LncNONMMUG027912 alleviates lipid accumulation through AMPKα/mTOR/SREBP1C axis in nonalcoholic fatty liver

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Research Article

Keywords: Long non-coding RNA NONMMUG027912, AMPKα, mTOR, SREBP1C, lipid metabolism

Posted Date: May 18th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1218577/v2

Abstract

Various metabolic diseases are closely related to lipid metabolism disorders, but the regulatory effect of long noncoding RNAs (lncRNAs) on the function of lipids has been poorly elucidated. Previous our work has found that lncNONMMUG027912 (abbreviated as lnc027912) involved in cholesterol metabolism. Here, we further explored the novel function of lipid metabolism-associated lnc027912. We found that upregulated lnc027912 in AML12 cells treated with oleic acid (OA) and palmitic acid (PA) showed a significant decrease in lipid accumulation, triglyceride (TG) levels, and lipid biosynthesis genes. In terms of regulatory mechanisms, lnc027912 increased the expression of p-AMPKα, inhibited p-mTOR levels, decreased the expression of SREBP1C in nuclei, decreased the promoter activity of SREBP1C, and inhibited the expression of lipid synthesis genes. Most importantly, lnc027912 could reduce lipid accumulation and liver inflammation through AMPKα/mTOR signal axis in nonalcoholic fatty liver disease (NAFLD) mice model. Altogether, our study revealed a novel molecular mechanism of lnc027912 in lipid metabolism through the AMPKα/mTOR/SREBP1C signaling axis and highlights the potential of lnc027912 as a new treatment target for lipid disorder diseases (such as NAFLD).

Background

Lipid metabolism is the process of lipid biosynthesis, distribution, accumulation and secretion [1]. Lipids synthesized by the liver are in a dynamic balanced state, including synthesis and catabolism [2]. The biosynthesis of lipids is regulated by many transcription factors, such as cholesterol regulating element binding protein (SREBP), which regulates the levels of many key enzymes, such as fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC), and stearoyl-CoA desaturase (SCD) [3]. Recent studies have demonstrated that SREBP-1C plays a central role in regulating hepatic lipogenic genes [4,5]. Furthermore, SREBP-1C induced fatty acid (FA) synthesis, and upregulated SREBP-1C caused hepatic triglyceride (TG) accumulation and lipid metabolism disorders [6,7]. Additionally, the control of lipid homeostasis is crucial for treating lipid disorder diseases, such as obesity, type 2 diabetes, nonalcoholic fatty liver disease (NAFLD), dyslipidemia, and arteriosclerotic cardiovascular disease. However, the regulatory strategies of lipid metabolism have been very limited so far.

Long noncoding RNAs (lncRNAs) are noncoding transcripts more than 200 nucleotides in length with no protein-coding capacity [8]. As the function of lncRNAs continues to grow, it has become more important that they are key regulators of many biological processes, particularly lipid homeostasis. A large number of works have reported that lncRNAs are involved in various lipid metabolism processes, such as synthesis, transport and secretion [9]. LncHULC acts on peroxisome proliferator activated receptor alpha (PPARα) to regulate long-chain acetyl-CoA synthetase 1 (ACSL1), which is involved in cellular fatty acid metabolism [10]. Moreover, lncRNAs, including MALAT-1[10], HOTAIR [11], H19 [12], HR1 [13], LeXis [14] and Gm16551 [15], were found to be related to lipid metabolic disorders. Recent studies have demonstrated that lncH19 suppresses lipid metabolism, which may act as a therapeutic target for atherosclerosis [16]. These results suggested that lncRNAs played a vital role in maintaining lipid homeostasis. Our previous work found that the lncRNA NONMUG027912 (lnc027912)

is implicated in the process of cholesterol metabolism [17]. However, the function of lnc027912 in regulating lipid metabolism has not been investigated.

In this study, the role of lnc027912 in regulating lipid metabolism was further explored. Upregulated lnc027912 significantly reduced lipid accumulation. Mechanistically, lnc027912 inhibited SREBP1C promoter activity and reduced the expression of SREBP1C in nuclei by activating p-AMPKα and restraining mTOR. More importantly, lipid accumulation and inflammation was inhibited in liver special knockin lnc027912 mice by activating AMPKα/mTOR signal axis. Together, our findings provide new insight into the molecular function of lnc027912 in lipid metabolism and highlight the potential of lnc027912 as a new treatment target for NAFLD.

Materials And Methods

2.1 Cell cultured and transfection

AML12, Hepa1-6 and HEK-293T cells were obtained from the Stem Cell Bank, Chinese Academy of Sciences (Shanghai, China). AML12 cells were cultured in Dulbecco's modified Eagle/F-12 medium (DMEM/F12) (Gibco BRL, Gaithersburg, MD, USA) with 10% (v/v) fetal bovine serum (FBS) (Ginimi, USA), 1% (v/v) ITS liquid media supplement (100×) (Thermo Fisher, USA), and 40 μg/μl dexamethasone (Sigma, Germany). HEK-293T and Hepa1-6 cells were cultured in DMEM (Gibco, BRL, Gaithersburg, MD, USA) and Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco BRL, Gaithersburg, MD, USA), respectively, with 10% (v/v) fetal bovine serum (FBS) (Ginimi, USA) and 1% penicillin/streptomycin (Beyotime, Shanghai, China). All cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2.

According to the Lipofectamine™ RNAiMAX (Invitrogen, USA) manufacturer's protocols, Hepa1-6 cells were transfected with 50 nM siRNA (RiboBio, Suzhou, China). Si-lnc027912 sequence: GUGUAUUGCUUUGAGUUCATTUGAACUCAAAGCAAUCCACTT; si-control sequence: UUCUCCGAACGUGUCACGUTTAC GUGACACGUUCGGAGAATT.

2.2 Construction of overexpression cell line

After mixing the plasmids (pCDH-GFP-lnc027912 and pCDH-GFP) and pPACKH1-plasmid Mix, they were transfected into HEK-293T cells, the culture medium was collected, and the virus particles were precipitated with PEG-it. Puromycin was added to AML12 cells to screen out the cell line stably expressing lnc027912.

2.3 Animal

All mice were in C57BL/6 background in the study. The wild-type (WT) mice were purchased from the experimental animal center of Wenzhou Medical University in the experiment. The transgenic mice liver-specific knockin lnc027912 (CKI-lnc027912) were generated by Cyagen through CRISPR / cas9 technology.

Wildtype (WT) and CKI-lnc027912 male and female mice were randomly divided into two groups. One group was fed with control diet (Madison; #MD12051), and the other group was fed with High fat diet (HFD) for 3 months (45% kcal from fat, Madison; #12032).

All animal experiments were conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and in line with the global 3R (reduce, reuse, and recycle) initiative. All protocols were approved by the Committee on the Ethics of Animal Experiments of Wenzhou Medical University (No: xmsq 2021-0079).

2.4 Oleic acid (OA) and palmitic acid (PA) treatment and Oil red O staining

AML12-GFP and AML12-lnc027912 cells were seeded in 12 wells. The cells were treated with 200 μM OA (Sigma, USA) or PA (Sigma, USA) for 24 h. Cells and livers samples were fixed with 4% (wt/vol) paraformaldehyde. Then, samples were stained with Oil red O (Sigma, USA), as previously described [18].

2.5 Hematoxylin eosin (HE) staining

The liver samples were fixed in 4% formaldehyde, then gradient dehydration of liver tissue by ethanol. The detail steps were performed, as previously described [17].

2.6 Measurement of triglyceride (TG)

AML12-GFP and AML12-lnc027912 cells were treated with OA (200 μM) and PA (200 μM) for 24 h. Then, TG was measured according to the TG manufacturer's protocols (Jiancheng Bioengineering Institute, Nanjing, China).

2.7 Quantitative RT–PCR

RNA was isolated using RNAiso Plus (Takara, Dalian, China). cDNA was synthesized according to HiScript II 1st Strand cDNA Synthesis Kit protocols (Vazyme, Nanjing, China). Then, the relative expression level of genes was analyzed using real-time PCR. These results was detected using the ΔΔCT method. GAPDH expression was a control to normalize the data. All primer sequences used are listed in supplemental Table S1.

2.8 Western blot analysis

Cellular protein was obtained using cell lysis buffer. Then, the protein samples were separated via sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Immunoblotting experiments were performed using specific primary antibodies. Finally, the expression of gene signals was detected using a ChemiDoc^{TM} Imaging System (Bio–Rad, USA). The antibodies used are listed in supplemental Table S2. GAPDH was used as a control.

2.9 Immunofluorescence

AML12-GFP and AML12-lnc027912 cells were treated with OA and PA. Then, the cells were fixed and further permeabilized with Triton X-100. Primary FAS (ab216991, Cambridge, UK, 1:100), SREBP1C (Proteintech, 1:100, Wuhan, China), Alexa Fluor 555-labeled donkey anti-mouse IgG (H+L) (Beyotime, 1:250, Shanghai, China) and Cy3-labeled goat anti-mouse IgG (H+L) (Beyotime, 1:250, Shanghai, China) antibodies were incubated with the cells. 4',6-Diamidino-2-phenylindole (DAPI) was used to stain nuclei (Solarbio, Beijing, China). The images were captured using a laser confocal microscope (Nikon, Tokyo, Japan), and these pictures were further quantified using ImageJ software.

2.10 Luciferase reporter assays

The SREBP1C promoter containing the far-upstream element sequence (approximately -2000-0 bp) was subcloned, and then, sequences were inserted into the pGL3-basic vector (Promega, Beijing, China). The recombinant plasmid (pGL3-SREBP1C-promoter vector) was obtained from MiaoLingBio. HEK-293T cells were seeded onto 48-well plates for 24 h and then transfected with pCDH-lnc027912, pGL3-SREBP1C, and phRL-TK vectors for 36 h using Lipofectamine 3000 Reagent (Invitrogen, USA). After the cells were treated with PA or OA (200 μM) for 24 h, a dual-luciferase reporter assay (Promega, Beijing, China) was performed according the manufacturer's protocols.

2.11 Statistical analysis

All experiments were repeated at least three times, and the data are presented as the mean ± SD (standard deviation). All statistical analyses were created using GraphPad Prism 8.0 (San Diego, CA). Multiple results were compared using one-way ANOVA. P <0.05 was considered statistically significant.

Results

3.1 Upregulated lnc027912 reduced the total triglyceride level in hepatic cells

Our previous studies have demonstrated that lnc027912 plays a key role in regulating cholesterol homeostasis [17]. Cholesterol levels are closely related to lipid metabolism, inducing lipid biosynthesis, transport or secretion [19]. To further explore the function of lnc027912 in regulating lipid metabolism, virus particles (obtaining pCDH-lnc027912-GFP and pCDH-GFP vectors) were used to infect AML12 cells, as shown in Fig. 1A, and more than 95% of cells showed green fluorescence. Compared with the control group, the CT value of lnc027912 was decreased by 10 (Fig. 1B). These results suggest that the lnc027912 overexpression cell line was successfully constructed. Additionally, the total triglyceride (TG) level was analyzed. As shown in Fig.S1A, overexpression of lnc027912 significantly inhibited the TG level. To further explore the role of lnc027912, silencing lnc027912 was performed in Hepa1-6 cells, and the results showed that the lnc027912 level decreased by 54% (Fig. S1B). However, we noticed that the TG level was not changed (Fig. S1C).

To further demonstrate the effect of lnc027912 on lipid levels, cells were treated with OA and PA, and TG levels were assessed by oil red O staining and TG kits. As shown in Fig. 1C, compared

with the control group, upregulation of lnc027912 in cells resulted in a decrease in oil red O-positive lipids. Importantly, overexpression of lnc027912 could significantly reduce lipid accumulation induced by OA and PA. Then, the number of lipid drops was measured using Image-Pro Plus 6.0, showing that lipid drops were significantly reduced in the lnc027912 upregulation groups (Fig.S1D). Furthermore, we used a TG kit to measure the TG level, and the results also showed that the TG decreased by 32.7% (OA-treated, Fig.S1E) and 32.3% (PA-treated, Fig. S1F) compared with the control group. Together, these results strongly suggested that lnc027912 inhibited lipid accumulation in cells, while molecular mechanism need to be further explored.

3.2 Lnc027912 regulates triglyceride metabolism mechanism

To further investigate the mechanism by which lnc027912 influences lipid levels, the expression of triglyceride metabolism-related genes was analyzed. Overexpression of lnc027912 significantly inhibited the lipogenesis genes SREBP1C and FAS; concurrently, the genes of MTTP were induced by RT–qPCR (Fig. S2). Importantly, lnc027912 inhibited lipid biosynthesis and increased lipid transport when cells were treated with OA or PA (Fig. 2A, 2B and Fig. S3A, 3B). Together, these results sufficiently confirmed that lnc027912 could inhibit OA- or PA-induced lipid accumulation in hepatic cells.

SREBP1C is a key transcription regulator of fatty acid biosynthesis [20]. Therefore, we reasoned that lnc027912 may be involved in regulating SREBP1C activity or location to modulate lipid metabolism. First, to investigate the effect of lnc027912 on the location of SREBP1C, after lnc027912 was upregulated in AML12 cells, the expression of SREBP1C in the nuclei was decreased (Fig. 2C, as shown by white arrow), which may inhibit the expression of lipid metabolism-related genes. Additionally, the fluorescence density of SREBP1C was significantly inhibited in lnc027912-upregulated cells (Fig. S3C). Furthermore, a dual-luciferase reporter assay was performed to investigate the effect of lnc027912 on the promoter activity of SREBP1C. As shown in Fig. 2D, as expected, SREBP1C promoter activity was significantly inhibited with or without OA and PA stimulation when lnc027912 was upregulated in AML12 cells.

To confirm the mechanism of lnc027912 in lipid metabolism, the FAS level was detected by immunofluorescence (Fig. 2E). FAS was significantly inhibited in lnc027912-overexpressing cells. Concurrently, we found that lnc027912 can effectively reduce the elevation of FAS caused by OA or PA (Fig. 2E and Fig. S3D). To further explore the mechanism of lnc027912 regulation of SREBP1C, AMPKα and mTOR levels were detected. As shown in Fig. 4D and 4E, the p-mTOR (Ser2448) levels were inhibited after overexpression of lnc027912, while the levels of p-AMPKα (Thr172) was significantly increased. These results suggested that lnc027912 inhibited the expression of lipid synthesis genes and lipid accumulation through activating p-AMPKα and inhibiting mTOR signals.

3.3 Lnc027912 alleviate liver inflammation in HFD-induced NAFLD mice

To further investigate the mechanism of lnc027912 in involving in lipid metabolism *in vivo*, the transgenic mice liver-specific knockin lnc027912 (CKI-lnc027912) were generated by Cyagen through CRISPR / cas9

technology. First, the lnc027912 level was analyzed in mice liver. As shown in Fig.3A, compared with the control group, the CT value of lnc027912 was decreased by 10 (Fig.3A), indicated that the liver specific knockin lnc027912 mice was successfully constructed. Then, mice was seed with high fat diet (HFD, 45% fat) for 3 months to build NAFL mice model. HE staining results shown that HFD feed induced NAFLD (as black arrows indicates steatohepatitis and vacuolation), while lnc027912 significantly reduced steatohepatitis and vacuolation in hepatic cells (Fig.3B). Additionally, oil red O results also further demonstrated that lnc027912 decreased lipid accumulation, showing smaller lipid deposits in CKI-mice liver (Fig.3C, as yellow arrows indicates). These results have shown that the key role of lnc027912 in alleviating HFD-induced steatohepatitis. To further explore the mechanism of lnc027912 regulation of lipid metabolism, SREBP1C, FAS, AMPKα, and mTOR protein levels were detected in liver tissue. As shown in Fig.3D and supplemental Fig. S4, the SREBP1C, FAS and p-mTOR (Ser2448) levels were inhibited in CKI-lnc027912 mice, while the levels of p-AMPKα (Thr172) was induced. Altogether, these results further confirmed that lnc027912 alleviated lipid accumulation by inhibiting the expression of SREBP1C and FAS, through increased of p-AMPKα and decreased of p-mTOR level *in vivo*.

In summary, these findings thoroughly demonstrated that lnc027912 decreased the expression of SREBP1C in nuclei by inducing p-AMPKα and reducing the phosphorylation of mTOR, then decreased the activity of the SREBP1C promoter, resulting in decreased lipid synthesis, then alleviated triglyceride accumulation *in vitro* and *in vivo* (Fig. 4).

Discussion

Excessive lipids could disrupt lipid synthesis and transport balance in the liver [21, 22]. Lipid homeostasis disorders not only result in NAFLD but also many metabolic syndromes with obesity, dyslipidemia, hyper viscosity, and hyperinsulinemia diseases [23-25]. In the previous study, increased of lncRNA Gm15622 may involving in regulating lipid metabolism by targeting several transcription factors, such as SERBP1C [4], thereby control fatty acid and cholesterol synthesis. Although the molecular mechanism of NAFLD has not been clearly known, some researches have shown that lncRNAs may be used as potential targets for treatment of NAFLD. Our previous work found that lnc027912 is involved in the regulation of cholesterol metabolism by modulating the expression of farnesyl-diphosphate farnesyltransferase 1 (Fdft1), which is a key enzyme for cholesterol biosynthesis [17]. However, lnc027912, which is involved in lipid metabolism, has not yet been elucidated.

Some works have demonstrated that lncRNAs could affect lipid synthesis by regulating the level of SREBP1C. LncMALAT1 could inhibit PA-induced upregulation of SREBP1C and hepatic lipid accumulation through inhibition of FAS, which is the principle target of SREBP1C [26]. Overexpression of lncHR1 could decrease TGs by inhibiting the activation of SREBP1C and FAS in oleic acid (OA)-induced hepatic cells [13]. These results indicated that lncRNAs played an important role in inhibiting lipid accumulation through control SREBP1C in OA- and PA-treated cells. In this study, we found that lnc027912 could abrogate PA- or OA-induced lipid accumulation through a mechanism of action that decreased SREBP1C activity. Importantly, further mechanistic exploration showed that lncRNA027912

could inhibit the promoter activity of SREBP1C. Additionally, studies have revealed that SREBP1C transits into cell nuclei and is involved in regulating lipid biosynthesis [27]. However, lncRNA-induced SREBP1C transfer into the nucleus has rarely been found. Interestingly, our findings showed that lnc027912 decreased the SREBP1C protein level in the nucleus.

The key role of AMPK in maintaining metabolic homeostasis, so which is widely recognized for treatment of various metabolic diseases [28]. The ability of AMPK to inhibit lipid biosynthesis is regulated in large part by inhibition of the mTOR [29]. So, the activity of AMPK and mTOR acts as a switch to control anabolism and catabolism [30]. In the present study, our findings were consistent with previous studies showing that lnc027912 activated p-AMPKα and inhibited mTOR phosphorylation and then reduced lipid accumulation *in vitro* and *in vivo*. These results also indicated that lnc027912 may be useful for regulating the AMPK-targeted therapeutics to treat metabolic related diseases.

Altogether, our study demonstrated that lnc027912 inhibited SREBP1C activity through the AMPKα/mTOR signaling pathway and was involved in lipid metabolism balance *in vivo* and *in vitro*. Thus, lnc027912 plays a vital regulatory role in the regulation of lipid metabolism homeostasis and liver disease progress, which may be an effective target for the regulation of lipid metabolism diseases, such as NAFLD.

Declarations

Conflicts of interest

The authors declare that they have no competing interests.

Acknowledgement

This study was supported by Wenzhou Science and Technology Bureau Foundation (Y2020216, ZY2021011).

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Figures

Figure 1

Lnc027912 inhibited lipid accumulation in AML12 cells. (A) AML12 cells were transfected with pCDH-GFP or pCDH-GFP-lnc027912 virus particles. Images were obtained using laser confocal microscopy. (B) RT-

qPCR analysis of the level of lnc027912 in pCDH-GFP-lnc027912 AML12 cells. (C) Oil red O staining was used to analyze lipid levels in cells under OA and PA treatment. (n=3, ****P<0.001)

Figure 2

Lnc027912 regulate lipid metabolism mechanism. Changes in key genes related to lipid metabolism were analyzed by Western blot after OA (A) and PA (B) treatments. (C) The SREBP1C location was detected by immunofluorescence (red: SREBP1C antibody staining; blue: DAPI-stained nucleus, arrow indicates the location of SREBP1C) (n≥3). (D) Changes in SREBP1C promoter activity were detected by dual-luciferase reporter assay. The fluorescence intensity of FAS was analyzed using Image J (E). (F) The expression levels of AMPKα, p-AMPKα-Thr172, mTOR and p-mTOR-Ser2448 were analyzed by Western blot. Data are presented as the mean ± SD. (n≥3, **P<0.01, ***P<0.001).

Figure 3

Lnc027912 alleviate liver inflammation and lipid accumulation in NAFL mice. (A) The level of lnc027912 was measured in WT and CKI-lnc027912 mice livers (n=3). (B) Liver H-E staining was performed in control or HFD diets mice (WT and CKI-lnc027912 mice) (n=3, black arrows indicates liver inflammation and vacuolation). (C) The control or HFD diet mice liver section was stained with oil red O (n = 3, black arrows indicate big lipid drops, yellow arrows indicate small lipid drops). (D) In HFD diet mice liver tissues, the expression levels of SREBP1C, FAS, AMPKα, p-AMPKα-Thr172, mTOR and p-mTOR-Ser2448 were analyzed by Western blot (n=3). OA, Oleic acid; PA, palmitic acid.

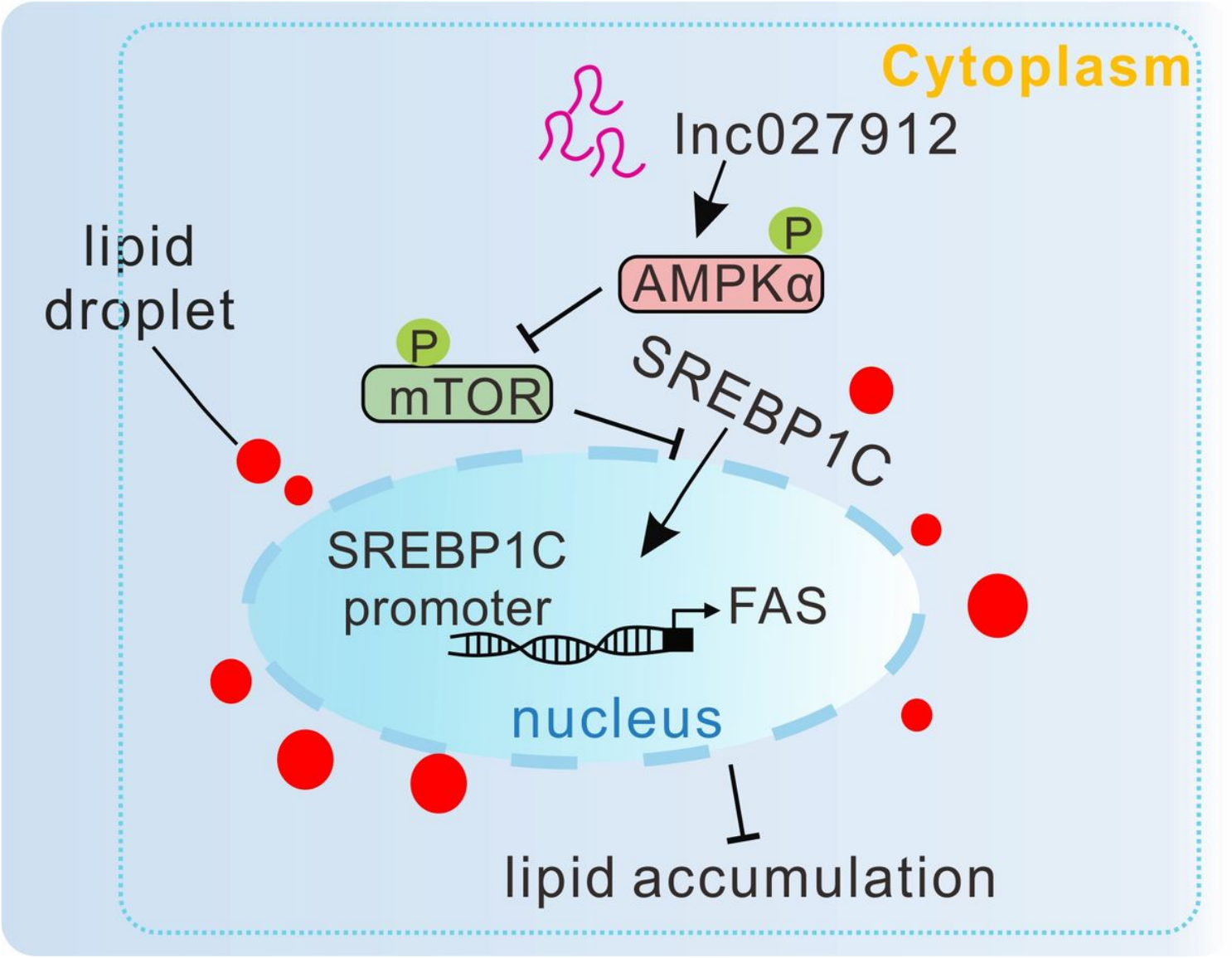

Figure 4

Diagram of the role of lnc027912 in regulating lipid metabolism. Lnc027912 could decrease the expression of SREBP1C in nuclei through increased p-AMPKα and decreased p-mTOR, inhibited SREBP1C promoter activity, then inhibited lipid synthesis gene-FAS and reduced lipid accumulation.

Supplementary Files

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