



# Acetylation of H3K27 activated lncRNA NEAT1 and promoted hepatic lipid accumulation in non-alcoholic fatty liver disease via regulating miR-212-5p/GRIA3

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## Abstract

Non-alcoholic fatty liver disease (NAFLD) was a world-wide health burden. H3K27 acetylation, long non-coding RNA (lncRNA), and miRNA were all implicated in NAFLD regulation, yet the detailed regulatory mechanism was not well understood. lncRNA NEAT1, miR-212-5p, and GRIA3 expression were detected both in high fatty acid-treated hepatocytes cells and NAFLD patients. Lipid droplets were stained and analyzed by oil red O staining. Expression of fatty acid synthase (FASN), acetyl-CoA carboxylase (ACC), and GRIA3 was detected by qRT-PCR and western blot. RNA level of lncRNA NEAT1 and miR-212-5p was analyzed by qRT-PCR. The binding sequences of lncRNA NEAT1/miR-212-5p and miR-212-5p/GRIA3 were predicted bioinformatically and validated through luciferase assay. ChIP was performed to analyze H3K27 acetylation on the promoter of lncRNA NEAT1. lncRNA NEAT1 and GRIA3 was upregulated, while miR-212-5p was downregulated in NAFLD patients. FFA promoted lncRNA NEAT1 and GRIA3 expression while suppressing miR-212-5p and promoted lipid accumulation as indicated by increased oil red O staining and FAS and ACC expression. ChIP indicated enrichment of H3K27 on NEAT1 promoter. Inhibition of H3K27 acetylation suppressed lncRNA NEAT1 level. Luciferase results indicated direct interaction of NEAT1/miR-212-5p (which was confirmed by RIP) and miR-212-5p/GRIA3. lncRNA NEAT1 knockdown upregulated miR-212-5p level and inhibited FFA-induced lipid accumulation while suppressing GRIA3 expression. Such function was antagonized by miR-212-5p inhibition and GRIA3 knockdown counteracted with miR-212-5p inhibition. H3K27 acetylation was enriched within the promoter of lncRNA NEAT1 and promoted lncRNA NEAT1 transcription. lncRNA NEAT1 could then interact with miR-212-5p and suppress its cellular concentration.

**Keywords** H3K27 · NAFLD · Acetylation · lncRNA NEAT1 · GRIA3 · miR-212-5p

## Abbreviations

H3K27ac Acetylation on lysine 27 of histone 3  
ACC Acetyl-CoA carboxylase

FASN	Fatty acid synthase
FBS	Fetal bovine serum
FFA	Free fatty acid
Hcy	Homocysteine
LXR $\alpha$ , NR1H3	Liver X receptor alpha
lncRNA	Long non-coding RNA
WT	Wild type
MUT	Mutated type
NAFLD	Non-alcoholic fatty liver disease
RIP	RNA-binding protein immunoprecipitation
RISC	RNA-induced silencing complex
ChIP	Chromatin immunoprecipitation
SCD1	Stearoyl-CoA desaturase-1
TG	Triglyceride
T2DM	Type 2 diabetes

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## Introduction

Non-alcoholic fatty liver disease (NAFLD) had become a global healthy issue, posing remarkable burden on patients' health and society. NAFLD referred to a clinical syndrome progressing from steatosis to fibrosis and necro-inflammation, which could finally lead to steatohepatitis in patients without alcoholic history [1]. Accumulation of free fatty acids (FFAs) in hepatocytes induces lipotoxicity, leading to NAFLD [2]. Patients with NAFLD had higher risk for multiple disease including cirrhosis and even hepatic cancer. The common risks factors for NAFLD included obesity, type 2 diabetes (T2DM), metabolic syndrome, hyperlipidemia, and hypertension [3]. The incidence of NAFLD was increasing in recent years, due to rising prevalence of obesity and T2DM [4]. Therefore, it is urgent to develop clinical management strategies in patients with NAFLD risks to prevent NAFLD development. However, the mechanism of NAFLD development was not well elucidated. This study aimed to explore the regulatory mechanism during the development of NAFLD.

Long non-coding RNA (lncRNA) referred to non-coding RNA with 200 nt in length, which could regulate gene expression and participate in regulation of multiple biological processes, including development of NAFLD. For instance, Zhao and colleagues demonstrated that lncRNA *Blnc1* was strongly associated with NAFLD development through regulating LXR-SREBP pathway [5]. lncRNA *MALAT1* was associated with NAFLD and fibrosis by influencing *CXCL5* signal [6]. lncRNA *AK012226* was associated with lipid accumulation in NAFLD mice and FFA-treated hepatocytes [7]. lncRNA *NEAT1* was reported upregulated in NAFLD [8].

Histone acetylation was an important type of post-translational modification. Acetylation could neutralize the positive charge of lysine and affect the structure and conformation of histone, through which alters density and the space structure of chromatin, promoting gene transcription [9]. Acetylation on lysine 27 of histone 3 (H3K27ac) was one crucial site. It was reported that H3K27ac was highly enriched in active promoter and enhancers, leading to elevated gene transcription [10]. H3K27ac could also influence expression of lncRNA. For instance, H3K27ac enhanced lncRNA *EIF3J-AS1* and promoted proliferation in colon cancer and activated lncRNA *TINCR* in breast cancer [11, 12]. Recent studies implicated the role of H3K27ac in NAFLD. For instance, liver X receptor alpha (LXR $\alpha$ , NR1H3) could alter H3K27ac and affect NAFLD development via influencing inflammation and fibrotic signal pathways [13]. Besides, *Snail1* could mediate deacetylation of H3K27 and suppress fatty acid synthase (FASN) expression, through which prevents NAFLD

in obesity [14]. Another report mentioned that homocysteine (Hcy) could increase H3K27ac and modulate steatosis [15]. However, the roles and mechanisms of H3K27ac in NAFLD were not well explained, due to the tremendous genes it might affect. As it was reported that lncRNA *NEAT1* was upregulated in NAFLD [8], but whether this upregulation was associated with H3K27ac was not clear.

MicroRNA (miRNA) are single-stranded RNA with around 22 nt in length, which are involved in many crucial biological processes, such as proliferation, apoptosis, and differentiation [16]. miRNA was also involved in NAFLD development, either by directly modulating NAFLD-related factors or by promoting metabolic disorders that contributed to NAFLD [17]. For example, miR-132, which was considered as a key regulator of hepatic lipid homeostasis, could promote lipogenesis and NAFLD [18]. miR-212-5p was also related with lipid accumulation by affecting FAS and stearoyl-CoA desaturase-1 (SCD1) [19]. We noticed that there were binding sites between miR-212-5p and lncRNA *NEAT1*; hence, we were interested about whether there was a regulatory network between these factors in NAFLD. Bioinformatics analysis revealed that *GRIA3* was a potential target of miR-212-5p. *GRIA3* could promote EMT in lung cancer [20]; however, the function of *GRIA3* in NAFLD was never reported.

This study aimed to evaluate the roles of lncRNA *NEAT1* in hepatic lipid accumulation regulation and to explore their regulatory mechanisms. We uncovered that acetylation on H3K27 within lncRNA *NEAT1* promoter upregulated lncRNA *NEAT1*. lncRNA *NEAT1* could then interact with miR-212-5p and suppress its expression. *GRIA3* was a direct target of miR-212-5p. Decreased miR-212-5p resulted in *GRIA3* upregulation and finally promoted lipid accumulation. When *GRIA3* was inhibited, inhibiting miR-212-5p could not promote lipid accumulation any more. This study would deepen our understanding about hepatic lipid accumulation regulation and shed new light upon the signal axis from H3K27ac to lncRNA *NEAT1*, miR-212-5p, and *GRIA3* in regulating hepatic lipid accumulation. Elevated serum FFAs and hepatic lipid accumulation are features of NAFLD. These findings might provide theoretical support for future studies or development of new therapeutic methods of NAFLD.

## Materials and methods

### Clinical samples

This study was approved by The First Affiliated Hospital of University of South China Ethical Committee and all experiments were designed and conveyed following the Declaration of Helsinki. Ten patients with NAFLD and ten healthy

controls from The First Affiliated Hospital of University of South China were enrolled in the present study. Blood samples were collected by vein puncture from all subjects following 12 h of fasting. The whole blood was centrifuged at 4000 rpm for 10 min and then the supernatant serum was isolated and stored at  $-80^{\circ}\text{C}$  until analysis. Expression of lncRNA NEAT1, miR-212-5p, and GRIA3 mRNA was detected by qRT-PCR in these serum samples from healthy controls and NAFLD patients.

### Cell culture and treatment

HepG2 and Huh-7 cells were obtained from cell bank of Chinese Academy of Sciences and cultured in DMEM + 10% FBS (fetal bovine serum) under  $37^{\circ}\text{C}$  in a humidified incubator with 5%  $\text{CO}_2$ . Reagents were obtained from Thermo fisher. For free fatty acid (FFA) treatment, 0.5 mM FFA (oleate and palmitate, 2:1) was added into the culture medium for 48 h.

### Plasmid construction

Plasmid for overexpression of lncRNA NEAT1 (oe-NEAT1) was constructed by cloning the DNA sequence encoding lncRNA NEAT1 into pcDNA 3.1. Luciferase system containing wild type (WT) or mutated (MUT) lncRNA NEAT1 or the 3' UTR of GRIA3 was constructed by cloning corresponding DNA sequences into pRL luciferase reporter system (Promega, USA). The shRNA for lncRNA NEAT1 and GRIA3 was purchased from Genepharma Company. The mimics and inhibitor of miR-212-5p were obtained from Genepharma.

### RNA extraction and real-time reverse transcription-PCR (qRT-PCR)

We extracted total RNA from cells using Trizol (Thermo fisher). Concentration and integrity of RNA were evaluated by NanoDrop (Thermo fisher) OD260/OD280 between 1.8 and 2.0 and Agilent 2100 Bioanalyzer (Agilent Technologies) 28S:18S ratio between 1.8 and 2.0. The cDNA was synthesized by reverse transcription kit (Thermo fisher) obtained from Thermo fisher and qPCR was performed on ABI Q7 platform (Thermo fisher). GAPDH was used as internal reference for mRNA and U6 was used as reference for miRNA. The reaction system was prepared using TB Green® Premix Ex Taq™ II (Takara) following manufacturers' instructions. Briefly,  $95^{\circ}\text{C}$  for 30 s followed by 40 cycles of  $94^{\circ}\text{C}$  for 5 s,  $60^{\circ}\text{C}$  for 34 s, and  $72^{\circ}\text{C}$  for 30 s. Relative expression was calculated using  $2^{-\Delta\Delta\text{Ct}}$  method. Primer sequences were GAPDH-forward, 5'-CGTGTT CCTACCCCAATGT-3' and reverse, 5'-TGTCATCAT ACTTGGCAGGTTTCT-3'; U6-forward, 5'-CTCGCTTCG

GCAGCACA-3' and reverse, 5'-AACGCTTCACGAATT TGGGT-3'; FASN-forward, 5'-GGAGGTGGTGATAGC CGGTAT-3' and reverse, 5'-TGGGTAATCCATAGAGCC CAG-3'; GRIA3-forward, 5'-TCCGGGCGGTCTTCTTTT TAG-3' and reverse, 5'-TCCACCTATGCTGATGGTGT-3'; and ACC-forward, 5'-ATGTCTGGCTTGACCTAGTA-3' and reverse, 5'-CCCCAAGCGAGTAACAAATTCT-3'.

### Western blot

Cells were harvested and lysed with RIPA buffer (Millipore). Total proteins were extracted and the concentration was measured by BCA kit (Thermo fisher). Samples were then loaded onto SDS-PAGE (10 ug per well) and separated. After that, proteins were transferred onto PVDF membranes (Millipore). Membranes were blocked with 5% BSA, incubated with primary antibody at  $4^{\circ}\text{C}$  overnight, washed 3 times with TBST, and incubated with secondary antibodies in dark for 1 h at room temperature. The signal was detected using ECL Western Blotting Substrate (Thermo fisher, #32106). The expression intensity was analyzed using imageJ (V1.53 for mac). Primary antibodies used in this study were GRIA3 (abcam, #ab40845), FASN (CST, #3189), and ACC (CST, #3662) and were all diluted 1:1000. HRP-linked secondary antibodies were goat anti-rabbit IgG (1:1000, CST, #7074).

### Oil red O staining

Briefly, cells were fixed with 10% formalin at room temperature for 1 h and washed by 60% isopropanol. Then, 0.2% oil red O was used to stain the cells at room temperature for 10 min. Cells were then washed 3 times with ddH<sub>2</sub>O. Excessive oil red O was removed by incubating cells with 1 ml isopropanol on a mild shaker for 10 min at room temperature.

### Triglyceride detection

Detection of triglyceride (TG) concentration was performed using commercial kit (Abcam, #ab65336) following manufacturers' instructions. Briefly, cells were collected and lysed. The lysates were loaded into a 96-well plate and incubated with assay buffer and lipase for 20 min. TG reaction mix was then added into each well and incubated for another 60 min. OD 570 nm was detected using a plate reader (Tecan).

### Dual-luciferase reporter assay

The assay was conveyed using dual-luciferase reporter assay system from Promega following manufacturers' instructions. Cells were seeded and cultured in a 24-well plate and

transfected with indicated luciferase plasmids. Signals were detected 48-h post-transfection.

### RNA immunoprecipitation (RIP) assay

We performed RIP assay using RIP kit from Millipore (#17-700) following manufacturers' instructions. Briefly, cells were lysed with lysis buffer and incubated with Ago2 antibody or IgG control. After immunoprecipitation, qRT-PCR analysis was performed to detect the enrichment of lncRNA NEAT1.

### Chromatin immunoprecipitation (ChIP)

We performed ChIP assay using commercial kit from Cell signaling technology (CST#9003). Briefly, cells were fixed with formaldehyde for 10 min and harvested. Then cells were lysed and incubated with micrococcal nuclease to break down chromatin into fragments. Immunoprecipitation was performed using IgG or anti-acetyl-H3K27 antibody (CST#8173). qRT-PCR was then performed to detect H3K27ac enrichment in promoter region of lncRNA NEAT1.

### Statistical analysis

We conveyed student T test (for comparison between groups) and one-way ANOVA (for comparison among groups) analysis using GraphPad Prism 6 software. Correlation analysis

was analyzed by Pearson's correlation. Data represented as mean  $\pm$  SD and all experiments were repeated at least 3 times.  $P < 0.05$  was considered as statistically significant.

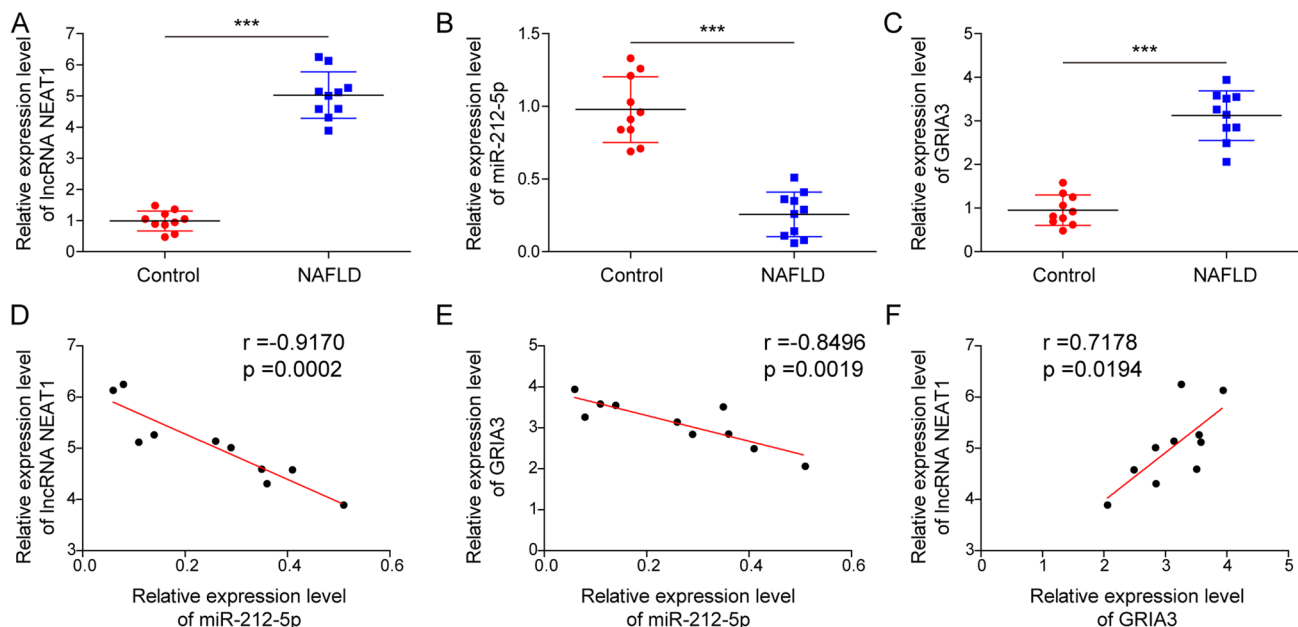
## Results

### lncRNA NEAT1 and GRIA3 was upregulated while miR-212-5p was downregulated in NAFLD patients

To evaluate whether NEAT1, GRIA3, and miR-212-5p was altered in NAFLD patients, we detected their expression by qRT-PCR in serum samples from 10 pairs of NAFLD patients and controls. While NEAT1 and GRIA3 were upregulated in NAFLD patients (Fig. 1A and C), RNA level of miR-212-5p was remarkably suppressed in NAFLD patients (Fig. 1B). In our further analysis, we also elucidated that NEAT1 was negatively correlated with miR-212-5p, miR-212-5p was negatively correlated with GRIA3, and NEAT1 was positively correlated with GRIA3 (Fig. 1D–F).

### FFA promoted lncRNA NEAT1 and GRIA3 expression while inhibiting miR-212-5p in hepatic cells

To evaluate whether lncRNA NEAT1, miR-212-5p, and GRIA3 participated in the development of NAFLD, we treated HepG2 and Huh-7 cells with free fatty acid (FFA) to induce lipid accumulation. The lipid droplets were analyzed



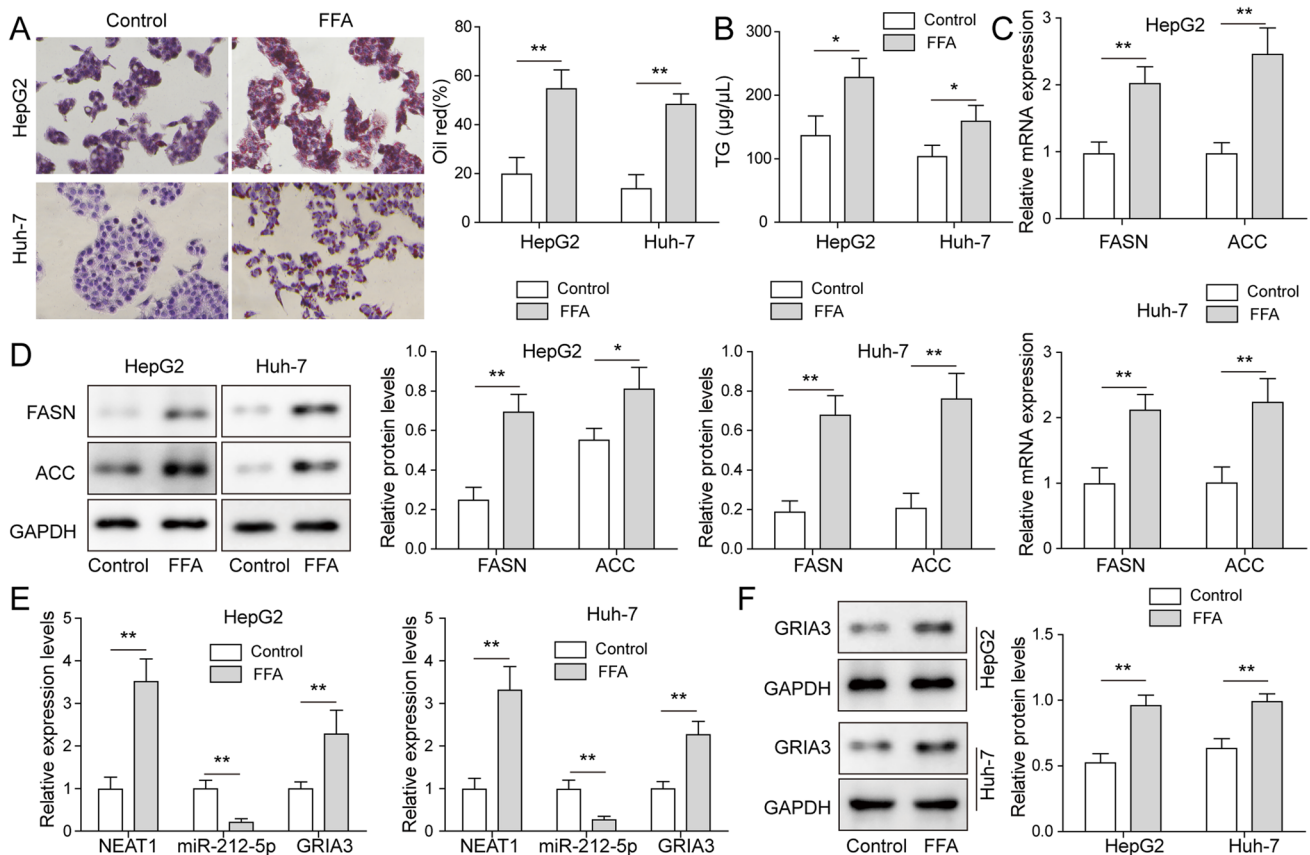
**Fig. 1** lncRNA NEAT1 and GRIA3 were upregulated while miR-212-5p was downregulated in NAFLD patients. Serum samples from 10 NAFLD patients and 10 controls were collected. Expression of A

NEAT1, B miR-212-5p, and C GRIA3 was detected by qRT-PCR. Correlation between D NEAT1 and miR-212-5p, E miR-212-5p and GRIA3, and F NEAT1 and GRIA3 was analyzed

by oil red O staining and TG level was detected by ELISA assay. More oil red O signal and TG were observed in FFA group. Our results indicated that FFA accumulated lipid accumulation within these hepatic cell lines (Fig. 2A and B). We also detected expression of lipid formation markers such as FASN and acetyl-CoA carboxylase (ACC) by both western blot and qRT-PCR. Both protein and mRNA level of these proteins were enhanced by FFA treatment (Fig. 2C and D). As we have confirmed that FFA could induce lipid accumulation in hepatic cells, we next tried to evaluate the trend of lncRNA NEAT1, miR-212-5p, and GRIA3 under FFA condition. The RNA level of lncRNA NEAT1 and GRIA3 was significantly promoted by FFA treatment, while miR-212-5p was significantly inhibited (Fig. 2E). Meanwhile, protein expression of GRIA3 showed similar trend and was promoted by FFA (Fig. 2F). These results implied abnormal expression of lncRNA NEAT1, miR-212-5p, and GRIA3 during lipid accumulation and indicated that they might be associated with NAFLD development.

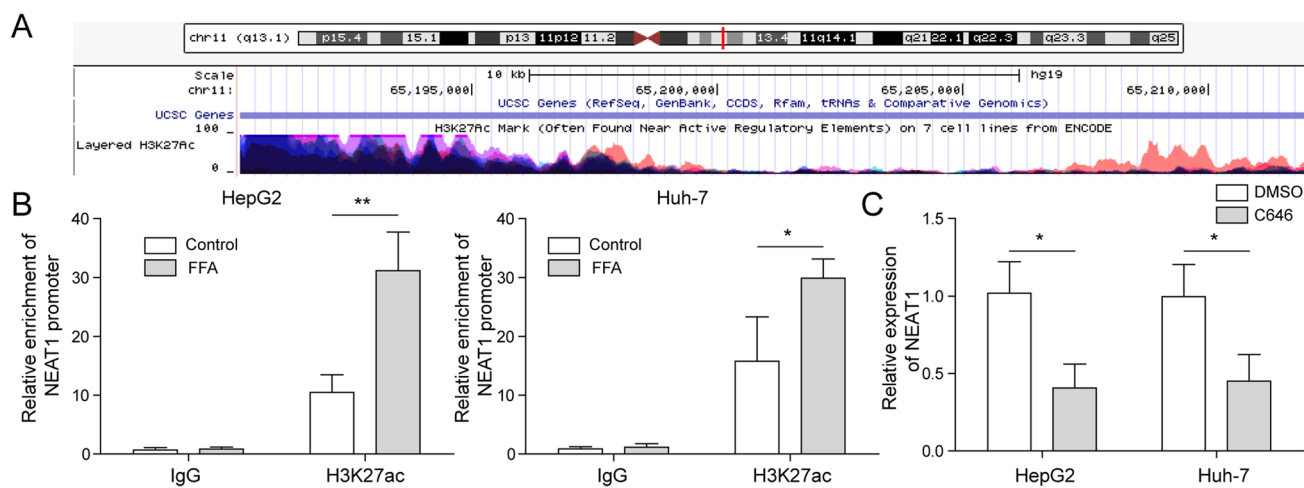
## Acetylation of H3K27 enhanced RNA level of lncRNA NEAT1 in FFA-induced hepatic cells

Highly acetylated region was predicted bioinformatically when we analyzed the promoter region of lncRNA NEAT1 (Fig. 3A). To validate this finding, we conveyed the following experiments in HepG2 and Huh-7 hepatic cells. ChIP assay was firstly performed using H3K27ac antibody (targeting acetylated H3K27). As shown in Fig. 3B, more DNA fragment was enriched by H3K27ac antibody after FFA treatment, indicating that FFA could enhance H3K27ac of lncRNA NEAT1 promoter. Next, we treated cells with C646, a histone acetyltransferase inhibitor, to suppress H3K27ac. Compared with control group, the RNA level of lncRNA NEAT1 was suppressed by C646 (Fig. 3C), indicating that H3K27ac was required for transcription of lncRNA NEAT1. All these results showed that acetylation of H3K27 enhanced RNA level of lncRNA NEAT1 in FFA-induced hepatic cells.



**Fig. 2** FFA promoted lncRNA NEAT1 and GRIA3 expression while inhibiting miR-212-5p in hepatic cells. HepG2 and Huh-7 cells were treated with FFA. **A** HepG2 and Huh-7 cells were stained with oil red O. **B** TG within these cells was analyzed by ELISA assay. **C** The mRNA level of FASN and ACC was detected by qRT-PCR. **D** The protein expression of FASN and ACC was analyzed by western blot. Statistical analysis of signal intensity was performed and displayed

in columns. **E** The RNA level of lncRNA NEAT1, miR-212-5p, and GRIA3 was analyzed by qRT-PCR. **F** The protein expression of GRIA3 was analyzed by western blot. Statistical analysis of signal intensity was performed and displayed in columns. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . Data are the means  $\pm$  SD from three independent experiments



**Fig. 3** Acetylation of H3K27 enhanced RNA level of lncRNA NEAT1 in FFA-induced hepatic cells. **A** Schematic display of acetylation analysis of the promoter region of lncRNA NEAT1. **B** HepG2 and Huh-7 cells were treated with FFA and subjected to ChIP with anti-H3K27ac antibody and IgG control. Relative enrichment of

lncRNA NEAT1 was analyzed and displayed in columns. **C** HepG2 and Huh-7 cells were treated with CC646 or DMSO control and the RNA level of lncRNA NEAT1 was analyzed by qRT-PCR. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . Data are the means  $\pm$  SD from three independent experiments

### Knockdown of lncRNA NEAT1 inhibited FFA-induced lipid accumulation in hepatic cells

We designed shRNA targeting lncRNA NEAT1 and transfected this shRNA into HepG2 and Huh-7 hepatic cells, aiming to reveal the role of lncRNA NEAT1 in FFA-induced lipid accumulation. While FFA originally enhanced lipid droplet signal in oil red O staining, sh-NEAT1 suppressed the signal (Fig. 4A). Besides, while FFA alone enhanced TG concentration, sh-NEAT1 restored TG to the similar level of control group (Fig. 4B). These results implied that sh-NEAT1 might inhibit FFA-induced lipid accumulation. We also detected RNA and protein levels of lipid formation markers FASN and ACC, which were as well inhibited by sh-NEAT1 (Fig. 4C and D), indicating that sh-NEAT1 could inhibit expression of lipid formation marker. Furthermore, expression of lncRNA NEAT1 and GRIA3 were both inhibited by sh-NEAT1 under FFA treatment (Fig. 4E and F). Besides, miR-212-5p was promoted by sh-NEAT1 under FFA condition (Fig. 4E). All these taken together demonstrated that sh-NEAT1 could inhibit FFA-induced lipid accumulation, and lncRNA NEAT1/miR-212-5p/GRIA3 axis was involved in the progression of NAFLD.

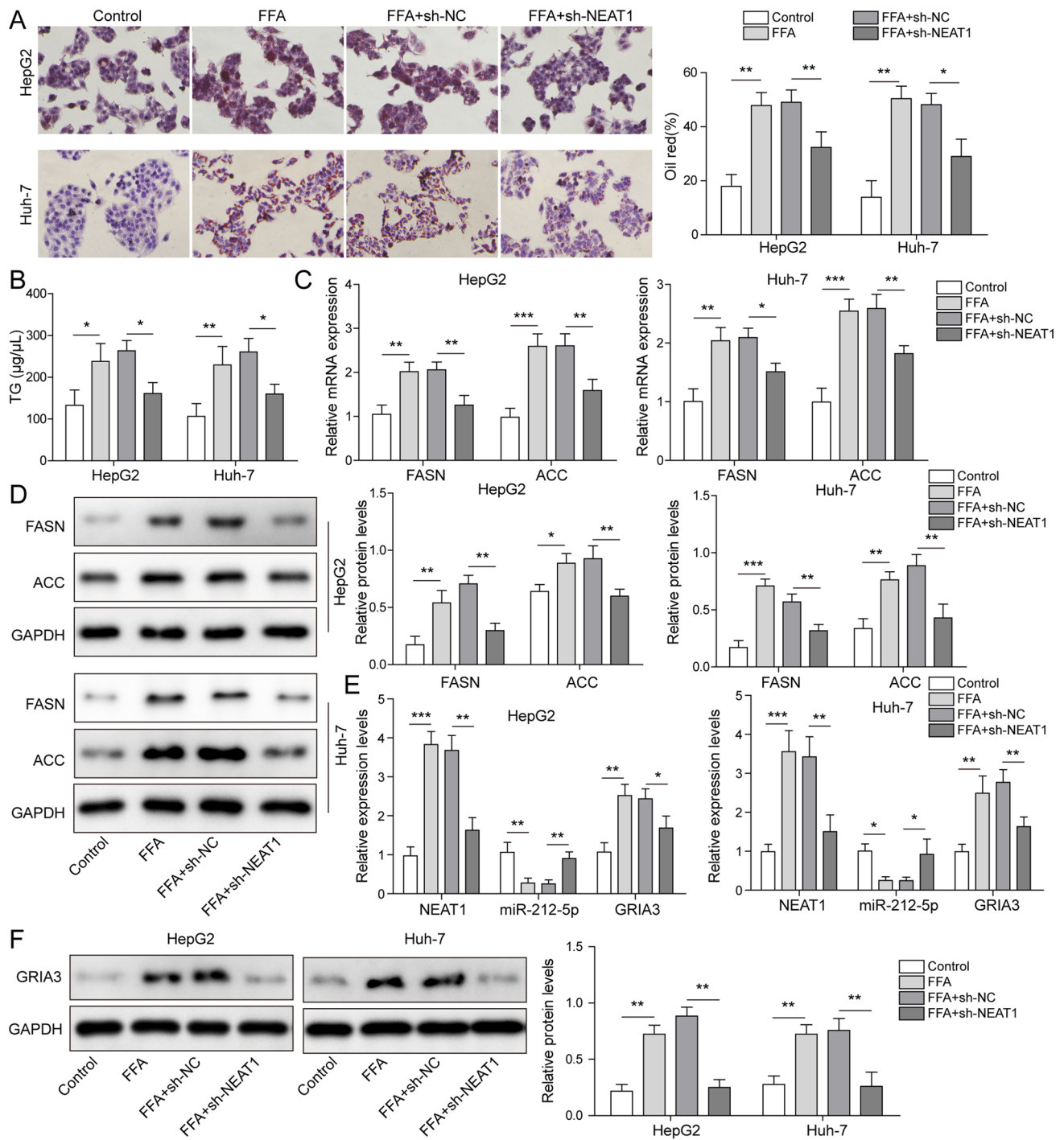
### lnc NEAT1 could directly suppress the level of miR-212-5p

As sh-NEAT1 affected miR-212-5p expression, we tried to reveal whether this effect was direct or indirect. Bioinformatics analysis of lncRNA NEAT1 and miR-212-5p sequences was performed and an interaction sequence was predicted (Fig. 5A). Dual-luciferase assay was then performed to

validate this finding. Luciferase system containing WT or mutated (MUT) binding sequence of lncRNA NEAT1 was designed and transfected into HepG2 and Huh-7 cells. In cells transfected with miR-212-5p mimics, the relative luciferase activity was significantly lower in NEAT1-WT group than that of NEAT1-MUT group (Fig. 5B), indicating that miR-212-5p could directly interact with lncRNA NEAT1. We also performed RIP experiment. In RIP assay we conveyed the pull down using antibody of Ago2, a key component of RNA-induced silencing complex (RISC). More lncRNA NEAT1 was recovered in cells with miR-212-5p mimics (Fig. 5C). This indicated that upregulation of miR-212-5p could promote interaction between Ago2 and lncRNA NEAT1. Next step we constructed lncRNA NEAT1 overexpression vector. The RNA level of miR-212-5p was significantly enhanced by sh-NEAT1, while inhibited by lncRNA NEAT1 overexpression (oe-NEAT1) (Fig. 5D). We could draw a conclusion that in hepatic cells, lncRNA NEAT1 could interact with miR-212-5p and suppress its level.

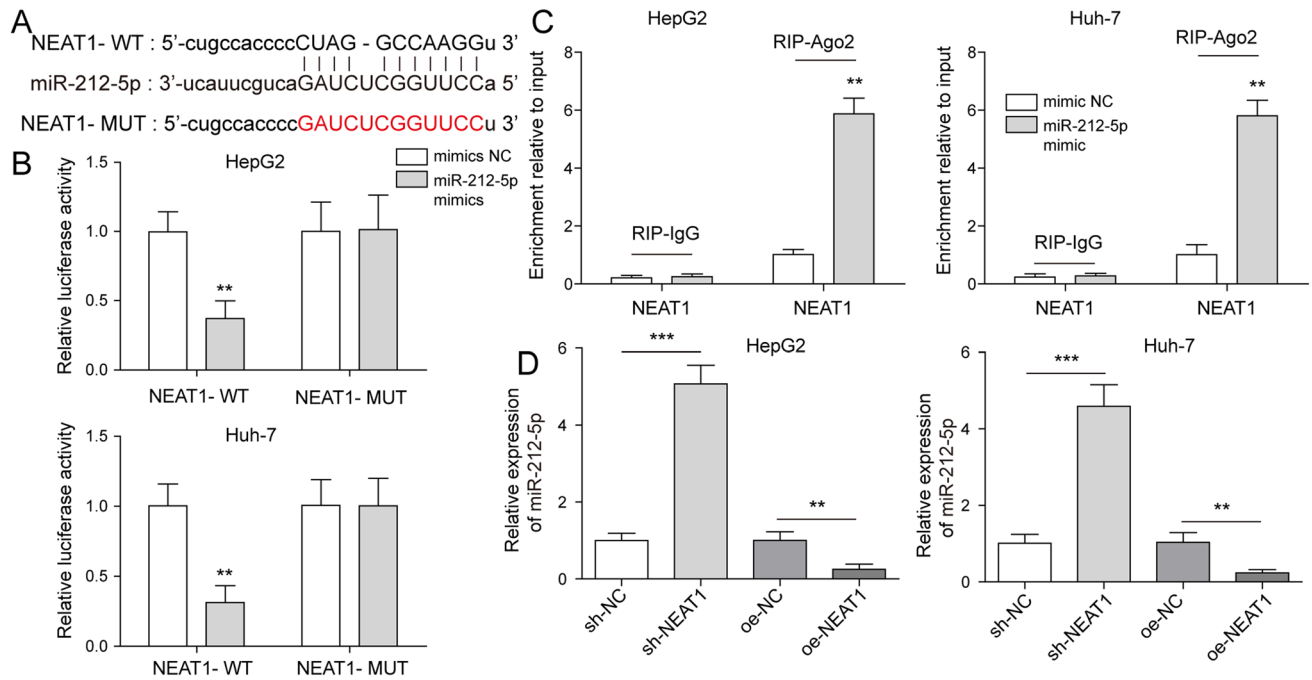
### Inhibition of miR-212-5p antagonized the inhibitory effect of sh-NEAT1 on FFA-induced lipid accumulation

In order to investigate whether miR-212-5p played critical role in regulating FFA-induced lipid accumulation, inhibitor of miR-212-5p was applied to HepG2 and Huh-7 cells. Sh-NEAT1 could originally inhibit oil red O signal and TG concentration, while miR-212-5p inhibitor boosted these two signals and antagonized the effect of sh-NEAT1 (Fig. 6A and B), indicating that miR-212-5p inhibitor could



**Fig. 4** Knock-down of lncRNA NEAT1 inhibited FFA-induced lipid accumulation in hepatic cells. HepG2 and Huh-7 cells were transfected with sh-NEAT1 and treated with FFA as indicated. **A** Lipid droplets were stained by oil red O, analyzed, and displayed in columns. **B** Concentration of TG within these cells were analyzed by ELISA assay. **C** The mRNA level of FASN and ACC was analyzed by qRT-PCR. **D** The protein level of FASN and ACC was analyzed

by western blot. The signal intensity was displayed in columns. **E** The RNA level of lncRNA NEAT1, miR-212-5p, and GRIA3 was analyzed by qRT-PCR. **F** Protein level of GRIA 3 was detected by western blot and signal intensity was displayed in columns. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . Data are the means  $\pm$  SD from three independent experiments



**Fig. 5** Lnc NEAT1 could directly suppress the level of miR-212-5p. **A** Schematic picture showing the bioinformatically predicted binding sequence between lncRNA NEAT1 and miR-212-5p. **B** HepG2 and Huh-7 cells were transfected with dual-luciferase reporter system containing WT/MUT NEAT1 and miR-212-5p mimics as indicated. Relative luciferase activity was analyzed by dual-luciferase assay

48-h post-transfection. **C** RIP analysis was performed in HepG2 and Huh-7 cells using anti-Ago2 antibody and enrichment of lncRNA NEAT1 was detected. **D** HepG2 and Huh-7 cells were transfected with sh-NEAT1 or oe-NEAT1 as indicated. The level of miR-212-5p was detected by qRT-PCR. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . Data are the means  $\pm$  SD from three independent experiments

promote FFA-induced lipid accumulation. Both mRNA and protein level of the lipid formation marker FASN and ACC were promoted by miR-212-5p inhibitor. And once again, the inhibitory effect of sh-NEAT1 on FASN and ACC was antagonized by the miR-212-5p inhibitor (Fig. 6C and D). Besides, miR-212-5p inhibitor boosted FFA-induced GRIA3 expression and antagonized the effects of sh-NEAT1 (Fig. 6E and F). Taken together, inhibition of miR-212-5p could promote FFA-induced lipid accumulation and reverse the inhibitory effect of sh-NEAT1 on this process.

### MiR-212-5p could target and inhibit GRIA3 expression

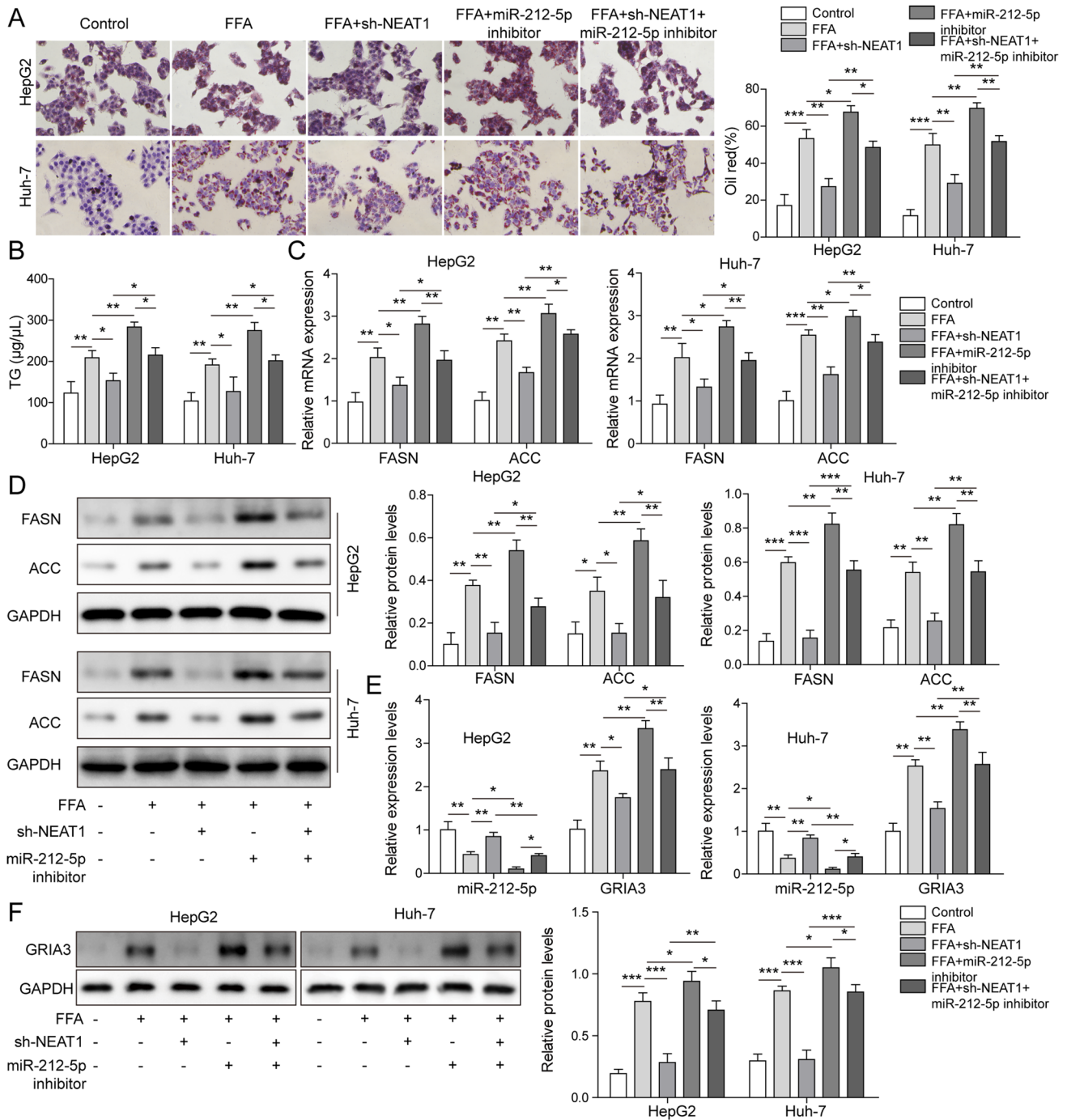
Bioinformatics analysis between miR-212-5p and 3'-UTR region of GRIA3 was conveyed and an interaction sequence was predicted (Fig. 7A). To validate this prediction dual-luciferase reporter assay was performed. Luciferase system containing WT/MUT GIRA3 3'-UTR was constructed and introduced into HepG2 and Huh-7 cells. In cells transfected with miR-212-3p mimics, the relative luciferase activity was inhibited in GRIA3-WT group instead of GRIA3-MUT group (Fig. 7C). This result demonstrated that miR-212-5p could indeed directly interact with GRIA3 and mediate its mRNA degradation. To confirm this finding, mRNA and

protein level of GRIA3 were detected by qRT-PCR and western blot, respectively, and they were both inhibited by miR-212-5p mimics (Fig. 7B and D). Besides, miR-212-5p inhibitor dramatically boosted GRIA3 expression (Fig. 7B and D). Through experiments we could conclude that miR-212-5p could directly target and inhibit GRIA3 expression.

### Knockdown of GRIA3 could antagonize the promoting effect on FFA-induced lipid accumulation mediated by miR-212-5p inhibitor

We constructed shRNA against GRIA3 (sh-GRIA3) aiming to exploring its role in FFA-induced lipid accumulation. Knockdown of GRIA3 by sh-GRIA3 in both HepG2 and Huh-7 cells resulted in hampered lipid accumulation under FFA condition (Fig. 8A and B), represented by decreased oil red O signal and TG concentration. Besides, shGRIA3 antagonized with miR-212-5p inhibitor, which could promote FFA-induced lipid accumulation (Fig. 8A and B). What's more, expression of the lipid formation marker FASN and ACC was suppressed by sh-GRIA3. Again, shGRIA3 antagonized with miR-212-5p inhibitor, which could promote expression of FASN and ACC (Fig. 8C and D). Besides, sh-GRIA3 somehow suppressed GRIA3 expression, also counteracted with miR-212-5p



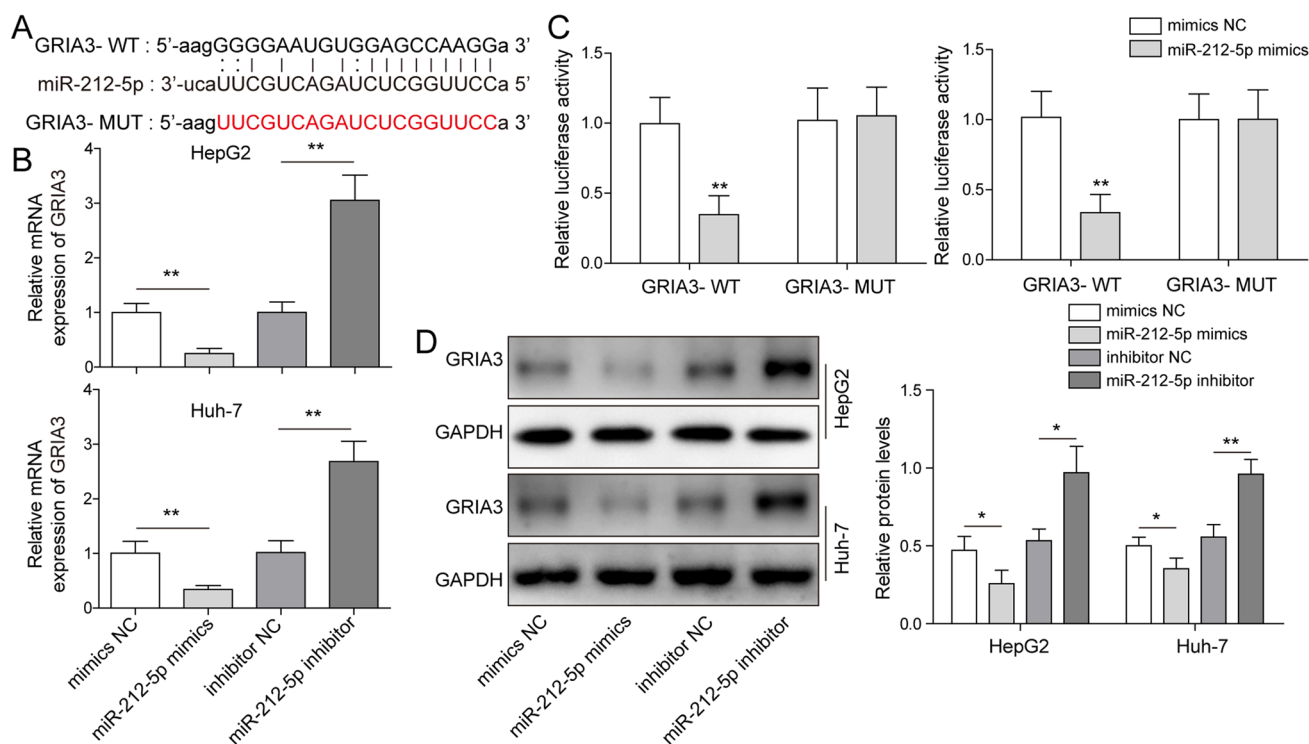


**Fig. 6** Inhibition of miR-212-5p antagonized the inhibitory effect of sh-NEAT1 on FFA-induced lipid accumulation. Sh-NEAT1 and miR-212-5p inhibitor were transfected into HepG2 and Huh-7 cells as indicated under FFA treatment. **A** Lipid droplet was stained by oil red O. **B** TG concentration was detected by ELISA assay. **C** The mRNA level of FASN and ACC was analyzed by qRT-PCR. **D** The

protein level of FASN and ACC was analyzed by western blot. The signal intensity was displayed in columns. **E** The RNA level of miR-212-5p and GRIA3 was analyzed by qRT-PCR. **F** Protein level of GRIA 3 was detected by western blot and signal intensity was displayed in columns. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . Data are the means  $\pm$  SD from three independent experiments

inhibitor, and indicated miR-212-5p inhibitor could promote GRIA3 expression (Fig. 8E and F). All these results indicated that knockdown of GRIA3 could antagonize the

promotive function of miR-212-5p inhibitor on lipid accumulation under FFA condition.



**Fig. 7** MiR-212-5p could target and inhibit GRIA3 expression. **A** Schematic picture showing the bioinformatically predicted binding sequence between miR-212-5p and GRIA3. **B** HepG2 and Huh-7 cells were transfected with miR-212-5p mimics or treated with miR-212-5p inhibitor as indicated. The mRNA level of GRIA3 was detected by qRT-PCR. **C** HepG2 and Huh-7 cells were transfected with miR-212-5p mimics and dual-luciferase system containing WT/

MUT GRIA3. Relative luciferase activity was detected 48-h post-transfection. **D** HepG2 and Huh-7 cells were transfected with miR-212-5p mimics or treated with miR-212-5p inhibitor as indicated. The protein level of GRIA3 was detected by western blot and signal intensity was displayed in columns. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . Data are the means  $\pm$  SD from three independent experiments

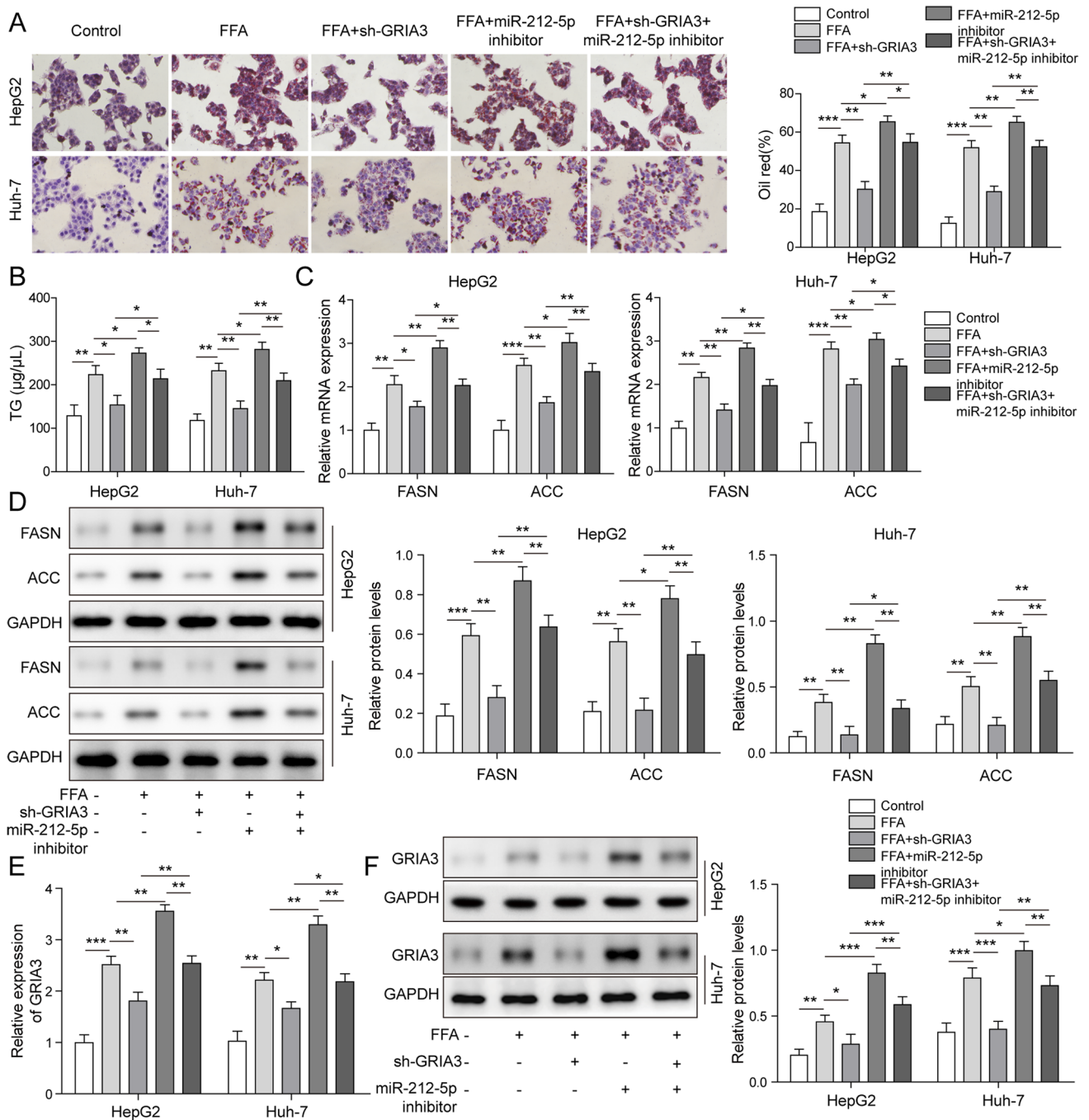
## Discussion

NAFLD had become a world-wide health burden and was the most common cause of chronic liver disease and even liver cancer [21]. Risk factors of NAFLD included obesity, cardiovascular, and metabolic diseases, suggesting that NAFLD was a multi-system disease involving multiple signal pathways [22]. Hence, studies about the regulatory mechanism of NAFLD was critical for development of treatment strategies and for revealing therapeutic targets. In this study we revealed that H3K27ac was enriched within the promoter of lncRNA NEAT1 and promoted lncRNA NEAT1 transcription. lncRNA NEAT1 could then interact with miR-212-5p and suppress its expression. MiR-212-5p could inhibit GRIA3, decreased miR-212-5p resulted in GRIA3 upregulation, and finally promoted lipid accumulation.

Acetylation was an important post-transcriptional modification, which could usually induce receptor dimerization or mediate protein–protein interaction [23]. Acetylation on histone, especially on H3K27, usually facilitated gene expression. For instance, H3K27ac enrichment in promoter of ERV in glioma [24]. H3K27ac could also activate transcription

of lncRNA [11, 25]. It was reported that H3K27ac enrichment activated expression of lncRNA-CCAT1, which contributed to esophageal squamous cancer regulation [25]. We confirmed FFA-induced enrich of H3K27 acetylation at the promoter of lncRNA NEAT1 and promoted its transcription. This fitted the well-accepted function of H3K27ac. We validated that H3K27ac induced lncRNA NEAT1 upregulation and promoted lipid accumulation that may cause NAFLD. Such finding was in agreement with other publications about how lncRNAs promoted NAFLD [8, 26, 27]. The mechanism by which FFA affected H3K27 acetylation was not studied, which is also the deficiency of our research. This will also become a new exploration direction in our future studies.

A common notion about lncRNA was the ceRNA theory that they could interact with target miRNAs and suppress their expression, resulting in miRNAs inhibition and upregulating the target genes of miRNA [28]. lncRNA NEAT1 was also involved in regulation of multiple miRNAs in various tissues. For instance, lncRNA NEAT1 could inhibit miR-204 and activate NF-kappaB signal in kidney [29]. In corneal fibroblast, lncRNA NEAT1 could target



**Fig. 8** Knock-down of GRIA3 could antagonize the promoting effect on FFA-induced lipid accumulation mediated by miR-212-5p inhibitor. HepG2 and Huh-7 cells were co-transfected with sh-GRIA3 and miR-212-3p inhibitor and FFA as indicated. **A** Lipid droplet was stained with oil red O. **B** TG concentration was analyzed by ELISA. **C** The mRNA level of FASN and ACC was analyzed by qRT-PCR. **D** The protein level of FASN and ACC was analyzed by western

blot. Signal intensity was analyzed and displayed in columns. **E** The mRNA level of GRIA3 was analyzed by qRT-PCR. **F** The protein level of GRIA3 was analyzed by western blot. Signal intensity was analyzed and displayed in columns. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . Data are the means  $\pm$  SD from three independent experiments

and bind with miR-1246, inhibiting its function and promoting inflammatory response [30]. In our study, lncRNA NEAT1 was upregulated in NAFLD patients; lncRNA NEAT1 harbored binding sequence and directly inhibited

miR-212-5p. MiR-212-5p played crucial role in lncRNA NEAT1 to mediate NAFLD development, and inhibition of miR-212-5p could promote FFA-induced lipid accumulation and reverse the inhibitory effect of sh-NEAT1 on this

process. This finding agreed with the ceRNA theory and extended the target spectrum of lncRNA NEAT1 in NAFLD regulation. Nevertheless, there might be other miRNAs associating with lncRNA NEAT1 signal network during NAFLD and we should pay more attentions in the following studies.

A ceRNA network involved a target protein besides lncRNA and miRNA. In this study we revealed GRIA3 as a target of miR-212-5p. GRIA3 was reported to be regulated by miRNAs, such as miR-330-3p in lung cancer [31] and miR-124 in depression [32]. We firstly identified GRIA3 as a direct target of miR-212-5p in hepatic cells and miR-212-5p-downregulated GRIA3 expression could inhibit FFA-induced lipid accumulation. This results shed new light on the regulatory mechanism of GRIA3.

To sum up, in this study we found that H3K27ac was enriched in the promoter region of lncRNA NEAT1 and promoted lncRNA NEAT1 transcription. LncRNA NEAT1 could interact with miR-212-5p and suppress its expression. MiR-212-5p could directly interact with and suppress the expression of GRIA3. Decreased miR-212-5p resulted in GRIA3 upregulation and finally promoting lipid accumulation. This study firstly revealed the fundamental role of signal axis lncRNA NEAT1/miR-212-5p/GRIA3 during NAFLD and demonstrated that acetylation of H3K27 could regulate this signal axis. These findings would support future studies and provide potential target to develop new therapeutic options to prevent NAFLD.

In this paper, we mainly studied the signal mechanism of lncRNA NEAT1 miR-212-5p/GRIA3. We found that lncRNA NEAT1 was highly expressed in the model of fat accumulation in liver cells treated by FFA. Because it has been found that the H3K27 acetylation could promote lncRNA expression, we explored whether the promotion of FFA on the lncRNA expression involves the modification of H3K27 acetylation.

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**Data availability** The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

**Code availability** Not applicable.

## Declarations

**Conflict of interest** The authors declared no conflict of interest.

**Ethical approval** This study was approved by The First Affiliated Hospital of University of South China Ethical Committee and all experiments were designed and conveyed following the Declaration of Helsinki. Ten patients with NAFLD and 10 healthy controls from The First Affiliated Hospital of University of South China were enrolled in the present study.

**Consent to participate** Not Applicable. This article does not contain any studies with human participants or animals performed by any of the authors.

**Consent for publication** Not Applicable.

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