

IGF2BP3 Mediates the mRNA Degradation of NF1 to Promote Triple-Negative Breast Cancer Progress via an m6A-Dependent Manner

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Abstract

N6-methyladenosine (m6A) is the most abundant reversible modification of eukaryotic mRNA. Emerging evidence has supported that m6A plays a vital role in tumorigenesis. As a crucial reader, IGF2BP3 usually mediates the stabilization of mRNAs in an m6A-dependent manner. However, the functions and m6A mechanism of IGF2BP3 have not been reported in triple-negative breast cancer (TNBC). Here we showed that IGF2BP3 was highly expressed in TNBC tissues and cell lines and was associated with poor prognosis. TET3-mediated IGF2BP3 promoter hypomethylation leads to upregulation of IGF2BP3 expression in TNBC. Knocking down IGF2BP3 markedly inhibited the proliferation and increased the apoptosis of TNBC *in vitro* and *in vivo*. Intersection co-assays revealed that IGF2BP3 decreased NF1 stabilization in an m6A-dependent manner. NF1 expression was negatively correlated with IGF2BP3, and knockdown of NF1 could rescue the phenotypes of IGF2BP3 knockdown cells partially *in vitro* and *in vivo*. Mechanistically, m6A reader IGF2BP3 promoted proliferation and inhibited the apoptosis of TNBC by destabilizing NF1 mRNA in an m6A-dependent manner. TET3-mediated IGF2BP3 promoter hypomethylation leads to upregulation of IGF2BP3 expression in TNBC. It suggested that IGF2BP3 has the potential to be the target for TNBC treatment.

Introduction

Breast cancer (BC) is the most common malignant tumor. The morbidity and mortality of breast cancer also rank first in female malignant tumors, posing a severe threat to the health and lives of women[1, 2]. Based on the expression of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), breast cancer is divided into four subtypes, including luminal A, luminal B, HER2-enriched and triple-negative breast cancer (TNBC)[3]. Different breast cancer subtypes show varied characteristics concerning biological properties, therapy strategies, and clinical prognosis[4]. Among them, TNBC is characterized by larger tumor size, higher grade, and poor survival, accounting for about 20% of all breast cancer cases[5, 6]. More than 70% of metastatic TNBCs patients did not survive after five years of diagnosis and showed the worst outcomes compared to other subtypes[7]. Therefore, it would be vital to research the molecular mechanisms of TNBC and develop novel treatment strategies.

At present, epigenetic modification plays an essential role in the occurrence and development of different tumors[8]. N6-methyladenosine (m6A) is the most common transcriptome modification in eukaryotic cells[9, 10] and influences mRNA splicing, localization, export, translation, decay, and stability[11–13]. m6A modification of mRNA is a dynamic and reversible process[14], introduced by methyltransferases ('writers') including methyltransferase-like 3 (METTL3), METTL14 and Wilms tumor 1-associated protein (WTAP), and fat mass and obesity-associated protein (FTO) and human AlkB homolog H5 (ALKBH5) induce the demethylation process('erasers')[15, 16]. In addition, m6A modification is functionally executed by 'readers'. YTH domain-containing proteins (YTHDC1, YTHDC2, YTHDF1, YTHDF2 and YTHDF3) and the insulin-like growth factor 2 mRNA-binding proteins (IGF2BP1, IGF2BP2, IGF2BP3) are primarily defined 'readers' to mediate the pre-mRNA processing, mRNA stability, degradation and translation processes[17–20].

IGF2BP3, also known as IMP3, was first discovered due to its high expression in pancreatic carcinoma[21]. After its initial discovery, IGF2BP3 was quickly interpreted to be the primary overexpressed among multiple tumors, such as lung cancer[22], ovarian cancer[23], liver cancer[24], osteosarcoma[25], bladder cancer[26] and breast cancer[27] and the abnormal upregulation showed its potential role in tumorigenesis. Previous studies have demonstrated that IGF2BP3 acts in various critical biological pathways and is involved in various critical cellular functions in breast cancer. For instance, IGF2BP3 facilitated TAZ activation by stabilizing Wnt Family Member 5B (WNT5B) mRNA in breast cancer [28]. It promoted chemoresistance by regulating ATP Binding Cassette Subfamily G(ABCG) expression in breast cancer [29]. Furthermore, IGF2BP3 promoted stem-like properties by regulating SLUG in TNBC[30]. IGF2BP3 also promoted metastasis through destabilization of progesterone receptors in breast cancer[31]. However, IGF2BP3 participated in tumor progression as an RNA-binding protein and m6A related mechanism was not involved in these studies.

Herein, we investigated the role of IGF2BP3 in TNBC and explored the deeper m6A related mechanisms that IGF2BP3 regulated the proliferation and apoptosis of TNBC. Firstly, we demonstrated that IGF2BP3 was upregulated and correlated with poor prognosis in TNBC compared with other breast cancer subtypes. We then found that TET3-mediated hypomethylation of IGF2BP3 promoter lead to upregulation of IGF2BP3 expression in TNBC. In addition, we found that IGF2BP3 was associated with TNBC cells proliferation and apoptosis *in vitro* and *in vivo*. mRNA-seq, RIP-seq and MeRIP-seq identified the potential targeting genes of IGF2BP3 in TNBC, and the multi-omics analysis identified the neurofibromin 1(NF1) as the direct target of IGF2BP3 in TNBC. Moreover, IGF2BP3 acted as an oncogene in TNBC by regulating NF1 stabilization through an m6A-dependent manner. In the present study, we discovered the specific mechanism of IGF2BP3 in TNBC proliferation.

Materials And Methods

Cell lines and cell culture

The human breast cancer cell lines MCF-7, ZR-75-1, BT474, SK-BR-3, MDA-MB-453, HCC-1806, MDA-MB-231, BT549 and breast epithelial cell line MCF-10A were purchased from American Type Culture Collection (ATCC, USA). Among them, MCF-7 and ZR-75-1 are considered luminal subtype. BT474 and SK-BR-3 represent the HER2-positive subtype, MDA-MB-453, HCC-1806, MDA-MB-231 and BT549 belong to the TNBC subtypes.

The cell lines were cultured in DMEM (Wisent, China), in addition, HCC-1806 were cultured in RPMI-1640 (Wisent, China), which were all supplemented with 10% FBS and penicillin-streptomycin (100 µg/ml). The cells were incubated at 37°C in a humidified atmosphere with 5% CO₂.

Lentivirus and siRNA transfection

Lentivirus were generated to knockdown and overexpress IGF2BP3. The MDA-MB-231 and HCC-1806 cells were infected with IGF2BP3 knockdown (termed as shIGF2BP3-1 and shIGF2BP3-2) lentivirus and

matched negative control vectors (termed as shRNA-NC), IGF2BP3 overexpress (termed as IGF2BP3) lentivirus and matched negative control vectors (termed as Vector), following the manufacturer's protocol (Obio Technology, China). We finally selected the stable cell lines with 3 µg/ml puromycin. Mechanistic studies used shIGF2BP3-1 structure with the higher efficiency named shIGF2BP3

The MDA-MB-231 and HCC-1806 cells were transfected with TET1-siRNA, TET2-siRNA, TET3-siRNA, NF1-siRNA (GenePharma, Shanghai, China) and non-silencing control. The IGF2BP3 negative control and knockdown TNBC cells MDA-MB-231 and HCC-1806 were transfected with NF1 siRNA and negative control vectors (shRNA-NC+Vector, shIGF2BP3+Vector, shRNA-NC+siNF1, shIGF2BP3+ siNF1). (GenePharma, China). The sequences of lentivirus and siRNAs were listed in Table S1.

qRT-PCR

Total RNA was extracted from clinical specimens or breast cancer cells using Trizol reagent (Takara, Japan). RNA was used to reverse transcription with PrimescriptTM reverse transcription reagent (Takara, RR036A, Japan) following the manufacturer's protocol. qRT-PCR was performed with the Roche LightCycler 480 RT-PCR system (Roche, Switzerland). The relative RNA expression level was calculated by $2^{-\Delta\Delta C_t}$ method and with the level normalized to β -actin. The specific PCR primers were purchased from Invitrogen Trading (Shanghai, China) and listed in supplementary Table S2.

Western blot analysis

Western blot was performed as previously described[32]. Briefly, cells were ruptured with RIPA (P0013C, Beyotime, China). Whole cell lysates were fractionated and transferred to a PVDF membrane (Millipore, USA). The primary antibodies were anti-rabbit IGF2BP3 (14642-1-AP, proteintech, China), caspase3 (19677-1-AP, proteintech, China), caspase9 (10380-1-AP, proteintech, China), NF1 (27249-1-AP, proteintech, China), anti-mouse β -actin (66009-1-Ig, proteintech, China). The dilutions of antibodies were according to the product usage information.

CCK-8 assay

Cell viability was detected using the CCK8 kit (Vazyme, Chian) as previously described[33]. Briefly, cells were plated in a 96-well plate at 2000 cells/well. The absorbance was measured by OD450 for 2 h after adding 10% CCK8 solution.

Colony formation assay

Colony-formation assay was conducted as previously described[34]. Cells were maintained in 6-well plates at 5×10^3 cells/well. After two weeks of culture, cells were stained with 0.1% crystal violet(C0121, Beyotime, China). The number of colonies was counted.

EdU assay

Following the manufacturer's instructions, EdU assays were performed using an EdU Cell Proliferation Kit (C0075, Beyotime, China). Briefly, cells were maintained in a 96-well plate at 2×10^4 cells/well and incubated with 50 μ M EdU per well at 37°C for 2 h. Then cells were fixed with 4% paraformaldehyde and stained with 1× Click Reaction Buffer and 1× Hoechst 33342 solution. In the end, the cells were observed under the Zeiss fluorescence photomicroscope.

Flow cytometry analysis

The apoptosis rate of cells was probed by Annexin V-APC/7-AAD apoptosis kit (MultiSciences Biotech, Hangzhou, China). Cells in different treatment groups were cultured for 24 h, and obtained 3×10^5 cells (including culture supernatant cells). Then resuspending cells with 300 μ l 1×Binding Buffer, each tube add 10 μ l 7-AAD and 5 μ l annexin V-APC. After vortexing gently and incubating in the dark for 15 minutes, samples were analyzed by FCM and the BDFACSCalibur™ system.

Animal Models

Balb/c nude mice (4-6 weeks) were randomly divided into 4 groups (8 mice per group). Stable shRNA-NC, shIGF2BP3, shRNA-NC+siNF1 and shIGF2BP3+siNF1 MDA-MB-231 cells (1×10^7 cells) were injected into nude mice. Tumor volumes were measured every 4 days [volume = $0.5 \times \text{length} \times (\text{width})^2$]. Animal experiments were carried out following the ethical standards of experimental animal institutions approved by the Animal Management Committee of Nanjing Medical University.

Primary tissue samples

Twenty-seven pairs of TNBC and matched adjacent regions were provided by the First Affiliated Hospital with Nanjing Medical University from 2015 to 2016, in according with the ethical guidelines of the Declaration of Helsinki and approved by the ethics and research committee of the First Affiliated Hospital of Nanjing Medical University.

Methylation-Specific PCR

The cells were extracted according to the instructions of QIAmp DNA Mini Kit (Qiagen). After diluting 1 μ L of DNA sample by 50 times, the DNA was converted to unmethylated cytosine bisulfite according to the instructions of the Epi Tect Bisulfite Kit (Qiagen). After the bisulfite modification, the DNA was eluted and purified and MS-PCR was then performed. Methylation specific primer sequences were shown in Table 2.

mRNA high-throughput sequencing

According to the manual's protocol, Trizol reagent (Takara, Japan) was used to isolate total RNA from stably IGF2BP3 knockdown or control MDA-MB-231 cells. After the samples were qualified, 3 μ g RNA was selected, and the Small RNA Sample Pre Kit was used to construct the library. The filtered sequences were then compared to the reference genome (hg38). The library establishment and next generation

sequencing (NGS) were performed by Beijing Allwegene (Beijing, China). Each group was sequenced in 3 replicates.

RIP sequencing

The RIP assay was performed using RNA immunoprecipitation lysis buffer (Millipore, USA). According to the manufacturer's instructions, MDA-MB-231 cell lysate was incubated with 5 µg of anti-rabbit IGF2BP3 (14642-1-AP, proteintech, China) or IgG at 4°C overnight. The RNA-protein complexes were adsorbed by protein A/G magnetic beads, and then performed RNA purification.

According to the manufacturer's protocol, index-encoded samples were clustered on the cBot Cluster Generation System using the TruSeq PE Cluster Kit v3-cBot-HS (Illumina). Then the library was sequenced using the PE 150 sequencing strategy on the Illumina Hiseq 4000 platform of Beijing Allwegene (Beijing, China).

MeRIP sequencing

Total RNAs were isolated from MDA-MB-231 cells. After fragmentation, cell lysate was incubated with m6A antibody (ab208577, Abcam, US) for immunoprecipitation according to the protocol of the MeRIP m6A kit (Millipore, Germany). The RNA fragments are first ligated at the 3' end before the chemical reaction. They are then reverse transcribed before the 3' adapters are ligated to the final cDNA. During reverse transcription, only fragments containing internal m6A sites are misincorporated, which can be further detected by high-throughput sequencing. The library establishment and NGS were performed by Beijing Allwegene (Beijing, China).

mRNA stability analysis

IGF2BP3 knockdown and overexpression MDA-MB-231 and HCC-1806 cell lines were plated into 6-well plates and then treated with actinomycin D (ActD) 5 µg/ml at 0 h, 2 h, 4 h and 6 h before collection. Total RNA was isolated to quantify the relative NF1 mRNA levels.

Luciferase assay

The MDA-MB-231 and HCC-1806 cells were transfected with negative pGL3 reporter and luciferase vector (NF1-A, NF1-B, NF1-C, NF1-D, NF1-E, NF1-B-mut, NF1-C-mut). Firefly and renilla luciferase activities were tested by the Dual-Luciferase reporter assay system (Promega, USA). The primers for vectors are listed in Table S3.

Statistical analysis

The data were analyzed using the SPSS 19.0 software. All experiments were performed in triplicate unless otherwise stated. The linear correlation analysis evaluated the correlation between IGF2BP3 and NF1. For all the continuous variables, Student *t*-test and two-way ANOVA were used for comparing differences between groups. **P*<0.05 was considered as statistically significant.

Results

IGF2BP3 was upregulated in TNBC and correlated with a poorer prognosis

To identify differentially expressed molecules in different breast cancer subtypes, we analyzed The Cancer Genome Atlas (TCGA) datasets between TNBC and non-TNBC for gene expression differences. The analyses revealed that IGF2BP3 and IGF2BP2 mRNA was obviously higher in TNBC than non-TNBC among the eight m6A' readers' (IGF2BP1, IGF2BP2, IGF2BP3, YTHDC1, YTHDC2, YTHDF1, YTHDF2, YTHDF3) (Fig 1a). Moreover, IGF2BP3 mRNAs were also upregulated in breast cancer tissues than in normal tissues (Fig 1b), especially in TNBC tissues (Fig 1c). We further proved that IGF2BP3 was highly expressed in TNBC tissues than adjacent normal tissues of our hospital patients (Fig 1d and 1e). IGF2BP3 expression in breast cancer cell lines was detected by qRT-PCR (Fig. 1f) and Western blot (Fig. 1g). Among the breast cancer cells, IGF2BP3 was highly expressed in TNBC cell lines (MDA-MB-453, HCC-1806, MDA-MB-231, BT-549), compared to non-TNBC cell lines (MCF-7, ZR-75-1, BT-474, SK-BR-3) both in mRNA and protein levels. Kaplan-Meier survival curves of TNBC indicated that high expression of IGF2BP3 was correlated with poor overall survival in TNBC patients (*P=0.02) (Fig.1h).

The promoter of IGF2BP3 hypomethylated in TNBC

IGF2BP3 expression is regulated by its DNA methylation status of promoter[35]. Moreover, we explored whether the upregulation of IGF2BP3 was related to the methylation status of its promoter in TNBC. The CpG islands in the IGF2BP3 promoter were shown in Fig. 2a. IGF2BP3 promoter methylation level was downregulated in TNBC tissues than in non-TNBC tissues (Fig. 2b). Moreover, to further investigate the transcriptional activity of IGF2BP3 in the different subtypes of breast cancer, the IGF2BP3 promoter luciferase construct pGL-IGF2BP3 was transfected into breast cancer cell lines. The results indicated that the luciferase activity was higher in TNBC cell lines than in non-TNBC cell lines (Fig. 2c). Similarly, Methylation-specific PCR (MSP) analysis revealed that IGF2BP3 methylation levels were substantially lower in TNBC cell lines than in non-TNBC cell lines (Fig. 2d).

The TET enzymes oxidize 5-methylcytosine to 5-hydroxymethylcytosine, which can lead to DNA demethylation[36]. To explore the potential roles of DNA demethylase in regulating IGF2BP3 promoter methylation level in TNBC, we knocked down TET1, TET2, and TET3 in MDA-MB-231 and HCC-1806 cells lines using specific siRNAs (Fig. S1a-c). We found knock down of TET3, but not TET1 and TET2, resulted in a decrease of IGF2BP3 expression (Fig. 2e-g). These results indicated that the high expression of IGF2BP3 was related to the hypomethylation of its promoter in TNBC.

Knockdown of IGF2BP3 inhibited the proliferation and promoted the apoptosis of TNBC *in vivo* and *in vitro*

To investigate the effect of IGF2BP3 in TNBC cells, MDA-MB-231 and HCC-1806 cell lines were stably transfected with knockdown IGF2BP3 and control lentivirus. The knockdown cell lines were named shIGF2BP3-1 and shIGF2BP3-2, and the matched control was named shRNA-NC. The expression of

IGF2BP3 was confirmed with qRT-PCR and western blot (Fig. 3a-b). The CCK8 assays, colony formation and EdU analysis assays indicated that knockdown of IGF2BP3 decreased cell proliferation (Fig. 3c-h and Fig S2a-b). Moreover, the flow cytometry analysis indicated the percentage of both early and late apoptotic cells based on Annexin V-APC/7-AAD staining were significantly increased upon knockdown of IGF2BP3 in MDA-MB-231 and HCC-1806 cell lines (Fig. 3i), and downregulation of IGF2BP3 increased the expression of cleaved-caspase 3 and cleaved-caspase 9 (Fig. 3j). In xenograft models, the tumor volume increased slower in the IGF2BP3 knockdown group than the control group (Fig. 3k). Four weeks later, tumor volume and weights in the IGF2BP3 knockdown group were both lower than the control group (Fig. 3l-m), which indicates that knockdown of IGF2BP3 inhibited tumor proliferation *in vivo*.

Overexpression of IGF2BP3 promoted proliferation and inhibited the apoptosis of TNBC *in vitro*

MDA-MB-231 and HCC-1806 cell lines were stably transfected with overexpression and control lentivirus, respectively named IGF2BP3 and Vector. We confirmed the IGF2BP3 expression using qRT-PCR and western blot (Fig. S3a-b). The proliferation ability of these infected cells was determined using CCK-8 assay, colony formation assay and EdU assay. IGF2BP3 overexpression increased cell proliferation in MDA-MB-231 and HCC-1806 cells (Fig. S3c-h). Moreover, the flow cytometry analysis indicated that early and late apoptotic cells significantly decreased upon overexpression of IGF2BP3 in MDA-MB-231 and HCC-1806 cell lines (Fig. S3i), and upregulation of IGF2BP3 decreased the expression of cleaved-caspase 3 and cleaved-caspase 9 (Fig. S3j).

Identification of the IGF2BP3 targets in TNBC

To explore the potential mechanisms of IGF2BP3 in TNBC, we firstly performed RNA-seq analysis on IGF2BP3 knockdown and controlled MDA-MB-231 cells. IGF2BP3 depletion resulted in 655 upregulated genes and 862 down-regulated genes (Fig. 5a). GO analysis and GSEA was used to investigate the role of IGF2BP3 in TNBC. GO analysis indicated that the differentially expressed genes (DEGs) regulated by IGF2BP3 related to the cell cycle, autophagy, TNF signaling pathway and TGF-beta signaling pathway (Figures 4b). GSEA analysis also showed that DEGs of IGF2BP3 were related to extrinsic apoptotic and intrinsic signaling pathway, RNA catabolic process and regulation of mRNA metabolic process (Fig. 4c-f), suggesting that IGF2BP3 could play an oncogenic role in TNBC. It is well known that IGF2BP3 acts as an m6A reader, IGF2BP3 functions by binding and regulating m6A methylated transcripts[37]. Thus, we applied MeRIP-seq and RIP-seq in MDA-MB-231 cells. MeRIP-seq analysis identified 26904 m6A peaks out of 10939 genes. The m6A peaks were appropriately detected with the m6A motifs ($P = 1 \times 10^{-191}$), mainly enriched in CDS regions (Fig. 4g-h).

NF1 was an m6A modification target of IGF2BP3 in TNBC

Overlapping the genes from RNA-seq, RIP-seq, and MeRIP-seq, we obtained 129 genes (Table S4) bound by IGF2BP3 were modified with m6A (Fig. 5a). We took the intersection and found NF1, which could affect the proliferation and apoptosis of the tumor. NF1 encodes neurofibromin and acts as a tumor suppressor with Ras-GAP activity[38]. We discovered that most IGF2BP3 binding sites in NF1 fit well with

the m6A modified sites (Fig. 5b). Fig. 5c also indicated a negative correlation between IGF2BP3 and NF1 expression in 27 TNBC patient tissues in our hospital. Kaplan-Meier survival curves of TNBC indicated that NF1 low-expression was correlated with poor overall survival in TNBC patients(*P=0.042) (Fig. 5d). Then the qRT-PCR and western blot analyses indicated that NF1 might be the potential target of IGF2BP3 (Fig. 5e-g).

IGF2BP3 regulated NF1 mRNA expression in an m6A-dependent manner

To investigate how IGF2BP3 regulates NF1 expression, IGF2BP3 knockdown and overexpression MDA-MB-231 cell lines were treated with 5 µg/ml Act D at various time points. Fig. 6a demonstrated that downregulation of IGF2BP3 expression increased the half-life of NF1 mRNA. On the contrary, overexpression of IGF2BP3 decreased the half-life of NF1 mRNA (Fig. 6b). Similar results were confirmed in HCC-1806 cell lines (Fig. 6c-d). These results indicated that IGF2BP3 could decrease NF1 expression by regulating its mRNA stability. Moreover, RIP assay was conducted in MDA-MB-231 (Fig. 6e) and HCC-1806 (Fig. 6f) cell lines. The results indicated that NF1 mRNA was tested in the IGF2BP3 and Input group but not in the IgG group. In addition, MeRIP assay was performed and the results showed that NF1 could be bound with the m6A sites (Fig. 6g-h). To investigate whether IGF2BP3 regulates the NF1 expression in an m6A-dependent manner, we conducted a dual-luciferase assay in MDA-MB-231 and HCC-1806 cells to confirm the m6A sites that were necessary for IGF2BP3 binding to NF1 mRNA. The site A, B, C, D and E containing m6A-rich sites were designed. Furthermore, pGL3 was designed as the negative control (Fig. 6i). The luciferase activity of the reporters carrying NF1- B and C were decreased by the knockdown of IGF2BP3. On the contrary, the NF1- A, D and E were not responsive to IGF2BP3 (Fig. 6j-k). Moreover, the luciferase activity of a reporter carrying NF1- B-mut and C-mut decreased compared with the control group (Fig. 6l-n). Generally, these results indicated that IGF2BP3 could directly bind to the NF1-B and C sites of NF1 mRNA to decrease NF1 expression in an m6A-dependent manner.

NF1 could reverse the inhibition of proliferation and promotion of apoptosis induced by IGF2BP3 knockdown

IGF2BP3 knockdown and the control groups of MDA-MB-231 and HCC-1806 cells were transfected siRNA to suppress NF1 expression. The transfection efficiency was certified by qRT-PCR and western blot (Fig. 7a-b). The CCK8 assay and colony formation assay indicated that the NF1 suppress group demonstrated a stronger ability of proliferation in MDA-MB-231 and HCC-1806 cells, while cell proliferation ability was decreased by knockdown of IGF2BP3 (Fig. 7c-f). Moreover, in the flow cytometry analysis, the NF1 suppress group could decrease the percentage of early and late apoptotic cells in MDA-MB-231 and HCC-1806 cell lines. In contrast, cell apoptosis was significantly increased by the knockdown of IGF2BP3 (Fig. 7g-h). In xenograft models, the tumor volume of the NF1 suppress group increased faster than that of the control group in the shRNA-NC group or shIGF2BP3 group (Fig. 7i-j), and the same results were shown in the control group tumor weights (Fig. 7k). These results indicated that NF1 could reverse proliferation inhibition and promote apoptosis induced by IGF2BP3 knockdown.

Discussion

m6A is the most abundant internal modification in human mRNA. Due to the development of high-throughput sequencing technology, researchers can pinpoint the exact m6A site and reveal its function in biological and pathological processes[39]. More and more evidence indicated that m6A modifications are related to different solid tumors[40–42]. TNBC has a higher probability of local recurrence and metastasis, which the prognosis of TNBC patients is still poor. Recently, epigenetic regulation has been reported to play a significant role in the occurrence and development of TNBC[43]. However, there have been few studies on the introduction of m6A and its specific potential effects in TNBC.

IGF2BP3 acts as an oncogenic role and is significantly high expression in multiple cancers, associated with poor survival[44]. This study revealed that IGF2BP3 was significantly upregulated in TNBC tissues compared with normal tissues. Moreover, IGF2BP3 was shown to be overexpressed in TNBC compared to other subtypes of breast cancer in both mRNA and protein levels, suggesting that IGF2BP3 might be an important oncogene selected during cancer development. IGF2BP3 is regulated by genomic alterations, post-translational modifications, and transcriptional control[45]. According to reports, part of pancreatic cancers and thyroid tumors had a specific chromosomal balanced translocation between the IGF2BP3 chromosomal locus on 7p15.3, resulting in stable overexpression of IGF2BP3[46]. Moreover, the IGF2BP3 promoter demethylated in CpG islands was a feature of intrahepatic cholangiocarcinoma compared with normal liver tissue[35]. Furthermore, TCGA also confirmed that the DNA methylation status of the IGF2BP3 promoter is negatively correlated with the expression of IGF2BP3 mRNA[46]. The recent studies showed that the IGF2BP3 promoter is hypomethylated in TNBC and indicated that the methylation status of specific CpG sites in the promoter region is crucial in regulating IGF2BP3. In addition, we discovered that knockdown of TET3 resulted in a decrease of IGF2BP3 expression, displaying that the upregulation of IGF2BP3 is associated with TET3-mediated hypomethylation of its promoter in TNBC.

The dysregulation of IGF2BP3 expression in TNBC suggested its potential role in tumorigenesis. For example, IGF2BP3 promoted proliferation ability by regulating the expression of MYC through mRNA stabilization in gastric cancer[47]. It also regulated cell cycle and angiogenesis by binding m6A modified VEGF and CCND1 in colon cancer[48]. However, there were few studies in breast cancer via an m6A-dependent manner. Accordingly, our studies demonstrated that knockdown of IGF2BP3 inhibited the proliferation and promoted apoptosis *in vitro*, whereas overexpression of IGF2BP3 displayed opposite effects. Moreover, the knockdown of IGF2BP3 decreased the tumorigenesis of TNBC cells *in vivo*. In order to clarify the molecular mechanism of how IGF2BP3 promotes tumorigenesis as an m6A' reader', we applied a multi-omics screening strategy by complexing MeRIP-seq, RIP-seq and mRNA-seq. Intersection co-analysis indicated that IGF2BP3 could recognize m6A methylation to regulate the tumorigenesis process, and NF1 was an essential target of IGF2BP3.

NF1 encodes a GAP that terminates Ras/MAPK signaling pathway by stimulating the hydrolysis of Ras•GTP to inactive Ras•GDP. The inactivation of NF1 results in the hyperactivation of Ras and its downstream signaling elements[49]. Functional studies showed that NF1 was involved in signaling

pathways and is responsible for different cellular processes[50, 51]. However, the relationship between NF1 and TNBC has not been reported. IGF2BP3 reads m6A modification by regulating targeted mRNA stabilization[18]. Correspondingly, we found that knockdown of IGF2BP3 increased the expression and half-time of NF1 mRNA. These results indicated that NF1 was negatively correlated with IGF2BP3 expression, which showed a poor prognosis in TNBC. Moreover, NF1 was necessary for the proliferation and apoptosis of TNBC cells. Moreover, knockdown of NF1 partially rescued the phenotypes of IGF2BP3-knockdown cells *in vitro* and *in vivo*, demonstrating the significance of NF1 in TNBC development. These findings indicated that IGF2BP3 could decrease NF1 stabilization in an m6A-dependent manner, leading to TNBC development.

Conclusions

In summary, our study demonstrated TET3-mediated IGF2BP3 promoter hypomethylation leads to upregulation of IGF2BP3 expression in TNBC. Moreover, IGF2BP3 recognized the m6A target on NF1 mRNA and decreased the stabilization of NF1. IGF2BP3 decreased NF1 expression in an m6A-dependent manner as an oncogenic regulator.

Accordingly, we provided a novel mechanism of the IGF2BP3-NF1 axis in regulating TNBC proliferation, which may be investigated for TNBC prognosis, diagnosis or treatment (Figs. 8).

Abbreviations

IGF2BP3: insulin-like growth factor 2 mRNA-binding protein 3

NF1: neurofibromin 1

TNBC: triple-negative breast cancer

m6A: N6-methyladenosine

mRNA: messenger RNA

RIP: RNA immunoprecipitation

MeRIP: Methylated RNA Immunoprecipitation

TCGA: The Cancer Genome Atlas

TET3: Tet Methylcytosine Dioxygenase 3

BC: Breast cancer

siRNA: small interfering RNA

Declarations

Availability of data and materials

All data during this study are included either in this article or in the supplementary information files.

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Competing interests

The authors declare that they have no competing interests.

Ethics approval and consent to participate

All procedures performed in studies involving human participants complied with the ethical standards of the Research Ethics Committee of the First Affiliated Hospital of Nanjing Medical University and the 1964 Declaration of Helsinki and its subsequent revisions. All informed consents participating in this study were written consent from patients with triple-negative breast cancer to collect samples from patients with triple-negative breast cancer.

Authors' contributions

Q.D. and J.F.-W. designed the study; X.Z., L.S., and H.D.-S. performed the experiments; F.X. conducted the statistical analysis; Z.W.-W. participated in the clinical specimens detection; X.Z. and J.F.-W. wrote and revised this manuscript. All authors read and approved the final manuscript.

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Supplementary Table

Table S4 is not available with this version

Figures

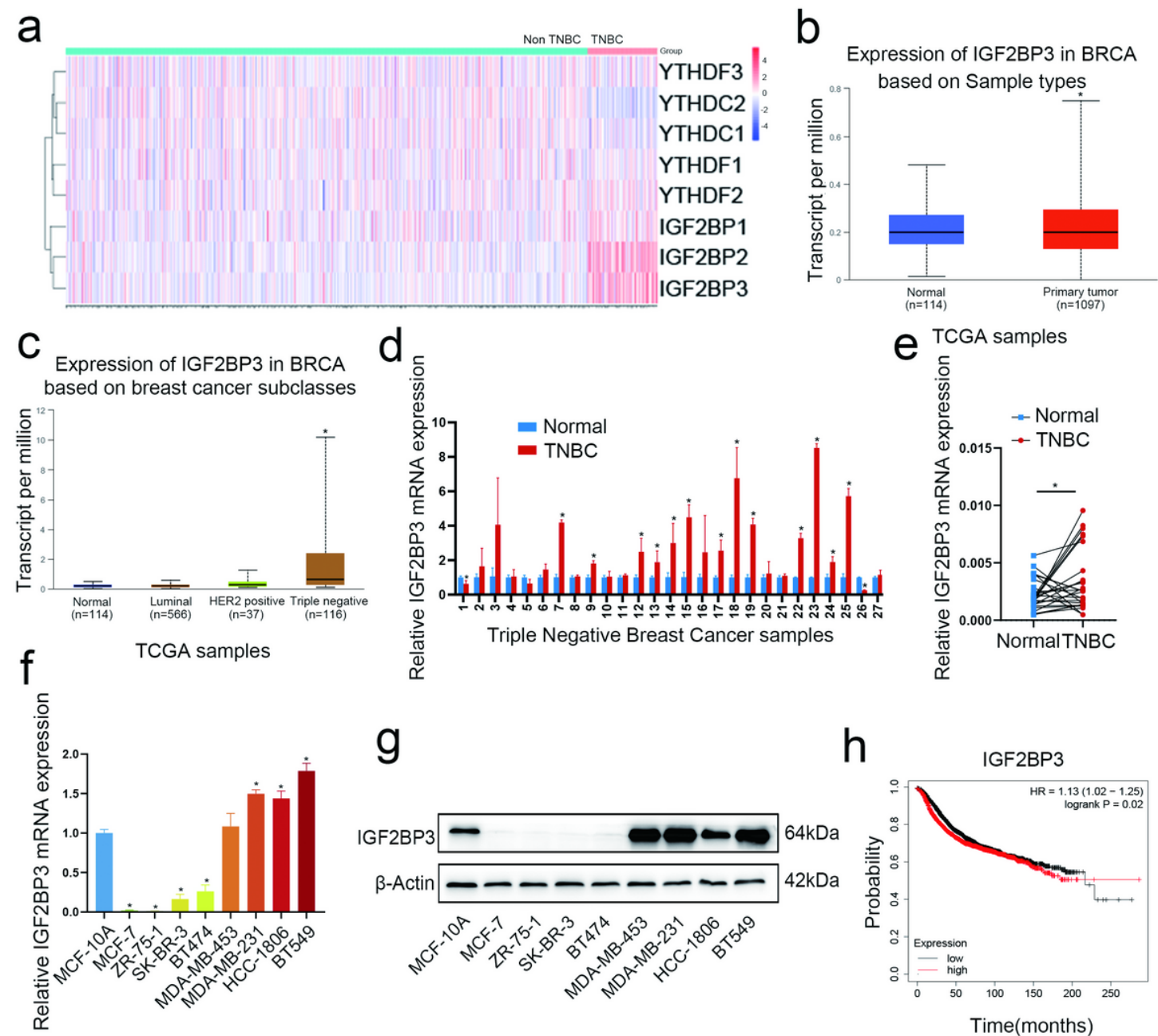


Figure 1

IGF2BP3 was upregulated in TNBC and correlated with a poorer prognosis

a Heatmap showing RNA differential expression of eight m6A' readers' between TNBC and non-TNBC. **b** Expression of IGF2BP3 in normal tissue (n=114) and breast cancer (n=1097) from TCGA dataset. **c**

Expression of IGF2BP3 in different molecular subtypes of breast cancer. **d** IGF2BP3 mRNA expression in 27 pairs of TNBC and normal tissues. **e** Average mRNA expression level of IGF2BP3 in 27 pairs of TNBC tissues and normal tissues. **f** IGF2BP3 mRNA expression of IGF2BP3 in different cell lines. **g** IGF2BP3 protein expression of IGF2BP3 in different cell lines. **h** Kaplan-Meier analysis of overall survival of TNBC patients. Data were shown as mean $\pm$ SEM, * p <0.05.

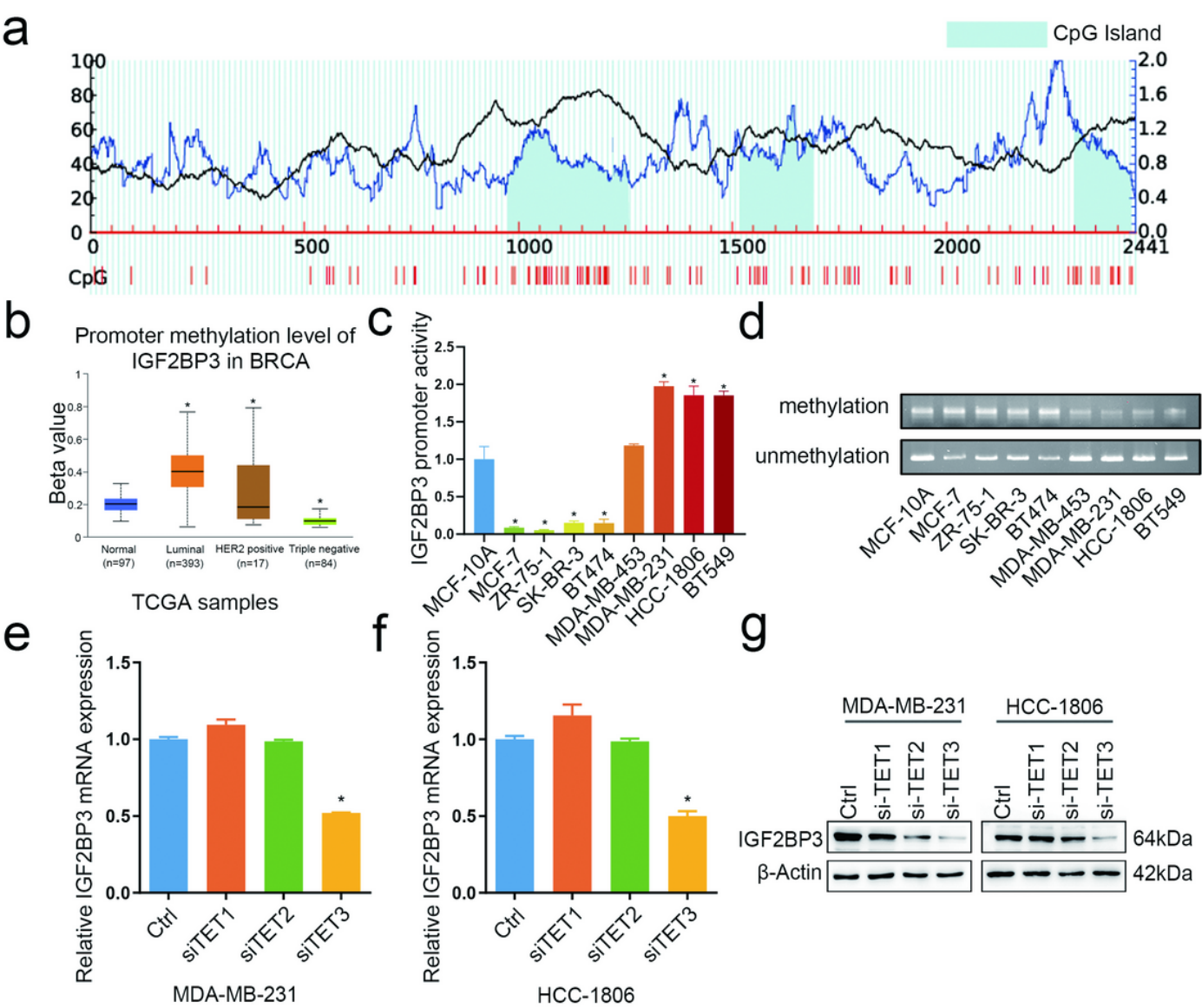


Figure 2

The promoter of IGF2BP3 was hypomethylated in TNBC

a Schematic representation of the CpG islands in the IGF2BP3 promoter. Red region, input sequence; Blue region, CpG islands. **b** Promoter methylation level of IGF2BP3 in different molecular subtypes breast cancer tissues from TCGA dataset. **c** Luciferase activity of IGF2BP3 promoter in different cell lines. **d** Methylation-specific PCR of the CpG island of the IGF2BP3 promoter region in different breast cancer cell

lines and matched normal breast cell line. **e-f** qRT-PCR was used to confirm the IGF2BP3 expression at mRNA levels. **g** Western blot was used to confirm the IGF2BP3 expression at protein levels. Data were shown as mean $\pm$ SEM, * p <0.05.

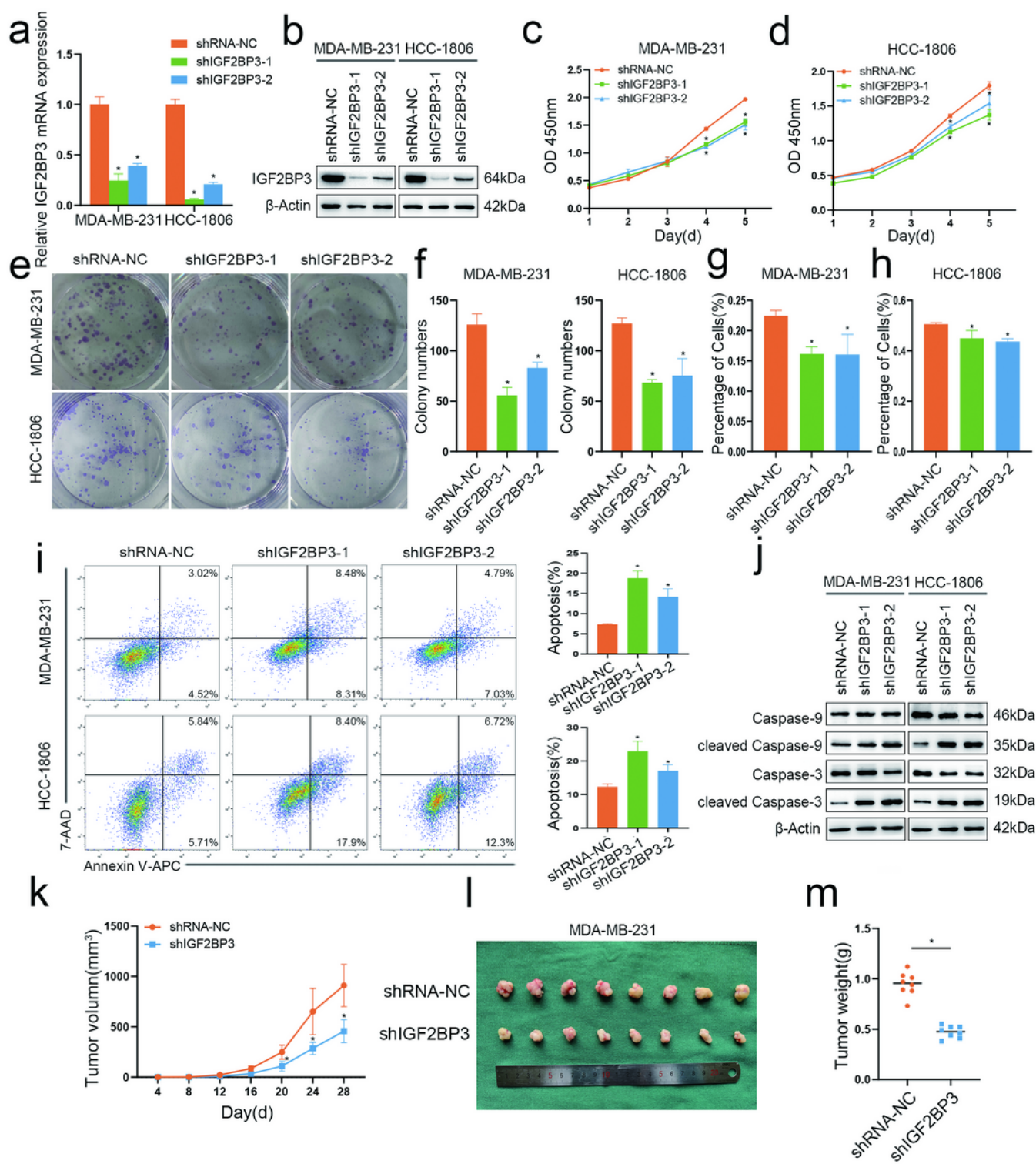


Figure 3

Knockdown of IGF2BP3 inhibited the proliferation and promoted the apoptosis of TNBC in *vivo* and in *vitro*

a-b MDA-MB-231 and HCC-1806 cell lines were transfected with lentivirus to knockdown IGF2BP3 expression (shIGF2BP3-1, shIGF2BP3-2). qRT-PCR (**a**) and Western blot (**b**) were applied to verify transfection efficiency. **c-d** CCK8 assay was performed in MDA-MB-231 and HCC-1806 cell lines. **e-f** Colony formation assay was performed in MDA-MB-231 and HCC-1806 cell lines. **g-h** Edu assay was performed in MDA-MB-231 and HCC-1806 cell lines. **i-j** Flow cytometry assay and western blot were used to confirm the apoptosis analysis induced by the knockdown of IGF2BP3. **k-l** Tumor volume in IGF2BP3-knockdown MDA-MB-231 cells compared with control every 4 days. **m** Tumor weight in IGF2BP3-knockdown MDA-MB-231 cells compared with control at 4 weeks. Data were shown as mean $\pm$ SEM, * $p < 0.05$.

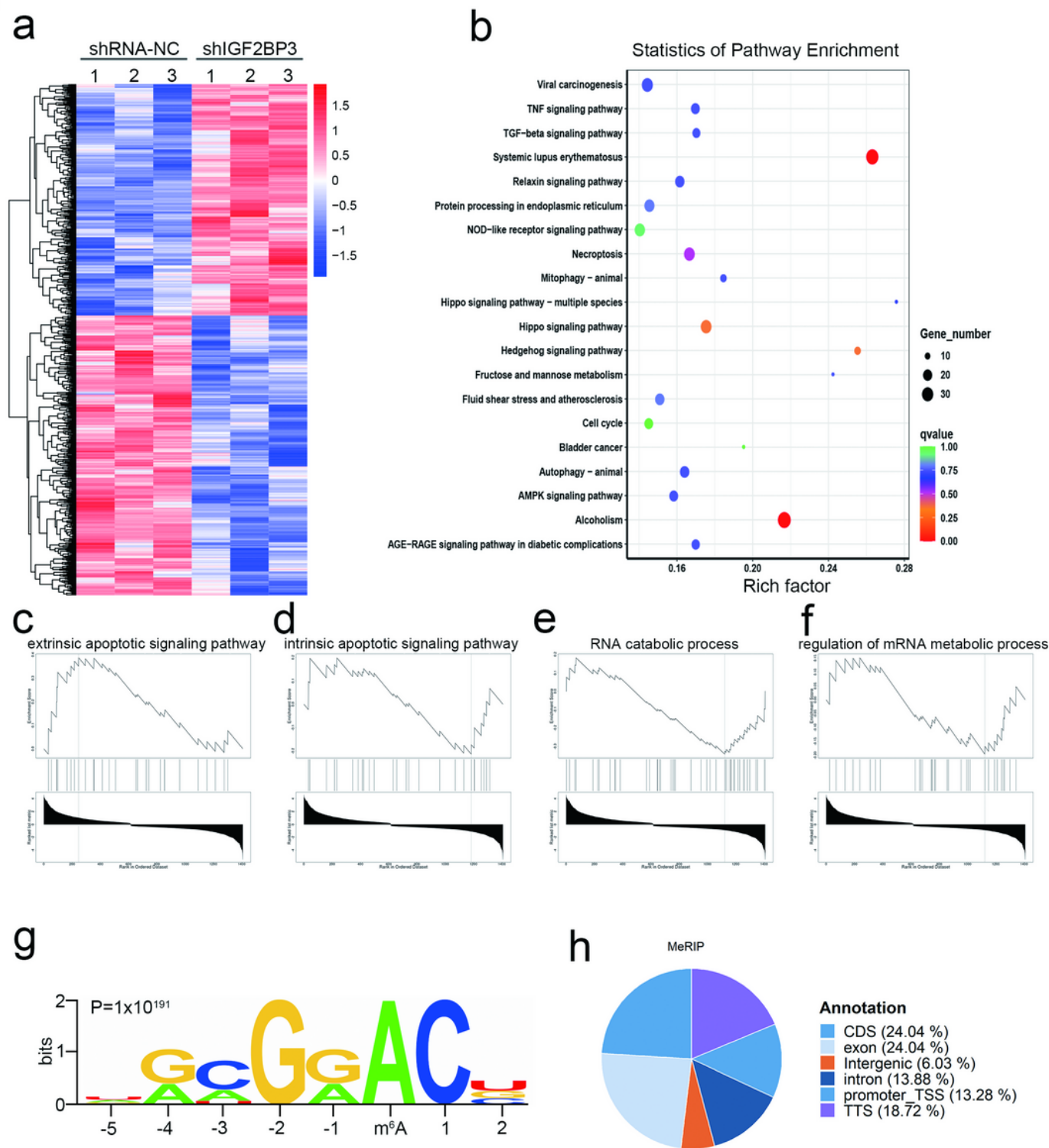


Figure 4

Identification of the IGF2BP3 targets in TNBC

a Heatmap of DEGs identified by RNA-seq. **b** GO enrichment analysis of DEGs. **c-f** GSEA plots showing the pathway of IGF2BP3-enriched DEGs involved. **g** m⁶A motif detection by DREME motif analysis and m⁶A-seq results. **h** Percentage of various RNA species modified by m⁶A.

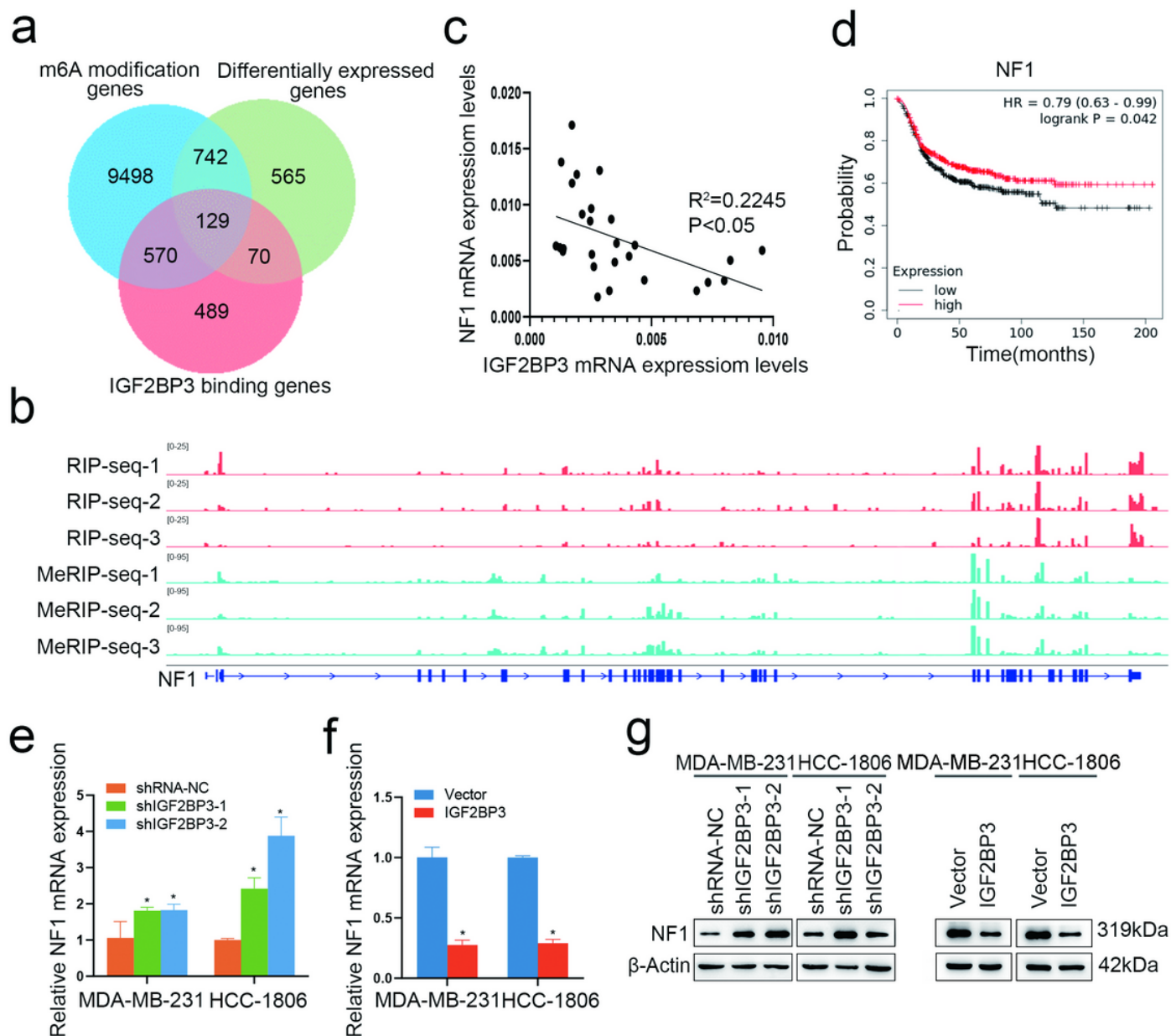


Figure 5

NF1 was an m6A Modification Target of IGF2BP3 in TNBC

a Overlapping analysis of genes identified by m6A-seq, RIP-seq, and RNA-seq. **b** Distribution of m6A peaks and IGF2BP3-binding peaks across transcripts. **c** Correlation analysis between IGF2BP3 and NF1 mRNA expression in TNBC tissues (n = 27). **d** Kaplan-Meier analysis of overall survival of TNBC patients. **e-g** Expression of NF1 was increased or decreased following IGF2BP3 knockdown or overexpression in MDA-MB-231 and HCC-1806 cells at mRNA (**e, f**) and protein levels (**g**). Data were shown as mean $\pm$ SEM, *p < 0.05.

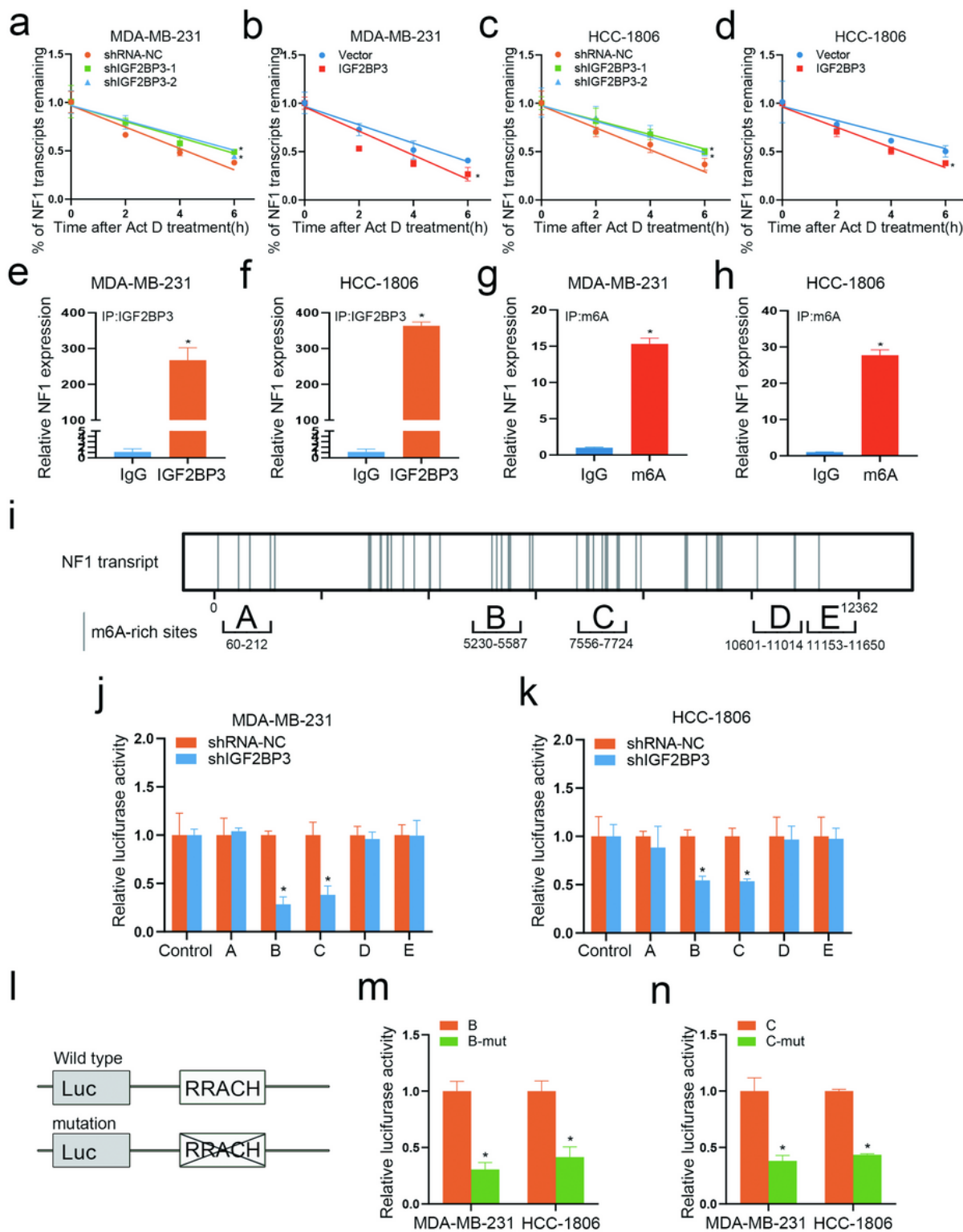


Figure 6

IGF2BP3 regulated NF1 mRNA expression in an m6A-dependent manner

a-d In MDA-MB-231 and HCC-1806 cell lines, IGF2BP3 knockdown prolonged the half-life of NF1 mRNA, while overexpression of IGF2BP3 shortened the half-life of NF1 mRNA. MDA-MB-231 and HCC-1806 cells were treated with 5 μ g/ml Act D for 0, 2, 4, and 6 h and followed by qRT-PCR analysis. **e-f** MDA-MB-231

and HCC-1806 cell lysates were immunoprecipitated with IGF2BP3 antibody or control IgG to detect NF1 mRNA expression. **g-h** MDA-MB-231 and HCC-1806 cell lysates were immunoprecipitated with m6A antibody or control IgG to detect NF1 mRNA expression. **i** Schematic of regions in the NF1 mRNA. **j-k** The luciferase activity for the reporter involving NF1-A, B, C, D, E and pGL3 was transfected by knocking down IGF2BP3 in MDA-MB-231 and HCC-1806 cells. **l** Schematic of mutation regions in the NF1 mRNA. **m-n** The luciferase activity for the reporter involving NF1-B, B-mut, C and C-mut was transfected in MDA-MB-231 and HCC-1806 cells. Data were shown as mean $\pm$ SEM, * $p < 0.05$.

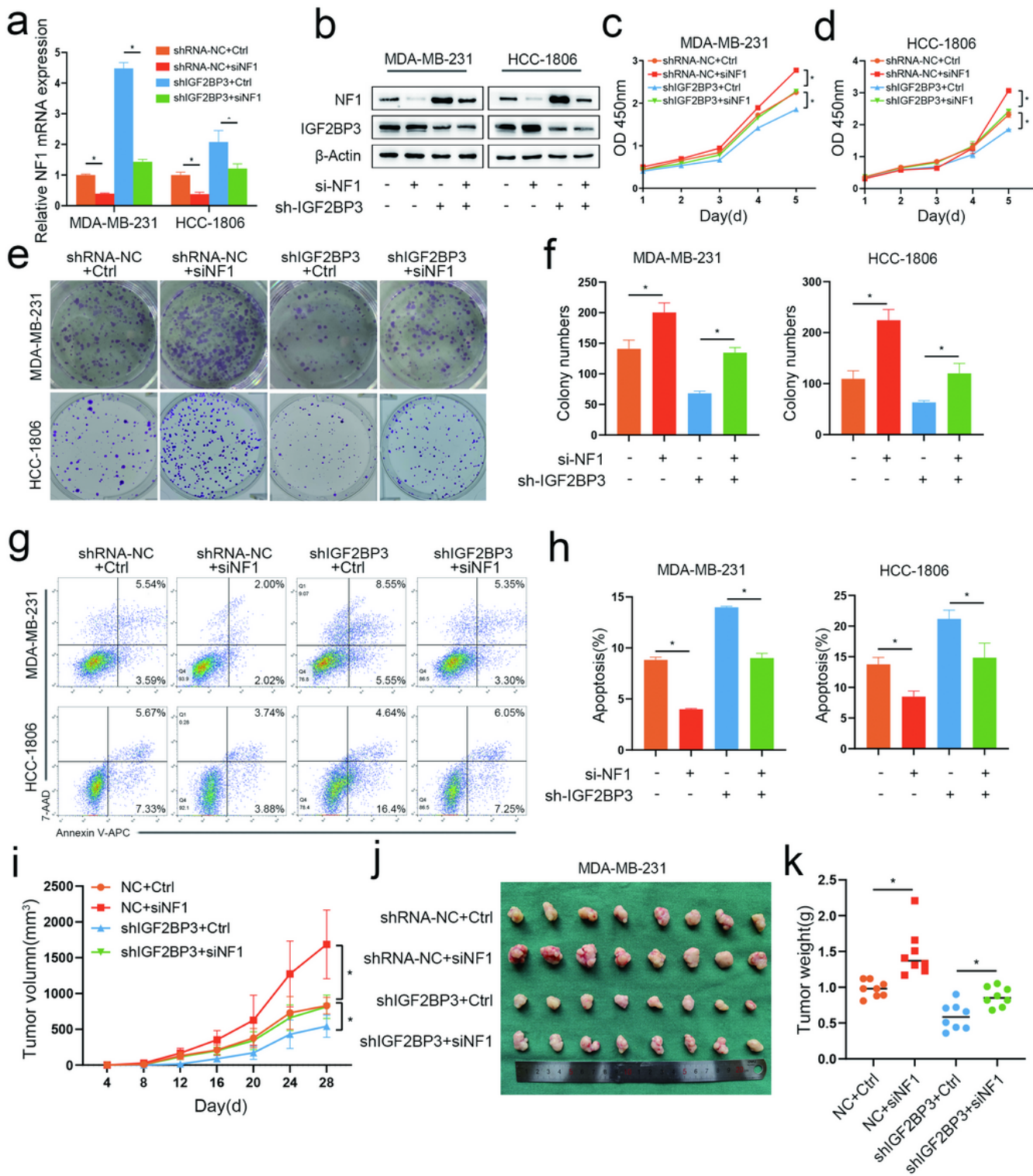


Figure 7

NF1 could reverse the inhibition of proliferation and promotion of apoptosis induced by IGF2BP3 knockdown

a-b IGF2BP3 knockdown and the control groups of MDA-MB-231 and HCC-1806 cells were transfected to knockdown NF1, followed by qRT-PCR and Western blots examination. **c-d** CCK8 assay was performed to detect the proliferation ability of MDA-MB-231 and HCC-1806 cells. **e-f** Colony formation assay was performed to analyze the proliferation ability of MDA-MB-231 and HCC-1806 cells. **g-h** Flow cytometry assay was used to detect the apoptosis analysis of knockdown of NF1 in shRNA-NC and shIGF2BP3 cells. **i-k** Tumor volume and weight in NF1 knockdown and control group in shRNA-NC and shIGF2BP3 MDA-MB-231 cells in nude mice in different time points. Data were shown as mean $\pm$ SEM, * $p < 0.05$.

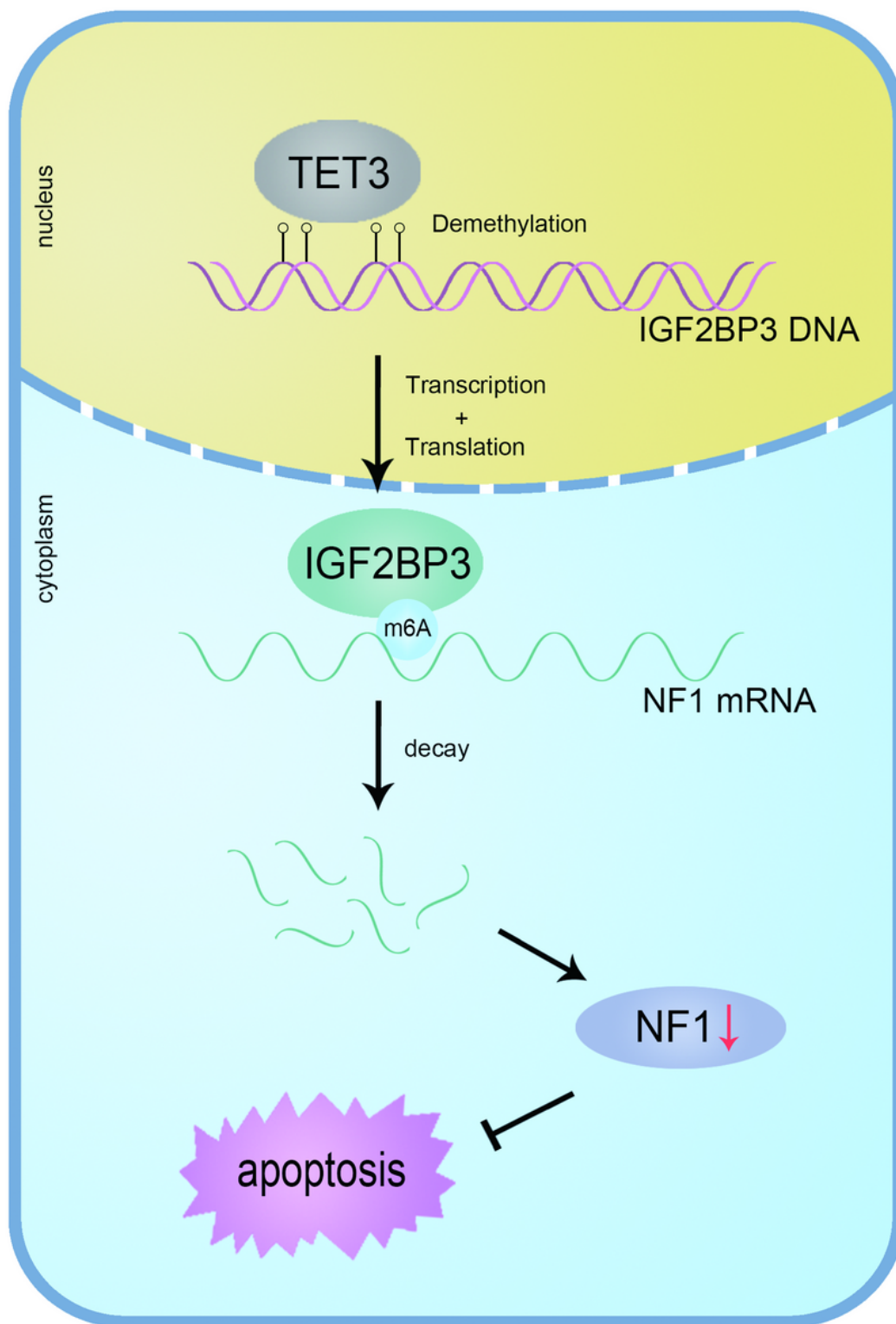


Figure 8

The graphic illustration of IGF2BP3 modulating TNBC proliferation and apoptosis via decreasing NF1 mRNA stability in an m6A-dependent manner

Supplementary Files

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