red O solution for 15 min. To eliminate background staining, destaining was performed using 60% isopropanol for an additional 5 min. The complete length of the aortic tree was then secured onto a black rubber plate and images were captured accordingly. All heart samples were rapidly frozen with liquid nitrogen and maintained at −80 °C for further study. Frozen sections obtained from the region around the aortic root were prepared and subsequently subjected to Oil Red O staining. ImageJ software was used to measure the area of Oil Red O-stained atherosclerotic lesions throughout the length of the aorta and aortic root.

2.5. Statistical analysis

Statistical analyses were performed using GraphPad Prism 8.0, and data were expressed as the mean ± standard deviation. A two-tailed Student's *t*-test was used for comparison between the two groups. Two-way analysis of variance (ANOVA) followed by Tukey's post hoc test and two-way ANOVA with Sidak's multiple comparison test were used to compare multiple groups. Differences were considered statistically significant at $P < 0.05$.

3. RESULTS

3.1. Identification of *GM47544* as a hepatic regulator of TG metabolism

Given that the ApoA1/C3/A4/A5 gene cluster serves as critical component in regulatinglipoprotein [28], we hypothesis that lncRNAs

derived from this gene cluster may participate in lipoprotein metabolism by modulating those genes expression. To explore the potential functionality of lncRNAs within the ApoA1/C3/A4/A5 cluster, we queried the Ensembl Genome Browser (www.ensembl.org/) database and obtained the gene information of all 12 annotated lncRNAs (Figure 1A,B). Liver tissues obtained from mice exposed to a high-fat diet (HFD) were subjected to Quantitative Real-time PCR (RT-qPCR) analysis, and tissues from chow-diet (CD)-fed mice were used for comparison. Among these (12 lncRNAs), the expression level of *GM47544*, a previously uncharacterized lncRNA, has increased significantly, which attracted our attention (Figure 1A). *GM47544* is derived from the natural antisense transcript of ApoA5 located on chromosome 9 of the mouse genome (Figure 1B). Moreover, higher *GM47544* expression was observed in the livers of db/db mice and in palmitic acid (PA)-treated AML-12 liver cells (Figure 1C,D). The findings indicated that *GM47544* potentially serves as a regulator of lipid metabolism.

We subsequently analyzed the protein-coding potential of *GM47544* using ORF Finder, which indicated the presence of two open reading frames (ORFs) (Supplementary Fig. 1A). However, both were confirmed to have no protein-coding ability, suggesting that *GM47544* is a noncoding transcript (Supplementary Figs. 1B—D). To determine the distribution of *GM47544* in tissues, we performed RT-qPCR, which showed that *GM47544* was widely distributed in multiple organs and tissues and extremely abundant in the liver (Figure 1E). Using fluorescence in situ hybridization (FISH) assays, we found that *GM47544* was mainly located in the cytoplasm (Figure 1F), which was further confirmed by RT-qPCR analysis of nuclear and cytosolic extracts (Figure 1G).

To determine *GM47544*'s function, we first generated AML-12 cell lines stably overexpressing *GM47544* (OE-*GM47544*) and negative control (OE-Ctrl) using lentiviral vectors (Figure 1H). RNA-seq analysis revealed that *GM47544* is implicated in adipogenesis, cholesterol homeostasis, and fatty acid metabolism (Figure 1I and Supplementary Fig. 1E). Subsequently, oil red staining and intracellular lipid content detection were employed to compare OE-*GM47544* and OE-Ctrl cells. The results indicated that overexpression of *GM47544* significantly downregulates intracellular TG content and reduces the number of lipid droplets within the cells (Figure 1J,K). Furthermore, we also observed that in cells overexpressing *GM47544*, the ability of low-density lipoprotein receptor (LDLR) to uptake LDL is enhanced, while the activity of intracellular hepatic lipase (HL) is also markedly increased, which demonstrated the lipid-lowering function of *GM47544* in facilitating lipid uptake and clearance (Figure 1L,M). By contrast, *GM47544* knockdown by ASO in AML-12 cells did not alter the levels of intracellular triglyceride or cholesterol (Supplementary Figs. 1F—H). These findings suggest that the overexpression of *GM47544* confers resistance to fatty acid-induced steatosis, highlighting its potential significance in hepatic triglyceride metabolism.

3.2. *GM47544* overexpression profoundly prevented diet-induced hypertriglyceridemia and hepatic steatosis

To better understand the contribution of *GM47544* to lipid metabolism *in vivo*, we generated adenovirus (Ad-*GM47544*) and adeno-associated virus 8 (AAV-*GM47544*) carrying *GM47544*, which were intravenously injected into C57BL/6 mice to induce *GM47544* overexpression in the liver. Initially, we conducted a lipid tolerance test in mice treated with Ad-*GM47544* by administering olive oil orally and monitoring plasma TG levels for a duration of 4 h. Ad-*GM47544* mice exhibited a notable reduction in the fluctuation of plasma TG levels (Figure 2A and Supplementary Fig. 2A).

Next, we sought to determine whether *GM47544* regulated plasma lipid levels and liver lipid deposition in long-term HFD-fed mice. Compared with the control group, *GM47544* overexpressed mice showed lower plasma TG and total cholesterol (TC) contents from the 2 nd week of AAV-*GM47544* injection in the HFD-fed group, and these effects lasted until 16 weeks of HFD feeding (Figure 2B,C). The fast protein liquid chromatography (FPLC) analysis of plasma revealed a significant reduction in circulating cholesterol and triglycerides specifically within the very-low-density lipoprotein fraction of *GM47544* overexpressed mice (Figure 2D). Interestingly, the *GM47544* overexpression group showed significant improvements in fatty liver parameters, such as body weight and liver weight (Figure 2E and Supplementary Fig. 2B). Lower levels of triglycerides were detected in the livers of *GM47544* overexpression group, whereas the levels of TC in the liver were similar in both groups (Figure 2F). Moreover, improvement in hepatic steatosis was confirmed by histological analysis of liver size and Oil Red O staining (Figure 2G). Hence, *GM47544* is a potential target for the advancement of innovative therapeutic strategies for the management of hyperlipidemia.

3.3. *GM47544* attenuated the development of atherogenesis

To investigate whether the overexpression of *GM47544* can attenuate the progression of atherosclerosis, *Apoe*$^{-/-}$ mice were treated with AAV8-*GM47544* (AAV-*GM47544*) or AAV8-Control (AAV-Ctrl) after high-cholesterol diet-feeding (HCD) for 16 weeks, followed by the assessment of plasma lipids and atherosclerotic lesions in the aorta. Compared to AAV-Ctrl *Apoe*$^{-/-}$ mice, AAV-*GM47544* *Apoe*$^{-/-}$ mice displayed lower serum cholesterol, free plasma cholesterol, and triglyceride levels (Figure 3A,B). FPLC analysis demonstrated that *GM47544* exhibited the potential to effectively decrease the plasma levels of total cholesterol and triglycerides, particularly in very low-density lipoprotein fractions (Figure 3C). Consistent with the observed decrease in circulating cholesterol levels, AAV-*GM47544* *Apoe*$^{-/-}$ mice demonstrated a 50% decrease in atherosclerotic plaques throughout the entire aorta and a 40% decrease in lesion areas within the aortic root (Figure 3D,E), indicating their enhanced resistance to atherosclerosis progression. In addition, *GM47544* relieved hepatic steatosis as evidenced by the reduced number of lipid droplets (LDs) and the decreased hepatic TG and TC content (Figure 3F,G). Taken together, these results indicate that *GM47544* overexpression improves liver steatosis and atherosclerotic plaque formation by lowering plasma lipid levels.

3.4. *GM47544* enhances plasma TG clearance by suppressing ApoC3 expression

Because lncRNAs can regulate the expression of adjacent genes, we hypothesized that *GM47544* may affect the expression of the ApoA1/C3/A4/A5 cluster and consequently regulate lipid metabolism. Intriguingly, we found that overexpression of *GM47544* markedly abolished ApoC3 protein levels in AML-12 cells, whereas the mRNA levels of the ApoA1/C3/A4/A5 cluster were barely changed in the *GM47544* overexpressed cells, confirming that *GM47544* regulates ApoC3 expression at the translational level (Figure 4A,B). The regulating effect of *GM47544* on APOC3 is concentration-dependent, with high expression levels of *GM47544* effectively downregulating APOC3 protein levels (Supplementary Figs. 3A and B). Furthermore, a significant reduction in ApoC3 protein levels was also detected in *GM47544* overexpressed primary hepatocytes, as well as in the livers of AAV-*GM47544* C57BL/6 and AAV-*GM47544* *Apoe*$^{-/-}$ mice fed with a high-fat diet, without changes at the transcriptional level (Figure 4C—H). By contrast, *GM47544* knockdown by ASO in AML-12 cells did not exert any significant impact on the ApoA1/C3/A4/A5 cluster (Supplementary

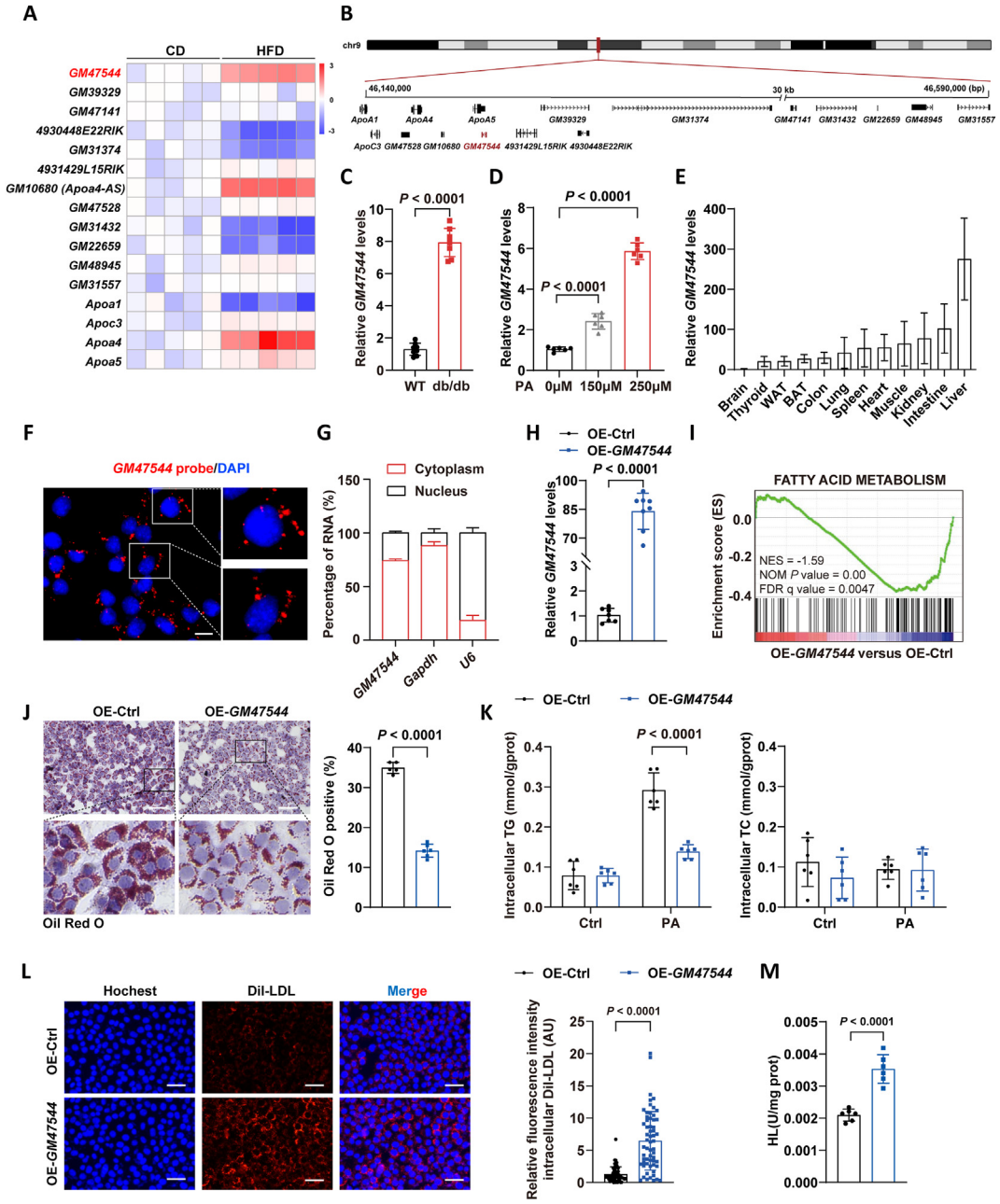

Figure 1: Identification of *GM47544* as a hepatic regulator of lipid metabolism.

(A) Representative heatmap showing the response of 12 differentially expressed lncRNAs in mice liver to HFD-feeding. Four highly expressed mRNAs (fold change $>$ 2; FDR-adjusted $P < 0.05$) located in the ApoA1/C3/A4/A5 gene cluster in the livers of HFD mice. The heatmap was drawn based on normalized expression levels (n $=$ 5).

(B) Schematic representation of the mouse ApoA1/C3/A4/A5 gene cluster locus.

(C) Relative RNA expression of *GM47544* in the livers of wide type (WT) or *db/db* male mice (n $=$ 8).

(D) Relative RNA expression of *GM47544* in AML-12 cells with the treatment of PA (250 μM) for 24 h.

(E) Relative RNA expression levels of *GM47544* in multiple mouse tissues (n $=$ 6).

(F) RNA FISH analysis of *GM47544* in AML-12 cells revealed that the majority of *GM47544* is located in the cytoplasm. Red: *GM47544*; blue: DAPI staining. Scale bar, 0.5 μm.

(G) Detection of *GM47544* in the cytoplasm and nucleus of AML-12 cells by qPCR. As a control, *GAPDH* was used for the cytoplasm and *U6* was used for the nucleolus.

(H) Relative expression levels of *GM47544* in AML-12 cell lines stably overexpressed *GM47544* (OE-*GM47544*) and the negative control (OE-Ctrl).

(I) Gene set enriched expression (GSEA) plots for fatty acid metabolic pathways. GSEA was performed based on average *GM47544* expression (OE-*GM47544* versus OE-Ctrl).

(J) Oil Red O staining and hematoxylin staining of lipid droplets in control and *GM47544*-overexpressed AML-12 cells treated with PA (250 μM, 24 h). Scale bar, 100 μm.

(K) Intracellular TG and TC levels of control and *GM47544*-overexpressed AML-12 cells with or without PA treatment (250 μM, 24 h).

(L) Left: Dil-LDL uptake was quantified under fluorescence microscopy in control and *GM47544*-overexpressed AML-12 cells treated with Dil-LDL (15 μg/mL) for 24 h. Red: Dil-LDL; blue: DAPI staining. Scale bar, 100 μm. Right: quantification of the relative fluorescence intensity of internalized Dil-LDL in control and *GM47544*-overexpressed AML-12 cells.

(M) Cellular HL activity in control and *GM47544*-overexpressed AML-12 cells was detected.

Data are presented as mean ± SD in C, D, H, J, K, L, and M, with statistical significance determined with unpaired two-tailed Student's *t*-test. Data are presented as mean ± SD in E and G.

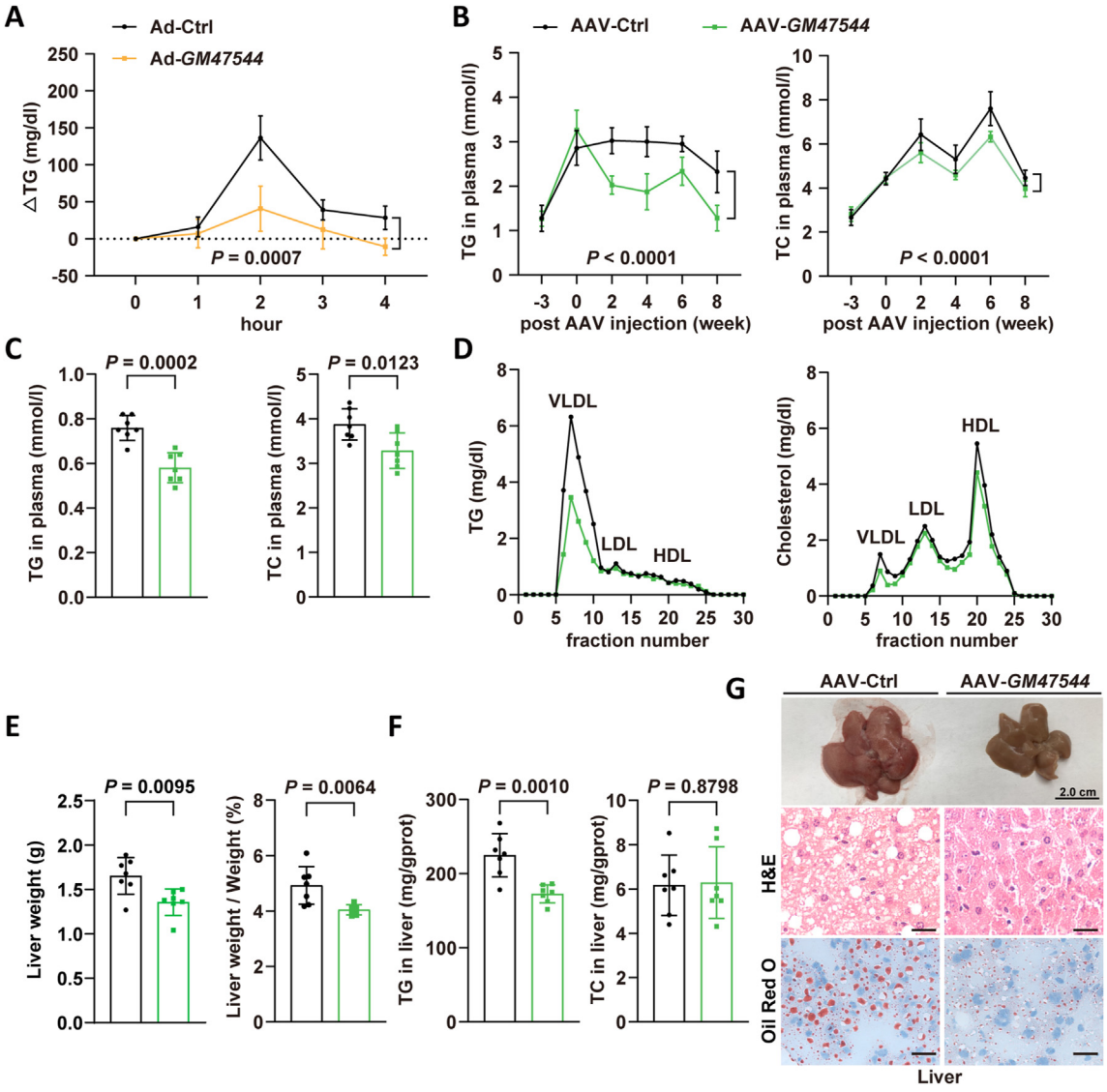

Figure 2: *GM47544* overexpression profoundly prevented diet-induced hypertriglyceridemia and hepatic steatosis.
(A) 8-week WT male mice were injected with adenovirus harbored GFP or *GM47544* (Ad-Ctrl or Ad-*GM47544*) via the tail vein. After 5 days of chow diet feeding, the mice orally received olive oil. Blood samples were collected each hour for 4 h post-oil administration. Plasma TG levels at indicated times in control and *GM47544*-overexpressed mice (n = 6).
(B) 11-week WT male mice were injected with AAV-Ctrl (Control) or AAV-*GM47544* (*GM47544* overexpression) via the tail vein after HFD feeding for 3 weeks. Blood samples were collected every 2 weeks. After continuous HFD feeding for 19 weeks, these mice were sacrificed. Dynamic changes in plasma TG and TC levels of control and *GM47544*-overexpressed mice during the first 11 weeks of HFD feeding (n = 7).
(C) Plasma TG and TC levels of control and *GM47544*-overexpressed mice after 19 weeks HFD (n = 7).
(D) Fast protein liquid chromatography (FPLC) analysis was performed on pooled plasma from control and *GM47544*-overexpressing mice, and triglyceride and cholesterol were determined in each of the eluted fractions.
(E) Liver weight and liver-to-body weight ratio of control and *GM47544*-overexpressed mice.
(F) Hepatic TG and TC levels of control and *GM47544*-overexpressed mice after 19 weeks HFD (n = 7).
(G) Macroscopic appearance of liver, H&E, and Oil Red O staining of liver sections from control and *GM47544*-overexpressed mice. Scale bar, 2 cm (upper), 100 μm (lower).
Data are presented as mean ± SD in C, E, and F, with statistical significance determined with unpaired two-tailed Student's *t*-test. Data are presented as mean ± SD in A and B, with statistical significance determined using a two-way analysis of variance (ANOVA).

Figs. 3C—E). The above results indicated that *GM47544* significantly downregulates the protein expression level of ApoC3 within hepatocytes, both *in vivo* and *in vitro*.

The liver is the primary organ responsible for ApoC3 synthesis, and it also serves as the primary source of circulating ApoC3 [13]. We examined and found that the plasma ApoC3 protein levels were markedly reduced in AAV-*GM47544* C57BL/6 and AAV-*GM47544* *Apoe*$^{-/-}$ mice compared to controls (Figure 4I,K). Meanwhile, we observed that exogenous LPL activity was strongly elevated in the plasma of AAV-*GM47544* C57BL/6 and AAV-*GM47544* *Apoe*$^{-/-}$ mice (Figure 4J,L). This activation of LPL is associated with a pronounced enhancement in systemic triglyceride (TG) clearance, leading to reduced circulating TG levels. Collectively, these findings provide robust support for the role of *GM47544* in reducing ApoC3 protein levels in the liver and plasma, thereby activating LPL, and subsequently enhancing plasma TG clearance.

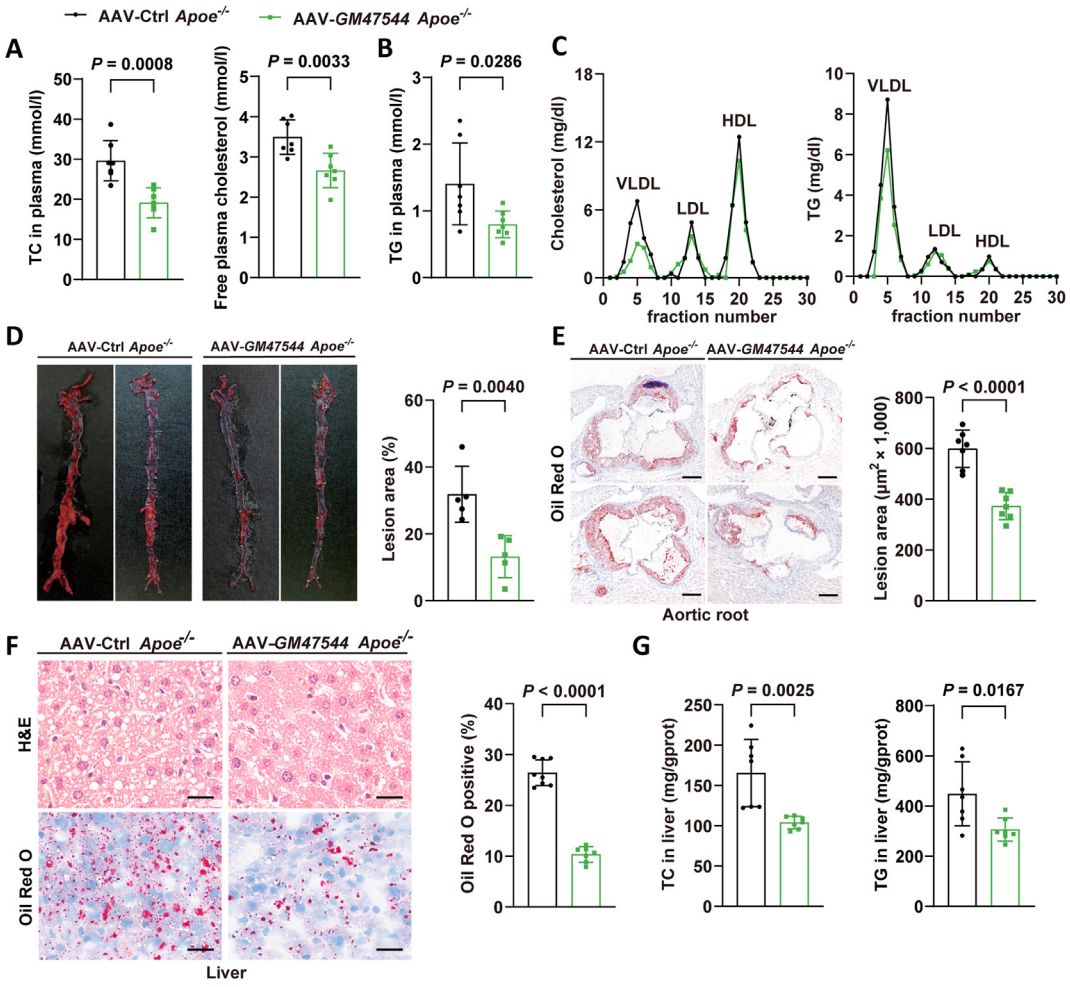

Figure 3: *GM47544* attenuated atherogenesis in *Apoe*$^{-/-}$ mice.

(A) 8-week *Apoe*$^{-/-}$ mice were injected with AAV8-Ctrl (Control) or AAV8-*GM47544* (*GM47544* overexpression) via the tail vein. After 16 weeks of HCD feeding, the mice were sacrificed. Plasma TC and free plasma cholesterol levels of control and *GM47544*-overexpressed *Apoe*$^{-/-}$ mice (n = 7).

(B) Plasma TG levels of control and *GM47544*-overexpressed *Apoe*$^{-/-}$ mice (n = 7).

(C) Fast protein liquid chromatography (FPLC) analysis was performed on pooled plasma from control and *GM47544*-overexpressed *Apoe*$^{-/-}$ mice, and triglyceride and cholesterol were determined in each of the eluted fractions.

(D) Representative images of *en face* aortas and quantification of plaque area in control and *GM47544*-overexpressed *Apoe*$^{-/-}$ mice.

(E) Oil-Red O staining and quantification of plaque in aortic root sections of control and *GM47544*-overexpressed *Apoe*$^{-/-}$ mice (n = 7). Scale bar = 200 μm.

(F) H&E and Oil-Red O staining of the liver in control and *GM47544*-overexpressed *Apoe*$^{-/-}$ mice. Scale bar = 100 μm.

(G) Hepatic TC and TG levels of control and *GM47544*-overexpressed *Apoe*$^{-/-}$ mice after 16 weeks of HCD feeding (n = 7).

Data are presented as mean ± SD in A, B, D, E, F, and G, with statistical significance determined with unpaired two-tailed Student's *t*-test.

3.5. *GM47544* promotes ubiquitin-dependent degradation of hepatic ApoC3

The ubiquitin—proteasome system is the principal proteolytic machinery responsible for regulating protein degradation in eukaryotic cells, which is essential for maintaining intracellular protein homeostasis and function [31]. Thus, we further explored the possibility that *GM47544* regulates ApoC3 protein through a ubiquitination modification pathway. Firstly, we demonstrated that in AML-12 cells overexpressing *GM47544*, the overall ubiquitin levels were markedly higher than those in the control group (Figure 5A). Additionally, we identified that ApoC3 has the potential to bind ubiquitin (Ub) molecules and can be degraded further via the ubiquitin-proteasome pathway (Supplementary Figs. 4A—B). Subsequently, we confirmed the interaction between Ub and ApoC3 by co-immunoprecipitation with or without *GM47544* treatment. As anticipated, overexpression of *GM47544* facilitated the ubiquitination of ApoC3 (Figure 5B). Subsequently, the effect of *GM47544* on ApoC3 stability was evaluated using the protein synthesis inhibitor CHX. After treatment with CHX, a significant decrease in ApoC3 protein synthesis was observed across all groups, indicating that overexpression of *GM47544* did not impact ApoC3 protein synthesis (Figure 5C). In addition, the proteasome inhibitor MG132 reversed the *GM47544* overexpression-induced ApoC3 protein decrease in a concentration-dependent manner, unlike the lysosome inhibitor NH_4Cl, suggesting that *GM47544* regulated ApoC3 stability in a proteasome-mediated manner (Figure 5D—E). These results suggest that *GM47544* facilitates the ubiquitination of ApoC3 after translation and is then degraded through the proteasome pathway.

Next, we explored the types of polyubiquitin chains linked to ApoC3 by *GM47544*. By co-transfecting ApoC3 with ubiquitin mutants in which

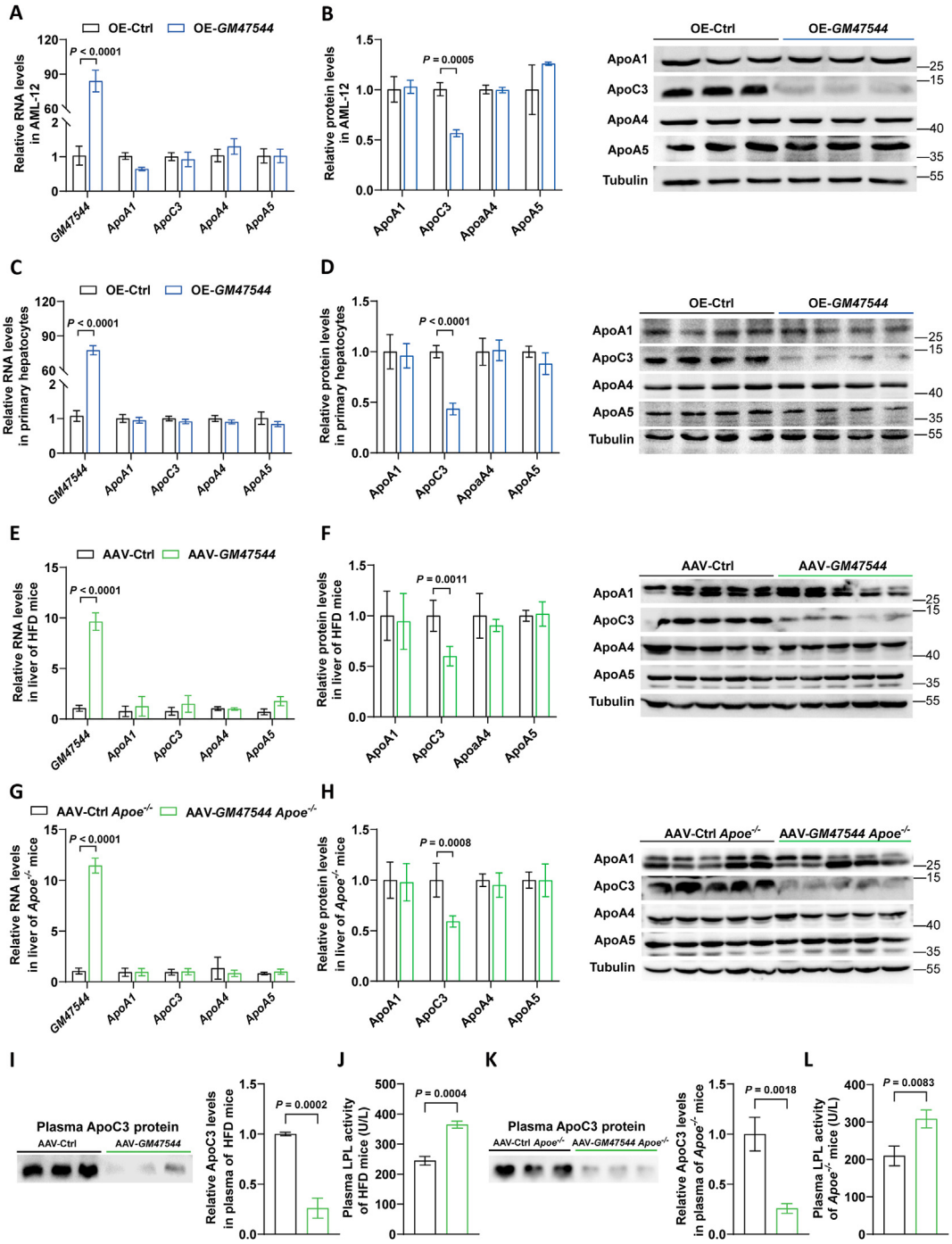

Figure 4: *GM47544* activated HL and LDLR by suppressing ApoC3 to enhance plasma TG clearance.

(A) Relative mRNA expression of *GM47544* and *ApoA1/C3/A4/A5* gene cluster measured by RT-qPCR.
(B) The ApoA1/C3/A4/A5 protein level was measured by western blot in control and *GM47544*-overexpressed AML-12 cells.
(C) Relative mRNA expression of *GM47544* and *ApoA1/C3/A4/A5* gene cluster measured by RT-qPCR.
(D) The ApoA1/C3/A4/A5 protein level was measured by western blot in the primary mouse hepatocytes transfected with empty vector (OE-Ctrl) and *GM47544* overexpression plasmids (OE-*GM47544*).
(E) Relative mRNA expression of *GM47544* and *ApoA1/C3/A4/A5* gene cluster measured by RT-qPCR.
(F) The ApoA1/C3/A4/A5 protein level was measured by western blot in the livers of control and *GM47544*-overexpressed mice.
(G) Relative mRNA expression of *GM47544* and *ApoA1/C3/A4/A5* gene cluster measured by RT-qPCR.
(H) The ApoA1/C3/A4/A5 protein level was measured by western blot in the livers of control and *GM47544*-overexpressed *Apoe*$^{-/-}$ mice.
(I) Plasma ApoC3 protein levels in control and *GM47544*-overexpressed mice.
(J) Plasma LPL activity stimulation in control and *GM47544*-overexpressed mice.
(K) Plasma ApoC3 protein levels in control and *GM47544*-overexpressed *Apoe*$^{-/-}$ mice.
(L) Plasma LPL activity stimulation in control and *GM47544*-overexpressed *Apoe*$^{-/-}$ mice.
Data are presented as mean ± SD in A, B, C, D, E, F, G, H, I, J, K, and L, with statistical significance determined using an unpaired two-tailed Student's *t*-test.

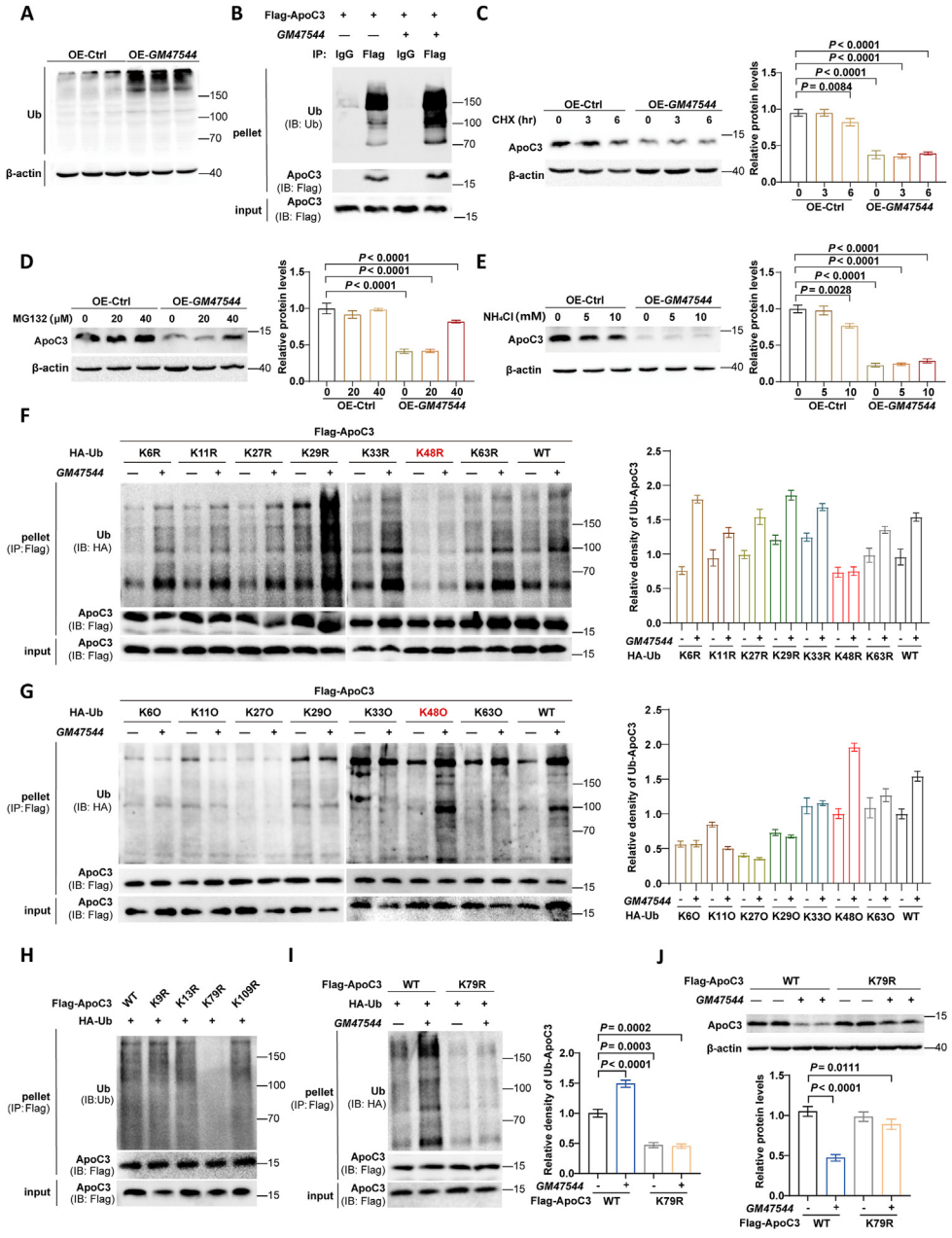

Figure 5: *GM47544* promotes ubiquitin-dependent degradation of hepatic ApoC3.

(A) Overall ubiquitin levels in the control and *GM47544*-overexpressed AML-12 cells.

(B) IP assays in AML-12 cells transfected with vectors expressing FLAG-ApoC3 were carried out using anti-FLAG antibodies, followed by western blotting analysis with antibodies against ubiquitin and ApoC3.

(C) ApoC3 protein levels in the control and *GM47544*-overexpressed AML-12 cells after treatment with cycloheximide (CHX, 20 μM).

(D) The control and *GM47544*-overexpressed AML-12 cells were cultured in the medium containing MG132 with the indicated dose for 6 h. Protein levels of ApoC3 were detected by western blot.

(E) The control and *GM47544*-overexpressed AML-12 cells were cultured in the medium containing NH_4Cl with the indicated dose for 6 h. Protein levels of ApoC3 were detected by western blot.

(F–G) The AML-12 cells, both control and *GM47544*-overexpressed, were transfected with the specified plasmids for 24 h. Afterward, ubiquitination assays were conducted using the indicated antibodies. WT, wild type; KR, K is mutated to R; KO, K only.

(H) AML-12 cells were transfected with the specified plasmids for 24 h before conducting ubiquitination assays using the designated antibodies.

(I) The AML-12 cells, both control and *GM47544*-overexpressed, were transfected with the specified plasmids for 24 h. Afterward, ubiquitination assays were conducted using the indicated antibodies.

(J) ApoC3 protein levels in the control and *GM47544*-overexpressed AML-12 cells transfected with the indicated plasmids for 24 h. IP, immunoprecipitation; IB, immunoblotting; IgG, immunoglobulin G.

Data are presented as mean ± SD in C, D, E, F, G, I and J, with statistical significance determined with unpaired two-tailed Student's *t*-test.

only one lysine residue was mutated to arginine (KR), we observed that *GM47544* failed to conjugate the polyubiquitin chains and ApoC3 exclusively when K48 was mutated to arginine (Figure 5F). By co-transfecting ApoC3 with ubiquitin mutants containing only a single lysine residue (KO), we found that *GM47544* facilitates the interaction between K48-linked polyubiquitin moieties and ApoC3 (Figure 5G). These results suggest that *GM47544* markedly promotes the K48-linked ubiquitination of ApoC3.

We further endeavored to identify the specific residues of ApoC3 that were modified by ubiquitination. To achieve this, we individually substituted all four lysine residues, which were predicted as potential sites for ApoC3 ubiquitination, with arginine and then assessed their modifications through ubiquitination assays. As shown in Figure 5H, there was a significant reduction in the ubiquitination of the ApoC3^{K79R} mutant compared to the other four mutants. Further investigations revealed that *GM47544* facilitated the ubiquitination of wild-type ApoC3 but failed to enhance the basal level of ubiquitination of the ApoC3^{K79R} mutant (Figure 5I). Consistently, *GM47544* downregulated the level of wild-type ApoC3, but not ApoC3^{K79R} (Figure 5J). Collectively, ApoC3^{K79R} is the critical ubiquitination site for ApoC3.

3.6. *GM47544* stem-loop 2 directly interacted with ApoC3 and promoted the degradation of ApoC3

To further probe the functional domain of *GM47544*, we constructed three truncated *GM47544* vectors and performed RNA pull-down experiments based on the distribution principle of stem-loop (SL) structure (Figure 6A). Only the full-length SL2 (142-278 nt) of *GM47544* bound closely to ApoC3 (Figure 6B). RNA

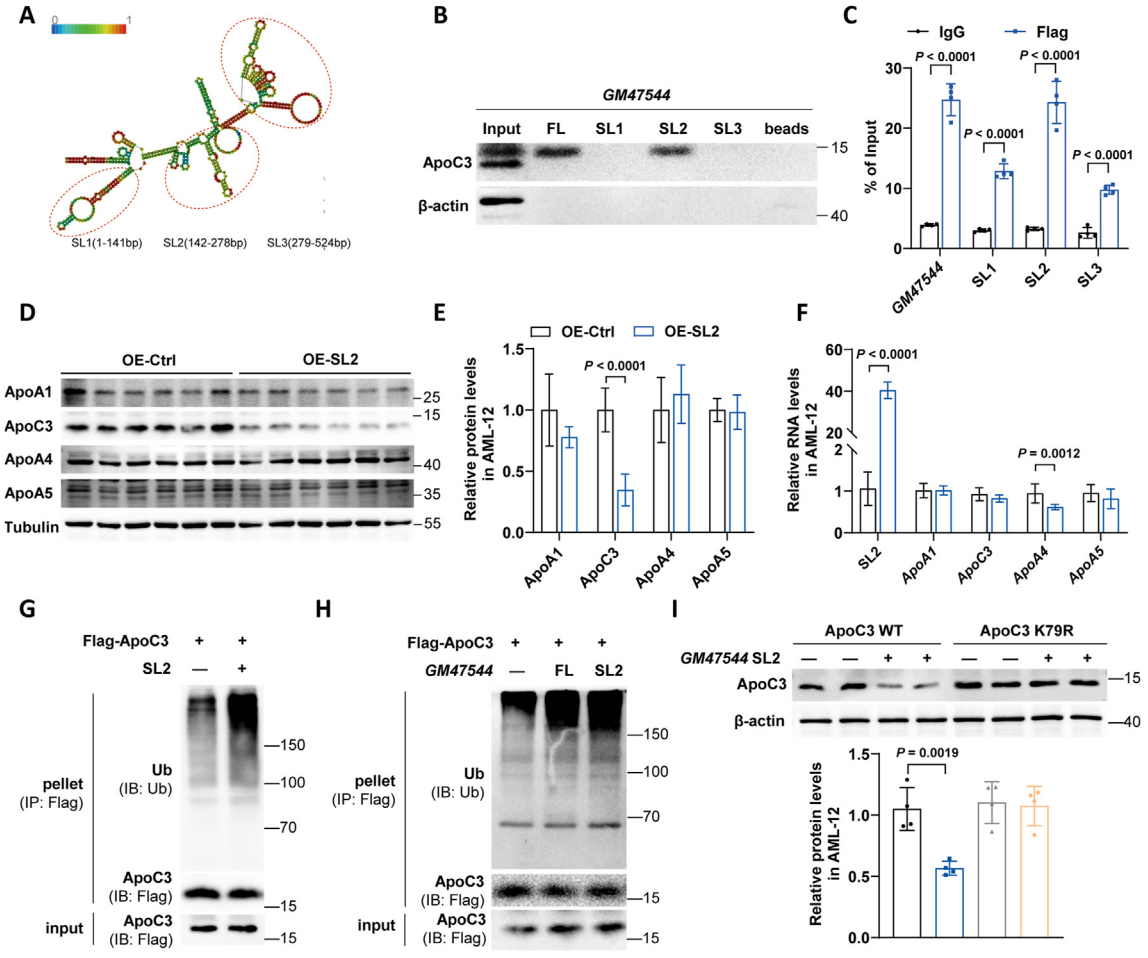

Figure 6: *GM47544* SL2 directly interacted with ApoC3 and promoted the degradation of ApoC3.

(A) The predicted secondary structure of *GM47544* RNA.

(B) Invitro-synthesized full-length (FL), stem-loop structure1 (SL1), stem-loop structure2 (SL2) and stem-loop structure3 (SL3) fragments of *GM47544* were incubated with AML-12 cell protein lysates. Subsequently, RNA pull-down and Western blotting assays were performed.

(C) RIP assays were performed using an anti-Flag antibody in AML-12 cells stably transfected with Flag-ApoC3. RT-qPCR analysis was employed to measure the enrichment of *GM47544* and its fragments.

(D—E) ApoC3 protein levels were measured by western blot in AML-12 cells transfected with pc3.1 (OE-Ctrl) or pc3.1-*GM47544* SL2 (OE-SL2).

(F) Relative expression of *GM47544* and *ApoA1/C3/A4/A5* were measured by RT-qPCR in control and *GM47544 SL2*-overexpressed AML-12 cells.

(G) IP assays were performed on AML-12 cells transfected with vectors expressing FLAG-ApoC3 and pc3.1-*GM47544 SL2* using anti-FLAG antibodies, followed by western blotting analysis with antibodies against ubiquitin.

(H) IP assays were performed on AML-12 cells transfected with vectors expressing FLAG-ApoC3, pc3.1-*GM47544,* or pc3.1-*GM47544* SL2 using anti-FLAG antibodies, followed by western blotting analysis with antibodies against ubiquitin.

(I) With or without *GM47544* full length or SL2, ApoC3 proteins were measured by western blot in AML-12 cells transfected with ApoC3 WT or ApoC3 K79R.

Data are presented as mean ± SD in C, E, F, and I, with statistical significance determined with unpaired two-tailed Student's *t*-test.

immunoprecipitation (RIP) also supported an interaction between *GM47544* SL2 and ApoC3 (Figure 6C). We constructed plasmids overexpressing *GM47544* SL2 and transfected them into AML-12 cells. Similar to the full-length *GM47544*, *GM47544* SL2 had no impact on the mRNA expression of the ApoC3-cluster, but it could significantly degrade the ApoC3 protein (Figure 6D—F). In line with this, the ubiquitylation level of the ApoC3 protein was increased by SL2 (Figure 6G,H). By contrast, *GM47544* SL2 did not regulate the protein content or ubiquitin levels of ApoC3 when K79 was mutated to arginine (K79R) (Figure 6I). The collective findings suggest that SL2 is the key region of *GM47544* that mediates the ubiquitination of APOC3 K79 and its subsequent degradation.

3.7. *AP006216.5*, human ortholog of *GM47544*, promoted the degradation of APOC3 and thus exerted lipid-lowering effect

By inspecting the syntenic regions of the human genome, we identified three potentially homologous long noncoding RNAs (Figure 7A). Similar to *GM47544*, the expression levels of these lncRNAs increased under PA stimulation in the human liver cells THLE-3 (Figure 7B). To further explore the ability of these lncRNAs to modulate APOC3 expression, we overexpressed them in THLE-3 cells using plasmids respectively. Notably, *AP006216.5* specifically reduced APOC3 protein levels rather than the mRNA levels of the APOA1/C3/A4/A5 cluster (Figure 7C,D and Supplementary Figs. 5A—D) and decreased the deposition of lipids in hepatic cells, especially the intracellular TG level (Figure E,F). *AP006216.5* also functioned in intracellular lipid regulation by upregulating LDLR and HL activity (Figure G,H).

We next tested whether *AP006216.5* could improve the ubiquitin-mediated degradation of APOC3 in human hepatic cells. Co-IP revealed that overexpressing *AP006216.5* significantly increased the ubiquitination of APOC3 (Figure 7I). Collectively, these observations indicated that the function of *GM47544* in TG regulation is conserved, at least in humans and mice. *AP006216.5,* a human ortholog of *GM47544,* could potentially be used to develop novel therapeutic strategies for the management of human hyperlipidemia.

4. DISCUSSION

In the present study, we identified *GM47544,* a previously uncharacterized functional lncRNA derived from the ApoA1/C3/A4/A5 cluster, is a crucial regulator of ApoC3. We found that *GM47544* abruptly decreased plasma triglyceride levels, thereby alleviating NAFLD and atherosclerosis in mice with acute and chronic lipid overload. Consistent with this finding, overexpression of *GM47544* significantly attenuated lipid accumulation *in vitro*. Furthermore, we found that SL2 of *GM47544* interacted directly with ApoC3 and facilitated the ubiquitination of K79 of ApoC3, which successively led to the degradation of the ApoC3 protein through the proteasome pathway. The inhibition of ApoC3, in turn, activates LPL/HL and LDLR, profoundly enhancing plasma TG clearance to attenuate hyperglyceridemia, NAFLD, and atherosclerosis. Interestingly, we identified *AP006216.5*, the human ortholog of *GM47544*, which promotes the degradation of ApoC3 and thus exerts a lipid-lowering effect in humans. Collectively, these findings suggest that *GM47544* plays a pivotal role in triglyceride regulation, promoting the decomposition of triglycerides through the ApoC3-LPL/HL pathway and uptake of triglycerides and cholesterol in the liver through the ApoC3-LDLR pathway.

The APOA1/C3/A4/A5 cluster is one of the most promising targets for the regulation of hyperglyceridemia. Several lncRNAs have been identified as regulators of the APOA1/C3/A4/A5 gene cluster. *APOA1-AS*, located in the antisense strand of APOA1, epigenetically controls the expression of this apolipoprotein gene cluster by recruiting histone-modifying enzymes [32]. Similarly, *APOA4-AS*, which interacts with mRNA-stabilizing protein HuR to enhance the stability of APOA4 mRNA, led to reduced levels of plasma TG and TC in ob/ob mice [33]. In our study, within the ApoA1/C3/A4/A5 cluster, the newly identified lncRNA *GM47544* interacted directly with ApoC3. *GM47544* facilitates ApoC3 degradation via the ubiquitin-proteasome pathway, ultimately lowering plasma and cellular TG accumulation. These results improve our understanding concerning studies of the biological functions of the APOA1/C3/A4/A5 cluster.

TRLs, particularly triglyceride-rich lipoproteins, are considered causal components of atherogenesis. They have been proposed as ideal targets for diet- or drug-based interventions to prevent hyperglyceridemia and ASCVD [34]. Novel targets for TRL-lowering therapy have emerged, including APOC3, angiopoietin-like proteins 3 and 4 (ANGPTL3/4), APOA5, and ATP citrate lyase [35,36]. Interestingly, based on the most important triglyceride regulatory gene loci of the APOA1/C3/A4/A5 cluster, APOC3 has been described as a classic triglyceride regulator that modulates hepatic TRL clearance through lipase-dependent and lipase-independent mechanisms [16,17,37]. Individuals with loss-of-function mutations in APOC3 exhibit a noteworthy 39% reduction in triglyceride levels, coupled with a substantial 40% decrease in the risk of ASCVD compared to noncarriers [38]. Moreover, epidemiological investigations have consistently demonstrated that APOC3 levels possess predictive value for ASCVD risk and cardiovascular mortality [17]. Targeting APOC3 is one of the most unique and potent approaches, resulting in a substantial reduction in TRLs [13,16—18]. In the current study, targeting ApoC3 by overexpression of *GM47544* strikingly decreased plasma triglyceride levels by up to 40% in HFD mice at 8 weeks, which conferred protection against hepatic lipid accumulation, hypertriglyceridemia, and atherosclerosis in HFD mice. In terms of mechanisms, we provided evidence that *GM47544* overexpression lost the inhibition of HL activity due to ApoC3 degradation, suggesting an association between liver *GM47544* and plasma triglyceride levels in a lipase-dependent manner. In addition, *GM47544* overexpression facilitated the assimilation of DiI-LDL in AML-12 cells, which represents a lipase-independent way of enhancing the uptake of TG in the liver, leading to the clearance of plasma TG. In summary, the effectiveness of *GM47544* in reducing plasma triglyceride levels underscores the importance of targeting ApoC3 and downstream lipase-dependent or lipase-independent pathways. However, we also observed that *GM47544* could not influence lipid metabolism at low concentrations or knock-down states. This may be attributed to the interaction between *GM47544* and ApoC3. Similar phenomena have been reported in previous studies. For example, overexpression of the lncRNA *HOTAIR* can upregulate EZH2 expression, whereas intervention with sh*HOTAIR* does not alter EZH2 protein levels. This discrepancy may be due to the dysregulation of the interaction between *HOTAIR* and EZH2 [39]. Therefore, we speculate that under basal conditions or when knocked down, *GM47544* may scarcely bind to ApoC3. Conversely, *GM47544* can effectively bind to ApoC3 and trigger ApoC3 degradation process only when its expression level is significantly higher than the basal level.

Targeted protein degradation (TPD) is an emerging and rapidly expanding strategy in drug discovery that utilizes small molecules to facilitate the rapid breakdown of disease-causing proteins [40]. TPD exploits the endogenous proteolytic pathways of cells to selectively eliminate proteins of interest (POIs), including disease-causing proteins, from cellular and tissue environments [41]. These technologies hold promise as novel therapies for unmet medical needs. They are much smaller and may have a higher membrane permeability and

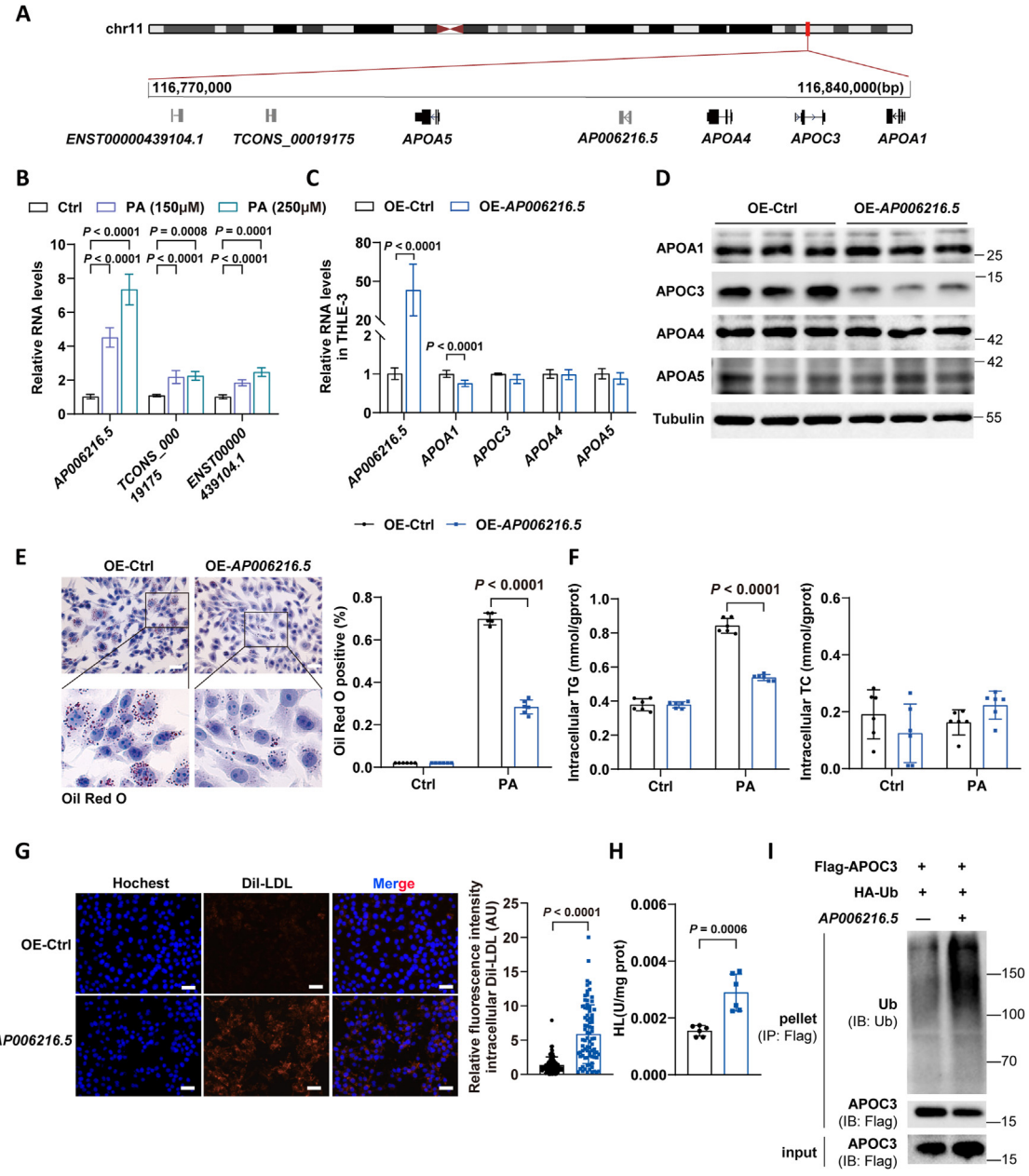

Figure 7: *AP006216.5*, a human ortholog of *GM47544*, promoted the degradation of ApoC3 and thus exerted a lipid-lowering effect.

(A) Schematic representation of the human APOA1/C3/A4/A5 locus.

(B) Relative expression levels of *ENST00000439104.1*, *AP006216.5,* and *ENST0000000457746.1* under PA stimulations in THLE-3 cells were detected by RT-qPCR analysis.

(C) Relative expression of *APOA1/C3/A4/A5* in control and *AP006216.5*-overexpressed THLE-3 cells was measured by RT-qPCR.

(D) APOA1/C3/A4/A5 protein levels in control and *AP006216.5*-overexpressed THLE-3 cells were measured by western blot.

(E) Oil Red O staining of control and *AP006216.5*-overexpressed THLE-3 cells.

(F) Intracellular TG and TC (F) levels of control and *AP006216.5*-overexpressed THLE-3 cells with PA treatment (250 μM, 24 h).

(G) Dil-LDL uptake was quantified under fluorescence microscopy in control and *AP006216.5*-overexpressed THLE-3 cells treated with Dil-LDL (15 μg/mL) for 24 h. Quantification of the relative fluorescence intensity of internalized Dil-LDL in control and *C* cells. Red: Dil-LDL; blue: DAPI staining. Scale bar, 100 μm.

(H) Cellular HL activity in control and *AP006216.5*-overexpressed THLE-3 cells was detected.

(I) THLE-3 was transfected with vectors expressing the FLAG-APOC3, with or without vectors expressing the *AP006216.5*. IP assays were performed using anti-FLAG antibodies, followed by western blotting analysis with antibodies against ubiquitin.

Data are presented as mean ± SD in B, C, E, F, G, and H, with statistical significance determined with unpaired two-tailed Student's *t*-test.

better cellular uptake [42]. In terms of lncRNA *GM47544*, we identified that SL2 of *GM47544* could directly bind to K79 of ApoC3, which promotes the ubiquitination of ApoC3, thus inducing anti-hyperglyceridemia and atherogenesis [43]. This represents a brand-new direction of research rather than the traditional strategies for lowering plasma triglycerides by targeting ApoC3 mRNA.

In summary, we demonstrated that lncRNA *GM47544* can directly bind to ApoC3 and promote its degradation of ApoC3 through the ubiquitin-

proteasome pathway, which strikingly lowered hyperlipidemia, attenuated NAFLD, and prevented atherosclerosis. These findings have set the stage for new therapeutic approaches aimed at lipoproteins and promise to advance CVD management substantially.

CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

Qianqian Xiao: Writing — review & editing, Writing — original draft, Visualization, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Luyun Wang:** Writing — review & editing, Writing — original draft, Resources, Project administration, Conceptualization. **Jing Wang:** Methodology, Investigation, Data curation. **Man Wang:** Methodology, Investigation, Data curation. **Dao Wen Wang:** Writing — review & editing, Supervision, Resources, Project administration. **Hu Ding:** Writing — review & editing, Supervision, Project administration, Data curation, Conceptualization.

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DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

DATA AVAILABILITY

Data will be made available on request.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2024.102011.

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