

***GUSBP11* Inhibited The Progression of Triple Negative Breast Cancer via Targeting The *miR-579-3p/SPNS2* Axis**

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Abstract

Objective: Growing evidences have exposed the important roles of long noncoding RNAs (lncRNAs) in the triple negative breast cancer (TNBC) inhibition. The function of glucuronidase beta pseudogene 11 (*GUSBP11*) in the TNBC occurrence remains obscure. To detect the function of *GUSBP11* in TNBC progression and explore its downstream molecular mechanism.

Materials and Methods: In this experimental study, using quantitative reverse transcription real-time polymerase chain reaction (RT-qPCR), we measured the *GUSBP11* expression in the TNBC cell lines. Gain-of-function assays, including colony formation, flow cytometry, and western blot were used to identify the probable effects of *GUSBP11* overexpression on the malignant behaviors of TNBC cell lines. Moreover, mechanism assays, including RNA immunoprecipitation (RIP), RNA pull down and luciferase reporter assays were taken to measure the possible mechanism of *GUSBP11* in the TNBC cell lines.

Results: *GUSBP11* expressed at a low RNA level in the TNBC cell lines. Overexpression of *GUSBP11* RNA expression inhibited the proliferation, migration, epithelial-to-mesenchymal transition (EMT) and stemness while elevated the apoptosis of the TNBC cell lines. *GUSBP11* positively regulated the expression of sphingolipid transporter 2 (*SPNS2*) via acting as a competing endogenous RNA (ceRNA) of *miR-579-3p*, thereby suppressing the development of TNBC cell lines.

Conclusion: *GUSBP11* impedes TNBC progression via modulating the *miR-579-3p/SPNS2* axis.

Keywords: *GUSBP11*, *miR-579-3p*, *SPNS2*, Triple-Negative Breast Cancer

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Introduction

Breast cancer (BC) is one of the most frequent cancers, which mostly occur in the females. The incidence rate is increasing, accompanied by the young age of BC patients in recent years (1, 2). Triple-negative breast cancer (TNBC) is a subtype of BC, which is featured by the absence of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor-2 (HER2) (3). Despite of improvements in the screening, operation, and chemo-radiotherapy methods, the TNBC patient's prognosis is still not optimistic (4, 5). Hence, it is necessary to explore novel potential therapeutic targets.

Non-coding RNAs (ncRNAs) mainly include long non-coding RNAs (lncRNAs) and microRNAs (miRNAs) (6, 7). In recent years, lncRNAs have been reported to act as important regulators in various biological processes of human cancers via various approaches, such as the regulation of transcription, translation, protein modification, and the formation of RNA-protein or protein-protein complexes (8). A large amount of evidence has suggested that lncRNAs act as tumor promoters or tumor suppressors in the TNBC development (9). In addition, many lncRNAs are identified as potential therapeutic targets for TNBC treatment (10). Even so, there are some lncRNAs underlying TNBC remain to be explored. In the current study, we mainly

focused on the role of a novel lncRNA glucuronidase beta pseudogene 11 (*GUSBP11*) in the TNBC.

MicroRNAs (miRNAs) are crucial regulators in the TNBC development (11). For example, *miR-29b-3p* contributes to the TNBC progression through the TRAF3 regulating (12); *miR-613* represses cell migration and invasion via inhibiting Daam1 in the TNBC (13). Besides, miRNAs can exert functions post-transcriptionally via degrading mRNA or inhibiting the translation via binding to the 3' untranslated region (3' UTR) of the targeted genes (14). The controller role of *miR-579-3p* in the melanoma progression has been revealed in a previous study (15), but we still don't know whether it can function in the TNBC and its underlying mechanism remain to be unveiled. In this study, we uncovered the involvement of *miR-579-3p* in the *GUSBP11*-mediated TNBC progression.

Our research group focused on the role of sphingolipid transporter 2 (*SPNS2*) in the TNBC progression. We speculated that *GUSBP11* inhibited TNBC cell malignancy via *miR-579-3p/SPNS2* axis. Therefore, we analyzed the expression pattern of the genes in the TNBC cell lines and examined the related biological functions. Collectively, this study was aimed to investigate the impacts of the *GUSBP11/miR-579-3p/SPNS2* axis in the TNBC progression.

Materials and Methods

Cell culture

In this experimental study, human BC cell lines (MDA-MB-231, MDA-MB-436, MDA-MB-453, SKBR3, MCF-7, BT-474, AU565, T-47D, ZR-75-1), HEK293T cell line and human breast non-tumorigenic epithelial cell line (MCF-10A) were all obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Human BC cell lines (CAL-120, SUM190 and SUM1315) were obtained from COBIOER (Nanjing, China). The HEK293T cell line was cultured in the ATCC-formulated Eagle's Minimum Essential Medium (EMEM, M0200, Sigma-Aldrich, St. Louis, MO, USA). MDA-MB-231, MDA-MB-436, MDA-MB-453 cell lines were cultured in the Leibovitz's L-15 Medium (11415049, Thermo Fisher Scientific, Rockford, IL, USA). The MCF-10A cell line was grown in the MEBM (CC-2151, LONZA, Basel, Switzerland). The SKBR3 cell line was grown in the McCoy's 5a Medium (16600108, Thermo Fisher Scientific, Rockford, IL, USA). MCF-7 and CAL-120 cell lines were grown in the Dulbecco's Minimum Essential Medium (A4192101, Gibco, Rockville, MD, USA). The BT-474 cell line was grown in the Hybri-Care Medium (ATCC46-X, ATCC, Manassas, VA, USA). AU565, T-47D, ZR-75-1 and SUM190 cell lines were cultured in the RPMI-1640 Medium (A4192301, Gibco, Rockville, MD, USA). The SUM1315 cell line was cultured in the Ham's F-12 medium (88424, Thermo Fisher Scientific, Rockford, IL, USA). All the culture mediums were treated with 10% fetal bovine serum (FBS, 16140071, Thermo Fisher Scientific, Rockford, IL, USA) and mixed with 1% penicillin and streptomycin (15140148, Thermo Fisher Scientific, Rockford, IL, USA). Cell culture was achieved in the 5% CO₂ at 37°C. The culture mediums were changed after 3-4 days of cultivation, and the cell lines went through passage every 7 days.

Cell transfection

For overexpression, the full-length cDNA sequences of *GUSBP11* were inserted into the pcDNA3.1 vectors (15042907, Sigma-Aldrich, St. Louis, MO, USA) to construct pcDNA3.1/*GUSBP11* plasmids. Likewise, the whole length of YY1, p300 and HDAC2 was separately inserted into pcDNA3.1 vectors to generate their overexpression vectors. Empty pcDNA3.1 vector was used as the negative control (NC) for all overexpression vectors. Besides, *miR-579-3p* and NC mimics, the specific shRNAs to *SPNS2* and nonspecific shRNAs (sh/NC) were purchased from GenePharma (Shanghai, China). Transfections were conducted using Lipofectamine 3000 (Invitrogen) and terminated after 48 hours. For rescue assays, we severally transfected pcDNA3.1, pcDNA3.1/*GUSBP11*, pcDNA3.1/*GUSBP11*+*miR-579-3p* mimics and pcDNA3.1/*GUSBP11*+sh/*SPNS2*#1 into MDA-MB-231 and MDA-MB-453 cell lines.

Quantitative reverse transcription real-time polymerase chain reaction

Total RNA was extracted from the cell lines using TRIzol Reagent (15596026, Thermo Fisher Scientific, Rockford, IL, USA). Next, PrimeScript RT master mix (RR036Q, Takara, Japan) was employed for reverse transcription of RNA. Then, SYBR Premix Ex Taq™ II (4309155, Applied Biosystems, Foster city, CA, USA) was utilized to examine the gene expression based on 2^{-ΔΔCt} method. *GAPDH* or *U6* was used as the internal reference. Samples were assayed in triplicate and results were obtained from three independent experiments.

Colony formation assay

Transfected TNBC cell lines (500 cells per well) were planted into 6-well plates. After 12 days, the culture medium was discarded and the cell lines were fixed with a Methanol solution (67-56-1, Bojing Chemical Co., Ltd, Shanghai, China) for 15 minutes, and stained by 0.5% crystal violet (V5265, Sigma-Aldrich, St. Louis, MO, USA) for 10 minutes at room temperature. The number of colonies was manually counted. Samples were assayed in triplicate and results were obtained from three independent experiments.

5-Ethynyl-2'-deoxyuridine

5-Ethynyl-2'-deoxyuridine (EdU) staining was performed using a BeyoClick™ Cell Proliferation Kit (C0075L, Beyotime, Guangzhou, China). Transfected TNBC cell lines were added with EdU and incubated for 2 hours at room temperature. After washing, cell lines were fixed with 4% paraformaldehyde. The nucleus was stained by DAPI (D9542, Sigma-Aldrich, St. Louis, MO, USA) and images were captured via using an inverted microscope (Olympus, Japan). Samples were assayed in triplicate and results were obtained from three independent experiments.

Terminal-deoxynucleotidyl Transferase Mediated Nick End labeling (TUNEL)

TUNEL reagent (12156792910, Roche, Basel, Switzerland) was commercially acquired for TUNEL experiment. Transfected cell lines (1×10⁴) were planted into the 96-well plates, fixed by 4% paraformaldehyde, permeabilized with 0.1% Triton-X100, and then treated with TUNEL kit (Merck KGaA, Darmstadt, Germany) for 1 hour. Finally, cell nucleus was subjected to DAPI staining and observed using fluorescence microscope (DMI8, Leica, Wetzlar, Germany). Samples were assayed in triplicate and results were obtained from three independent experiments.

Flow cytometry analysis

Transfected cell lines were collected and placed into the 6-well plates. Flow cytometer was used following the instruction (17-344, Sigma-Aldrich, St. Louis, MO, USA), and the Annexin V-FITC/PI double staining kit

(APOAF, Sigma-Aldrich, St. Louis, MO, USA) was purchased from Invitrogen. After staining for 15 minutes, cell lines were reaped for flow cytometry. Samples were assayed in triplicate and results were obtained from three independent experiments.

Transwell assay

Cell lines (5×10^4) were seeded into the upper chamber of the insert (pore size 8 μm ; 3428, Corning, NY, USA) and incubated in the serum-free DMEM medium. The DMEM medium containing 10% FBS was added to the lower chamber. After incubation for 24 hours in the 5% CO_2 at 37°C, the upper membrane cells were wiped, and the migrated cells through the membrane were fixed with 4% paraformaldehyde (E672002, Sangon Biotech, Shanghai, China) and stained with 0.1% crystal violet (V5265, Sigma-Aldrich, St. Louis, MO, USA). The images were observed via an inverted microscope (DMI1, Leica, Wetzlar, Germany). Samples were assayed in triplicate and results were obtained from three independent experiments.

Sphere formation assay

Cell lines were cultured in the serum-free DMEM medium treated with insulin (12643, Sigma-Aldrich, St. Louis, MO, USA), 20 ng/mL human recombinant epidermal growth factor (EGF, GF144, Sigma-Aldrich, St. Louis, MO, USA) and 10 ng/mL basic fibroblast growth factor (bFGF, 2255, Sigma-Aldrich, St. Louis, MO, USA). After 14 days of culture, the sphere formation was observed using a microscope (DMI1, Leica, Wetzlar, Germany). Samples were assayed in triplicate and results were obtained from three independent experiments.

ChIP assay

Following the protocol, an EZ ChIP Chromatin Immunoprecipitation kit (17-295; Millipore, Billerica, MA, USA) was applied to ChIP assay. Chromatin was cross-linked and sonicated to 200-1000-bp fragments, followed by immunoprecipitation with anti-YY1 or anti-IgG antibody (401455-2ML-M, Millipore, Billerica, MA, USA) which was selected as the NC. RT-qPCR was eventually carried out for enrichment detection. Samples were assayed in triplicate and results were obtained from three independent experiments.

Subcellular fractionation

RNA was separated from the nuclear or cytoplasmic fraction via a PARIS Kit (AM1921, Thermo Fisher Scientific, Rockford, IL, USA), followed by quantification with RT-qPCR. Here, *GAPDH* and *U6* were served as cytoplasmic and nuclear marker, respectively. Samples were assayed in triplicate and results were obtained from three independent experiments.

Fluorescence in situ hybridization

The cellular localization of *GUSBP11* was detected via

a FISH kit (F32952, Invitrogen, Carlsbad, CA, USA). The cell lines were cultured with the Digoxigenin-labeled *GUSBP11* probe (Ribobio, Guangzhou, China) in hybridization solution (H7782, Sigma-Aldrich, St. Louis, MO, USA). Nuclei were counterstained with DAPI (D9542, Sigma-Aldrich, St. Louis, MO, USA), and the cell lines were finally observed under a confocal laser-scanning microscope. Briefly, adding a few drops of DAPI dye to the prepared slides for 10 minutes. Then, the slides were gently rinsed with running water, and excess water absorbed by filter paper. Next, a drop of antifade mounting medium was added and the slides were observed under a fluorescence microscope. Samples were assayed in triplicate and results were obtained from three independent experiments.

RNA immunoprecipitation

RNA immunoprecipitation (RIP) assay was implemented via an RNA-binding protein immunoprecipitation kit (17-704, Sigma-Aldrich, St. Louis, MO, USA). Transfected cell lines were lysed, and then hatched with RIP buffer containing magnetic beads conjugated with anti-Ago2 antibody. After being washed and purified, the immunoprecipitated RNA was analyzed via RT-qPCR. Samples were assayed in triplicate and results were obtained from three independent experiments.

RNA pull down assay

GUSBP11 biotin probe (Ribobio, Guangzhou, China) or wild-type or mutant-type of *miR-579-3p* were transcribed into the cell lines. Transfected cell lysates were hatched with Dynabeads M-280 Streptavidin (11206D, Thermo Fisher Scientific, Rockford, IL, USA) overnight at 4°C according to the manufacturer's requirements. Then, the beads were washed and eluted. RNAs were extracted by TRIzol reagent (15596026, Thermo Fisher Scientific, Rockford, IL, USA) and evaluated by RT-qPCR. Samples were assayed in triplicate and results were obtained from three independent experiments.

Luciferase reporter assays

The sequence of *GUSBP11* promoter was sub-cloned into pGL3 vector (Promega, Madison, WI, USA). And, overexpression plasmids were co-transfected into the cell lines to evaluate the activity of *GUSBP11* transcription.

The *GUSBP11* fragment or *SPNS2* 3'UTR fragment covering the *miR-579-3p* binding site was inserted into the pmirGLO vector (Promega, Madison, WI, USA). And then, the cell lines were severally co-transfected with luciferase reporter vectors containing *GUSBP11*-Wt/Mut or *SPNS2* 3'UTR-WT/Mut and *miR-579-3p* mimics/NC mimics using Lipofectamine 3000 (L3000075, Invitrogen, Carlsbad, CA, USA). The Firefly and Renilla luciferase activity was measured at 48 hours after transfection with a dual-luciferase reporter assay kit (E1910, Promega, Madison, WI, USA). Samples were assayed in triplicate and results were obtained from three

independent experiments.

Statistical analysis

All experimental data were shown as mean \pm standard deviation (SD) of three independent experiments and analyzed by GraphPad Prism 5.0 (San Diego, CA, USA). The significant difference of the groups was assessed using Student's t test and one-way analysis of variance (ANOVA). Also, $P < 0.05$ indicated statistically significant data.

Results

Overexpression of *GUSBP11* in the RNA level suppresses the TNBC cell growth

Searching online database (<http://gepia2.cancer-pku.cn>), lncRNA *GUSBP11* was determined to be down-regulated in the all types of BC tissues in comparison with the normal tissues (Fig.1A-E). To further explore the potential role of *GUSBP11* in the specific cancer types, we evaluated its RNA level in the all subtypes of BC cell lines. In comparison with the human normal mammary cell line (MCF-10A), *GUSBP11* expression was only obvious down-regulated in the TNBC cell lines, including MDA-MB-436, MDA-MB-453 and MDA-MB-231 (Fig.1F), suggesting that *GUSBP11* down-regulation might be correlated with the TNBC progression. Next, we designed gain-of-function assays to identify the functional role of *GUSBP11* overexpression in the TNBC. At first, pcDNA3.1/*GUSBP11* was transfected into the MDA-MB-231 and MDA-MB-453 cell lines which presented the lowest RNA level of *GUSBP11* (Fig.1G). It was observed in the colony formation experiments that the number of colonies in the TNBC cell lines was decreased after the overexpression of *GUSBP11* (Fig.1H). Consistently, the EdU positive stained cells were lessened up to 30% when *GUSBP11* was up-regulated (Fig.1I). On the contrary, a rise of about 7% in the apoptosis rate was observed in MDA-MB-231 and MDA-MB-453 cell lines due to *GUSBP11* up-regulation (Fig.1J, K). All these data suggested that *GUSBP11* was down-regulated in the TNBC cells and its overexpression impeded cell growth.

GUSBP11 up-regulation represses invasion, migration and stemness of the TNBC cell lines

We continued to detect the effects of *GUSBP11* on other biological properties of the TNBC cell lines. Through Transwell assays, we found that the invasive and migratory abilities of TNBC cell lines were repressed by *GUSBP11* elevation (Fig.2A, B). Consistently, we observed that the overexpression of *GUSBP11* led to an increase in the protein expression of E-cadherin while a decrease in the protein expression of MMP2, MMP7, N-cadherin and Vimentin, which

indicated that *GUSBP11* up-regulation repressed the epithelial-mesenchymal transition, namely epithelial-to-mesenchymal transition (EMT) process in the TNBC cell lines (Fig.2C). Up-regulation of *GUSBP11* significantly suppressed sphere formation in the MDA-MB-453 and MDA-MB-231 cell lines, in different aspects including number and size (Fig.2D). Moreover, we also examined the RNA as well as protein levels of stemness markers using RT-qPCR and western blot. It was uncovered that the levels of *NANOG*, *OCT4* and *SOX2* were all decreased under *GUSBP11* overexpression (Fig.2E, F).

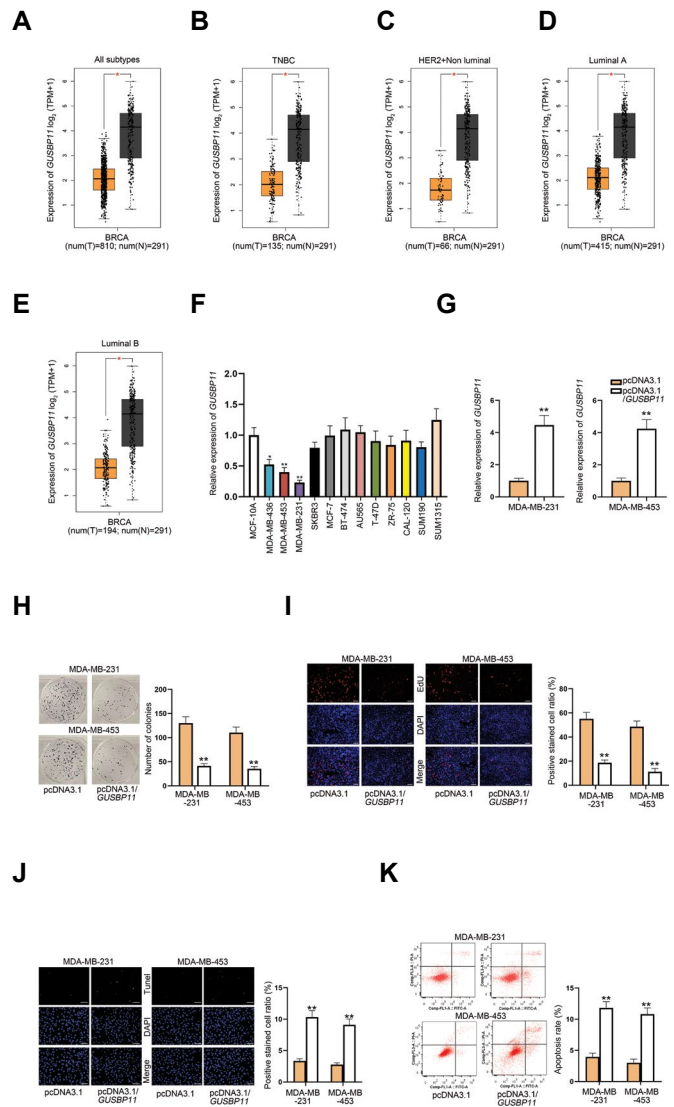


Fig.1: *GUSBP11* suppresses the TNBC cell lines growth. **A-E**. Box plot from GEPIA 2 database indicated the expression of *GUSBP11* in the different subtypes of breast cancer tissues. **F**. The expression of *GUSBP11* in the various types of breast cancer cell lines in comparison with human normal mammary cell line (MCF-10A). **G**. *GUSBP11* expression was enhanced in the TNBC cell lines via the transfection of pcDNA3.1/*GUSBP11*. **H**. The number of colonies in the pcDNA3.1/*GUSBP11*-transfected TNBC cell lines. **I**. The proliferation of the TNBC cell lines after *GUSBP11* elevation was evaluated by EdU assays (scale bar: 50 μ m). **J**, and **K**. TUNEL assay, along with flow cytometry was taken to assess the apoptosis of the TNBC cell lines upon *GUSBP11* elevation (scale bar: 50 μ m). Three independent experiments were conducted (n=3). TNBC; Triple negative breast cancer, *; $P < 0.05$, and **; $P < 0.01$.

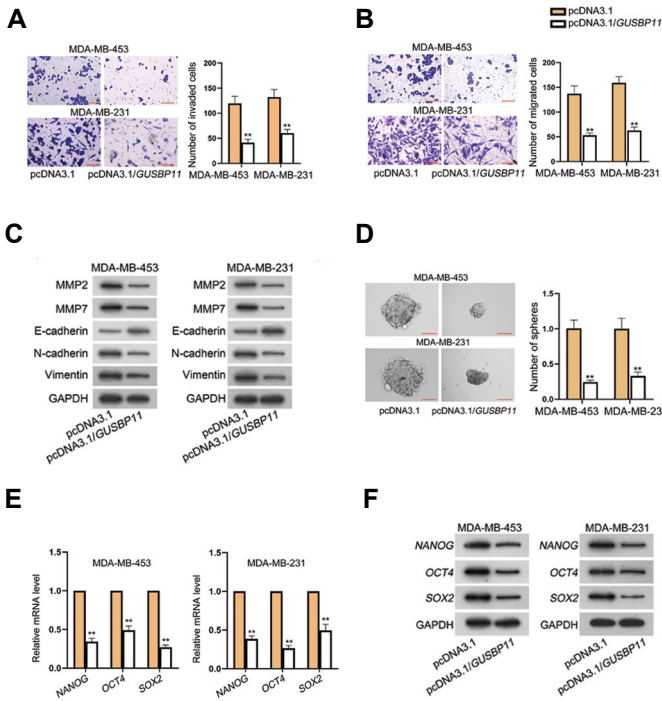


Fig.2: *GUSBP11* up-regulation represses cell migration, EMT and stemness in the TNBC cell lines. **A, B.** Transwell assays were performed to analyze the migration and invasion of the TNBC cell lines after *GUSBP11* overexpression (scale bar: 50 μ m). **C.** Protein levels of EMT markers as well as cell invasion-related factors were tested in the TNBC cell lines after *GUSBP11* overexpression. **D.** The sphere formation assay was taken to measure the effect of *GUSBP11* elevation on the stemness of the TNBC cell lines (scale bar: 100 μ m). **E, F.** The RNA level and protein level of stemness markers after *GUSBP11* overexpression. Three independent experiments were conducted (n=3). EMT; Epithelial-to-mesenchymal transition, TNBC; Triple negative breast cancer, and **; P<0.01.

YY1/p300/HDAC2 complex induces transcription inhibition of *GUSBP11* and suppresses its expression

To explore the upstream molecular mechanism of *GUSBP11* RNA in the TNBC, we used UCSC, an online database (<http://genome.ucsc.edu/>), and found that YY1 and p300 are two transcription factors that acted on the *GUSBP11* promoter. Through further screening in the JASPAR (<http://jaspar.genereg.net/>), we obtained three binding sequences of YY1 in the *GUSBP11* promoter (Fig.3A). Therefore, we constructed these three mutated sequences in order and then, verified the specific binding sites through luciferase reporter assay. Data revealed that YY1 might bind to the site 2 of *GUSBP11* promoter, where the luciferase activity of the HEK293T cell line showed an enhancement in the Site2-MUT group (Fig.3B). Through ChIP assay, it was verified that YY1 was enriched in the *GUSBP11* promoter in contrast to the control IgG group (Fig.3C). It has been reported that the YY1, p300 and HDAC2 can form a complex that regulates the development of colorectal cancer (16). Therefore, we conducted ChIP assay to verify the interaction of YY1/p300/HDAC2 axis and the *GUSBP11* promoter in the TNBC cell lines. Intriguingly, we uncovered that the enrichment of *GUSBP11* RNA in the immunoprecipitates conjugated to anti-YY1 was higher after overexpression of

those three factors (Fig.3D). Similarly, the result of luciferase reporter assay in the HEK293T cell line showed that the activity of *GUSBP11* promoter was decreased a lot after overexpression of YY1, HDAC2 and p300, whereas this decreased tendency was more obvious when they were all overexpressed (Fig.3E). Finally, the expression level of *GUSBP11* was found to be reduced after individually overexpression of YY1, p300 or HDAC2, while this tendency of *GUSBP11* expression was more evident after the co-overexpression of them (Fig.3F).

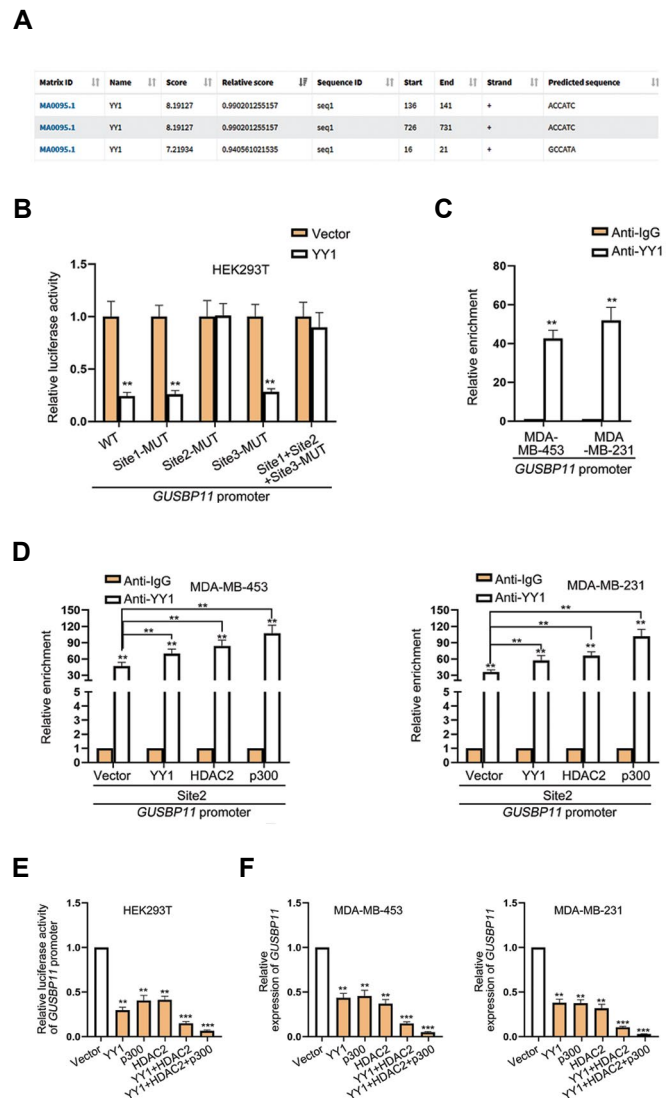


Fig.3: The YY1/p300/HDAC2 complex induces transcription inhibition of *GUSBP11* and suppresses its expression. **A.** Three binding sequences of YY1 in the *GUSBP11* promoter were obtained from JASPAR. **B.** The luciferase reporter assay was carried out to verify whether YY1 might bind to *GUSBP11* promoter. **C.** The affinity of YY1 in the *GUSBP11* promoter was verified by ChIP assay. **D.** ChIP assay was performed to measure the enrichment of YY1 upon YY1, HDAC2 and p300 overexpression. **E.** The luciferase reporter assay was performed in the HEK293T cell line when YY1, HDAC2 or p300 was overexpressed alone or they were overexpressed at the same time. **F.** RT-qPCR analysis of *GUSBP11* expression in the TNBC cell lines with individual or common overexpression of YY1, p300 and HDAC2. Three independent experiments were conducted (n=3). TNBC; Triple negative breast cancer, RT-qPCR; Quantitative reverse transcription real-time polymerase chain reaction, **; P<0.01, and ***; P<0.001.

GUSBP11 positively regulates *SPNS2* expression in the TNBC cell lines

In this part, we tried to verify the interaction between *GUSBP11* and *SPNS2*. According to GEPIA 2 database, we discovered that *SPNS2* expression was markedly declined in the TNBC tissues in contrast to normal tissues (Fig.4A). Meanwhile, a positive correlation between the *GUSBP11* expression and the *SPNS2* expression was observed (Fig.4B). Applying RT-qPCR, it was revealed that *SPNS2* was down-regulated in the TNBC cell lines in comparison with the MCF-10A cell line (Fig.4C). Afterwards, we explored whether *GUSBP11* and *SPNS2* could regulate each other. We found that *SPNS2* expression was up-regulated in the MDA-MB-231 and MDA-MB-453 cell lines transfected with pcDNA3.1/*GUSBP11* at both RNA and protein levels (Fig.4D). However, *SPNS2* overexpression did not affect the RNA expression of *GUSBP11* (Fig.4E, Fig.S1A, See Supplementary Online Information at www.celljournal.org). To further probe the potential mechanism of *GUSBP11* on the regulating *SPNS2* expression in the TNBC cell lines, we performed subcellular fractionation and FISH assays to determine the subcellular localization of *GUSBP11* in the TNBC cell lines. The results indicated that *GUSBP11* was majorly distributed in the cytoplasm, implying that *GUSBP11* regulated *SPNS2* at post-transcriptional level (17, 18, Fig.4F, G). To strengthen our hypothesis, we conducted luciferase reporter assay and determined that *GUSBP11* had no effect on the activity of the *SPNS2* promoter in RNA level (Fig.4H). As competitive endogenous RNA (ceRNA) mechanism is known as a common post-transcriptional regulatory method, we decided to explore whether *GUSBP11* may modulate the *SPNS2* expression through acting as a ceRNA to target certain miRNA in the TNBC cell lines. According to the result of RIP assays, both *GUSBP11* and *SPNS2* were highly enriched in the anti-Ago2 groups (Fig.4I), which supported the ceRNA model. Furthermore, a series of functional assays were taken to verify the effects of *SPNS2* overexpression in the TNBC cell lines, and results showed that increased *SPNS2* expression led to suppress cell proliferation, along with attenuated migration, invasion and EMT in the TNBC cell lines (Fig.S1B-J, See Supplementary Online Information at www.celljournal.org).

GUSBP11 positively regulates the *SPNS2* expression via interacting with *miR-579-3p*

We searched starBase (<http://starbase.sysu.edu.cn>) website to look for possible miRNAs combined with both *GUSBP11* and *SPNS2*. As illustrated in the Figure 5A, two miRNAs (*miR-579-3p* and *miR-664b-3p*) were observed at the intersection. Through RNA pull down assays, it was shown that *miR-579-3p* was abundantly

enriched in the *GUSBP11* biotin probe groups, while the other candidate *miR-664b-3p* showed no obvious change (Fig.5B). Therefore, *miR-579-3p* was chosen for further analyses. RIP data validated that *GUSBP11*, *miR-579-3p* and *SPNS2* were effectively abundant in the anti-Ago2 groups, indicating that these three RNAs co-existed in the RISCs (Fig.5C). Besides, we uncovered that the enrichment of *GUSBP11* and *SPNS2* was enhanced in the wild type of *miR-579-3p* group, while no obvious change was seen in the control group or the mutant group (Fig.5D). The respective binding sites of *GUSBP11* and *SPNS2* on the *miR-579-3p* were predicted via StarBase website (Fig.5E). We overexpressed *miR-579-3p* expression via the transfection of *miR-579-3p* mimics in the TNBC cell lines (Fig.5F), and it was then manifested from luciferase reporter assays that *miR-579-3p* mimics declined the luciferase activity of *GUSBP11*-WT and *SPNS2* 3'UTR-WT groups, while barely affected the *GUSBP11*-Mut and *SPNS2* 3'UTR-Mut groups (Fig.5G).

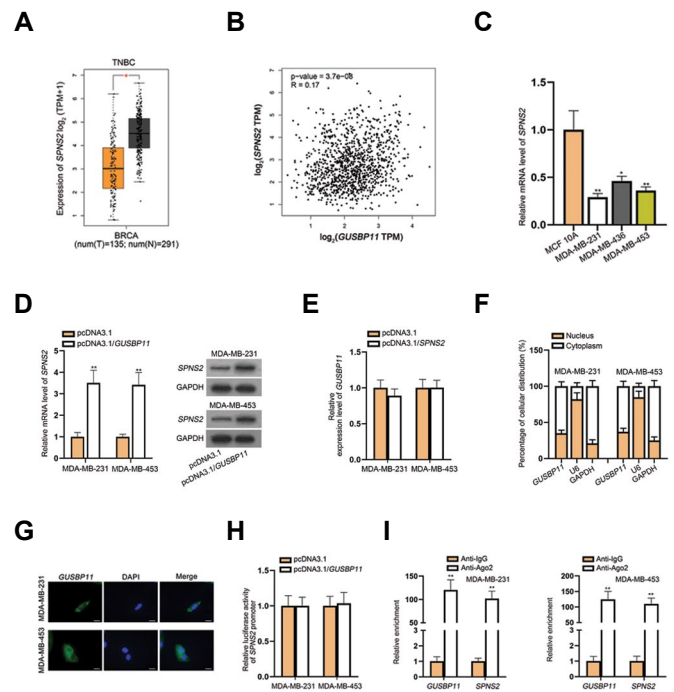


Fig.4: *GUSBP11* acts as a ceRNA to positively regulate *SPNS2* expression in the TNBC cell lines. **A.** Box plot from GEPIA 2 database indicated the expression of *SPNS2* in the 135 tumor TNBC tissues and 291 normal tissues. **B.** The correlation between *GUSBP11* and *SPNS2* expression was presented via GEPIA 2 database. **C.** The mRNA level of *SPNS2* in the TNBC cell lines MDA-MB-231, MDA-MB-436 and MDA-MB-453 in comparison with the MCF-10A cell line. **D.** *SPNS2* expression at both RNA and protein levels in the TNBC cell lines transfected with pcDNA3.1/*GUSBP11*. **E.** The *GUSBP11* RNA level in the TNBC cell lines with *SPNS2* overexpression. **F, G.** The cellular location of *GUSBP11* in the TNBC cell lines was determined by subcellular fractionation and FISH experiments (scale bar: 10 μ m). **H.** The luciferase activity of *SPNS2* promoter in the *GUSBP11*-overexpressed TNBC cell lines. **I.** The enrichment of *GUSBP11* and *SPNS2* in the Anti-Ago2 groups in contrast to the control IgG group was measured by RIP assays. Three independent experiments were conducted (n=3). TNBC; Triple negative breast cancer; *, P<0.05, and **, P<0.01.

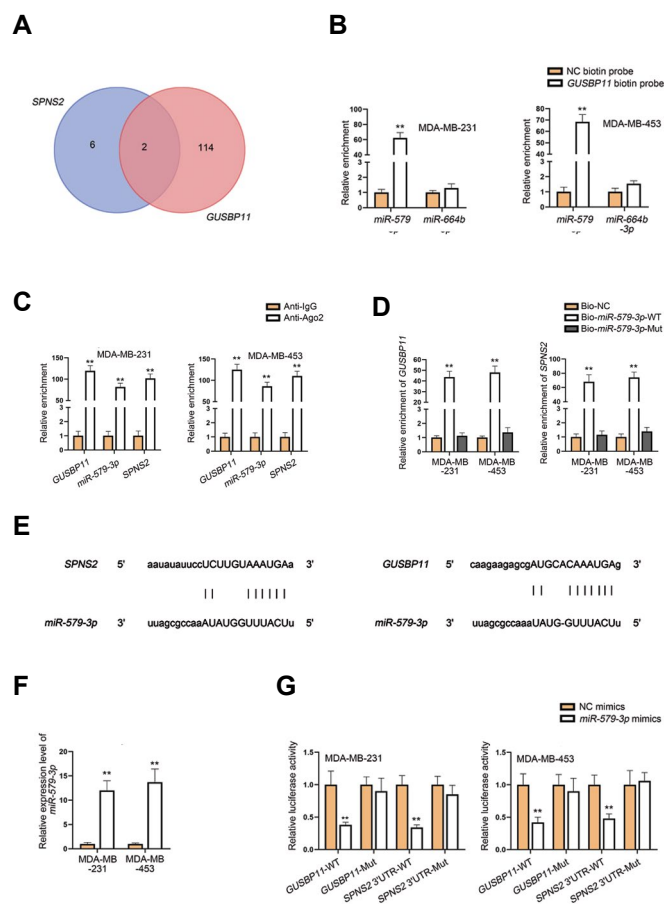


Fig.5: *GUSBP11* regulates *SPNS2* expression via interacting with the *miR-579-3p* in the TNBC cell lines. **A.** Potential miRNAs combined with the *GUSBP11* and *SPNS2* were predicted through starBase. **B.** The abundance of *miR-579-3p* and *miR-664b-3p* in the *GUSBP11* biotin probe groups was measured by RNA pull down assays. **C.** The enrichment of *GUSBP11*, *miR-579-3p* and *SPNS2* in the Anti-Ago2 groups was examined via RIP assays. **D.** The enrichment of *GUSBP11* and *SPNS2* in the bio-*miR-579-3p*-WT or bio-*miR-579-3p*-Mut groups was measured by RNA pull down assays. **E.** Respective binding sites of *GUSBP11* and *SPNS2* on the *miR-579-3p* were predicted via starBase website. **F.** *miR-579-3p* expression in the TNBC cell lines transfected with *miR-579-3p* mimics. **G.** The luciferase activity of *GUSBP11*-WT/Mut or *SPNS2* 3'UTR-WT/Mut in the TNBC cell lines when *miR-579-3p* was up-regulated. Three independent experiments were conducted (n=3). TNBC; Triple negative breast cancer and **; P<0.01.

***GUSBP11* restrains cell proliferation and promotes cell apoptosis in the TNBC cell lines via sponging *miR-579-3p* to elevate *SPNS2* expression**

We knocked down *SPNS2* in the MDA-MB-231 and MDA-MB-453 cell lines via the transfection of sh/*SPNS2*#1/2 (Fig.6A). After that, a series of rescue assays were taken to verify the regulatory mechanism of *GUSBP11/miR-579-3p/SPNS2* axis on the TNBC cell lines proliferation and apoptosis. As shown in the colony formation and EdU assays, overexpression of *GUSBP11* reduced the proliferation of the TNBC cell lines, while such effect was partially reversed by the co-transfection of *miR-579-3p* mimics or sh/*SPNS2*#1 (Fig.6B, C). In the TUNEL assays and flow cytometry analyses, the enhanced apoptosis rate induced by *GUSBP11* up-regulation was abolished after co-transfection of *miR-579-3p* mimics or sh/*SPNS2*#1 (Fig.6D, E).

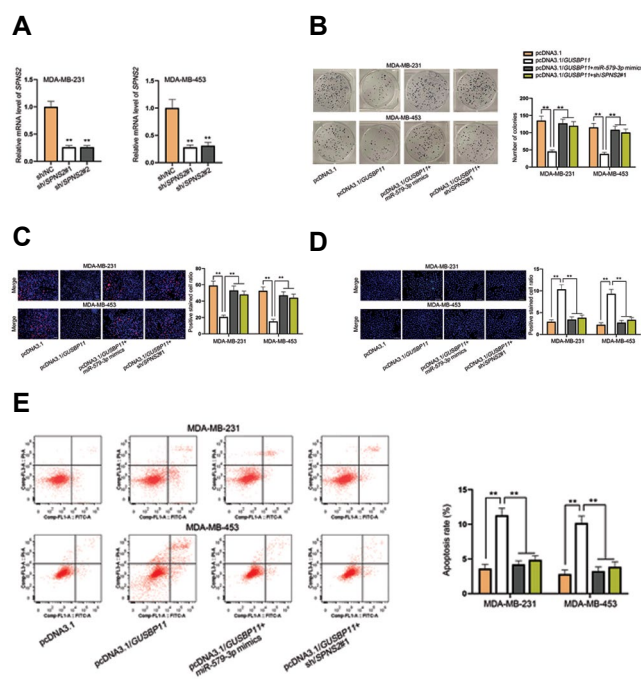


Fig.6: *GUSBP11* impedes the TNBC cell lines progression by targeting *miR-579-3p* to up-regulate the *SPNS2* expression. **A.** The mRNA level of *SPNS2* in the TNBC cell lines transfected with shRNAs targeting *SPNS2*. Rescue experiments were conducted in the TNBC cell lines transfected with different plasmids (pcDNA3.1, pcDNA3.1/*GUSBP11*, pcDNA3.1/*GUSBP11*+*miR-579-3p* mimics and pcDNA3.1/*GUSBP11*+sh/*SPNS2*#1). **B, C.** Colony formation and EdU (scale bar: 50 μm) assay were taken to analyze the proliferation of the TNBC cell lines under different transfection conditions. **D, E.** TUNEL (scale bar: 50 μm), together with flow cytometry was performed to measure the apoptosis of the TNBC cell lines in different groups. Three independent experiments were conducted (n=3). TNBC; Triple negative breast cancer and **; P<0.01.

***GUSBP11* represses cell migration, EMT and stemness in the TNBC cell lines via interacting with *miR-579-3p* to increase *SPNS2* expression**

The impacts of the *GUSBP11/miR-579-3p/SPNS2* axis on the cell migration, EMT and stemness were also determined. In Transwell assays, we found that the overexpression of *miR-579-3p* or the silencing of *SPNS2* could countervail the repressive cell migration and invasion in the TNBC caused by *GUSBP11* up-regulation (Fig.S2A, B, See Supplementary Online Information at www.celljournal.org). Meanwhile, the repressed EMT caused by *GUSBP11* overexpression was offset after the co-transfection of *miR-579-3p* mimics or sh/*SPNS2*#1 (Fig.S2C, See Supplementary Online Information at www.celljournal.org). Additionally, *miR-579-3p* elevation or *SPNS2* deletion could restore the reduced number of spheres that was mediated by the *GUSBP11* up-regulation (Fig.S2D, See Supplementary Online Information at www.celljournal.org). Meanwhile, the levels of stemness markers reduced by *GUSBP11* silencing were recovered after overexpression of *miR-579-3p* or knockdown of *SPNS2* (Fig.S2E, F, See Supplementary Online Information at www.celljournal.org).

Discussion

Recently, emerging evidences have shown that lncRNAs

are implicated in the development of TNBC (19). Thus, a better understanding of lncRNAs might contribute to effective treatment of the TNBC patients. According to recent studies, *GUSBP11* has been registered to be closely linked to gastric cancer (20) and neck squamous cell carcinoma (21). Nonetheless, the function of *GUSBP11* in the TNBC occurrence remains largely obscure. In our research, we discovered that *GUSBP11* was down-regulated in the TNBC cell lines. Overexpression of *GUSBP11* obviously inhibited cell growth, migration, EMT and stemness in the TNBC cell lines. All these data demonstrated that *GUSBP11* exerted anti-oncogenic functions on the TNBC progression.

Transcriptional regulation is a mechanism that can regulate RNA expression. Previous studies have reported that lncRNAs can be activated by their upstream transcription factors and thus up-regulating lncRNAs in the human cancers (22, 23). Additionally, lncRNAs can be down-regulated by their upstream transcription suppressors (24). Here, we also investigated the upstream mechanism of *GUSBP11* in the TNBC cell lines. YY1/p300/HDAC2 complex has been reported to be efficient in the gene transcription suppressing (25), so in our research, we predicted that YY1 and p300 may be two potential upstream regulatory factors for *GUSBP11*. Through mechanism experiments, we demonstrated that down-regulation of *GUSBP11* in the TNBC cell lines was induced by the YY1/p300/HDAC2 complex affinity to its promoter region.

Competitive endogenous RNA (ceRNA) mechanism is known as a common post-transcriptional regulatory method and lncRNAs have been extensively reported to affect cancer development via ceRNA model (26, 27). Accumulating evidence have pointed that lncRNAs can compete for miRNA response elements (MREs) with the driver genes to be involved in cancer development by acting as a ceRNA to interact with miRNA (28). Our study also demonstrated that *GUSBP11* functioned as a ceRNA to positively regulate *SPNS2* expression in the TNBC cell lines. As reported previously, *SPNS2* enhances proliferation, migration and invasion colorectal cancer cell line via controlling S1P/S1PR1/3 axis and Akt and ERK pathway (29). *SPNS2* plays crucial roles in repressing the migratory ability in the non-small cell lung cancer cell line (30). However, we found that *SPNS2* presented a low RNA level in the TNBC cell lines, and it was further validated that overexpression of *SPNS2* significantly suppressed the malignant cell behaviors in the TNBC. As known, miRNA is a key part of ceRNA mechanism and numerous miRNAs exert important roles in the TNBC progression (31). *MiR-221/222* enhances the Wnt/ β -catenin signaling to facilitate TNBC aggressiveness (32). *MiR-211-5p* inhibits tumor cell growth and metastasis in the TNBC cell lines as well as the TNBC xenograft model via targeting SETBP1 (33). In this study, we found that *miR-579-3p* was a common miRNA combined with *GUSBP11* and *SPNS2*. It has been documented that *miR-579-3p* is down-regulated in the squamous cell lung

carcinoma cell line, while its overexpression represses this progression (34). Moreover, *miR-579-3p* is related to melanoma progression and resistance to target treatments (15). In our study, we confirmed that *GUSBP11* inhibited the progression of TNBC via targeting the *miR-579-3p/SPNS2* axis.

However, due to the limited time and materials, there still existed several limitations in this study which required for further verification. Also, clinical data should be complemented to enrich the significance of our current study. We will make further clinical investigation in our future research.

Conclusion

GUSBP11 restrains cell proliferation and promotes cell apoptosis in the TNBC cell lines via sponging *miR-579-3p* to elevate *SPNS2* expression.

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Authors' Contribution

G.W.; Participated in study design, data collection and evaluation, drafting and statistical analysis. P.S.; Contributed to all experimental work, data and statistical analysis, and interpretation of data. C.Q.; Drafted the manuscript. All authors have read and approved the manuscript.

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