

The long noncoding RNA LINC00200 promotes the malignant progression of MYCN-amplified neuroblastoma via binding to insulin like growth factor 2 mRNA binding protein 3 (IGF2BP3) to enhance the stability of Zic family member 2 (ZIC2) mRNA

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ABSTRACT

Objectives: Neuroblastoma (NB) is one of the most common extracranial malignant tumors in children and remarkable heterogeneous tumor of the sympathetic nervous system. Long noncoding RNAs (lncRNAs) have been reported to be vital biological roles in the initiation and malignant progression of tumors. The aim of this study was to explore the biological role and the possible molecular mechanism of LINC00200 in NB.

Design & methods: The expression level of LINC00200 in NB tissues and cell lines were detected by real-time quantitative PCR (qRT-PCR). The biological effects of LINC00200 on NB cell proliferation, migration and invasion were examined by EdU and transwell assays. The molecular mechanism of LINC00200 in NB were explored and verified by bioinformatics analysis, RNA binding protein immunoprecipitation (RIP) assay and RNA pull down assay.

Results: The results showed that the expression of LINC00200 was significantly higher in NB tissues than in normal tissues. Besides, the expression of LINC00200 was higher in MYCN Amplified NB tissues than in MYCN non-Amplified NB tissues. Moreover, overexpression of LINC00200 could remarkably promote proliferation, migration and invasion of NB cell. Mechanistically, LINC00200 might bind to RNA binding protein (RBP) IGF2BP3 and promote the expression of ZIC2.

Conclusions: Overall, we showed that LINC00200 was upregulated in NB tissues and the LINC00200/IGF2BP3/ZIC2 regulatory axis might be the possible therapeutic target for NB.

1. Introduction

Neuroblastoma (NB) originates from embryonic neural crest cells and is the most common extracranial solid tumor in children [1]. NB accounts for approximately 10% of childhood tumors and is the leading cause of death from cancer in children aged 1–5 years [2]. NB has a complex clinical presentation and can occur anywhere along the sympathetic ganglion and shows heterogeneity between tumor locations [3]. The prognosis of children with NB is poor due to the clinical features of insidious primary site such as difficulty in early diagnosis, high

malignancy, rapid progression and early metastasis. Approximately 60% of NB patients exert metastases at the time of diagnosis, while bone marrow and cortical bone are the most common sites of metastasis [4]. Based on parameters of tumor histology, clinical stage, tumor cell ploidy, and MYCN oncogene amplification, patients with NB can be stratified into low-, intermediate-, and high-risk groups [5]. In the high-risk group of NB patients, amplification of the MYCN gene and mutations in the ALK gene have been identified as risk factors for their poor prognosis [6]. MYCN gene amplification exerts in approximately 20% of NB and is usually accompanied by segmental chromosome loss at

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the end of the short arm of chromosome 1 [7]. The treatments for NB include surgery, myeloablative chemotherapy, radiotherapy combined with immunotherapy, and stem cell transplantation [8]. Despite continuous improvements in diagnosis and therapy, the five-year survival rate for high-risk and relapsed NB patients is still less than 50% [9]. Therefore, it is clinically essential to investigate the underlying pathogenesis and possible therapeutic targets of NB.

In the human genome sequence, the majority of products in the transcription process are non-coding transcripts. Non-coding RNAs (ncRNAs) are RNA sequences that transcribed by genes but not ultimately translated into proteins, and generally greater than 200 nt in length [10]. In cells, lncRNAs can be located in the cytoplasm or nucleus and are rarely involved in encoding proteins and considered as null transcription products [11]. Studies have indicated that lncRNAs can regulate their target genes at the transcriptional, post-transcriptional, and translational levels [12]. lncRNAs have significant regulatory roles in cell proliferation, differentiation, development, and apoptosis of tumors [13]. lncRNAs can disrupt genomic stability and affect the function of related proteins through DNA methylation, chromatin remodeling, and genetic mechanisms of miRNAs, resulting in the initiation and progression of tumor [14,15]. Researchers have also identified many biological functions of lncRNAs, including genomic imprinting, X chromosome silencing, gene regulation, cell cycle regulation, and chromosomal dosage compensation effects [16].

Gene expression is a complex process that regulated by multiple proteins acting in concert to mediate biochemical reactions. Among these factors, RNA binding proteins (RBPs) can modulate downstream genes expression by directly binding to mRNAs and blocking microRNAs (miRNAs) biogenesis at the post-transcriptional level [17,18]. RBPs can bind to specific motifs of RNA sequences to form ribonucleoprotein (RNP) complexes that affect RNA structure and interactions and participate in a variety of cellular processes, particularly in gene transcription, RNA processing, localization, translation, and regulation of mRNA stability [19]. The family of insulin-like growth factor 2 mRNA binding proteins (IGF2BPs) are highly conserved RBP family that includes three members, IGF2BP1, IGF2BP2 and IGF2BP3, which share structural similarities, all containing two RNA recognition motifs and four KH structural domain [20]. The IGF2BP3 gene, located on human chromosome 7p15.3, encodes a 69 kDa protein that is involved in the initiation and development of different tumors by regulating mRNA stability [21,22].

In this study, we demonstrated that LINC00200 was upregulated in MYCN amplified NB tissues by analyzing the TARGET database. LINC00200 has previously been shown to be involved in tumor formation, metastasis and drug resistance as an oncogene in gastric cancer [23,24]. However, its biological role and molecular mechanism in NB remains unclear. We identified that the expression of LINC00200 in NB tissues was remarkably higher than that in normal control tissues. Moreover, the expression of LINC00200 in MYCN amplified NB tissues was also higher than that in MYCN non-amplified NB tissues. Inhibition of LINC00200 expression in NB cells could remarkably suppress cell proliferation, migration and invasion. Overall, the LINC00200/IGF2BP3/ZIC2 regulatory axis might be the potential pathogenic mechanism and therapeutic target of NB.

2. Materials and methods

2.1. TARGET database

The TARGET database (<https://ocg.cancer.gov/programs/target>) was established by the National Cancer Institute's (NCI) Office of Cancer Genomics (OCG) and Cancer Therapy Evaluation Program (CTEP) of the National Cancer Institute (NCI). It applies a comprehensive genomic approach to investigate the molecular mechanisms underlying the origin and progression of childhood cancer, with the aim of using big data analysis to guide the development of effective, less toxic treatments for

Table 1
Sequences of primers for transfection.

Name		Sequence
LINC00200 siRNA-1	Sense	5'-UCAUUUGGACACAAAUGGAG-3'
	Anti-sense	5'-CCAUUUGUGUCCAAAUGAAA-3'
LINC00200 siRNA-2	Sense	5'-UACUUUAAACUACUAGACUU-3'
	Anti-sense	5'-GUCAUAGUAGUUUAAAGUAGA-3'
LINC00200 siRNA-3	Sense	5'-AAAUUAGGGUGUGAUGUACGG-3'
	Anti-sense	5'-GUACAUCACACCAUUAUUUCA-3'
LINC00200 siRNA-NC	Sense	5'-UCGCACGCUUUGCGUAGAU-3'
	Anti-sense	5'-GCAAGUAUAGCGUAAGCA-3'
IGF2BP3 siRNA	Sense	5'-AAAAUACGAGAAAAACGAA-3'
	Anti-sense	5'-CGUUUUUUCUGUUAUUUUUU-3'
IGF2BP3 siRNA-NC	Sense	5'-AUAAUCUAGGAAAGCAUCAC-3'
	Anti-sense	5'-GAUGCUUUUCCUAGAUUAUUC-3'
ZIC2 siRNA	Sense	5'-UCCUUUUGUGGAUCUUGAGGU-3'
	Anti-sense	5'-CUCAAGAUCCACAAAGGACC-3'
ZIC2 siRNA-NC	Sense	5'-ACAAUAUCCACUUUACCAGAG-3'
	Anti-sense	5'-CUGGUAAGUGGAUAUUGUUG-3'

the disease. We analyzed lncRNA expression in 33 MYCN amplified NB tissues and 126 MYCN non-amplified NB tissues using the TARGET database.

2.2. Specimen collection

A total of 20 NB tissues (7 MYCN amplified-NB tissues as well as 13 MYCN non-amplified-NB tissues) and 20 normal control tissues resected intraoperatively from June 2016 to August 2020 from the Children's Hospital of Nanjing Medical University were collected in this study. According to the International Neuroblastoma staging system (INSS), there were 1 case in stage I, 3 cases in stage II, 6 cases in stage III, 9 cases in stage IV and 1 case in stage IVS. According to the primary location, there were 9 cases of left adrenal gland, 4 cases of right adrenal gland, 4 cases of retroperitoneal sympathetic nerve chain, 2 cases of thoracic cavity and 1 cases of pelvic cavity. The tissues were rapidly frozen in liquid nitrogen and stored in a -80 °C refrigerator after isolation. All specimens were confirmed as NB tissues by two experienced clinicopathologists, and all NB patients had no history of preoperative radiotherapy or chemotherapy and no history of other tumors. The experiment was approved by the ethics committee of the Children's Hospital of Nanjing Medical University (approval number, 202007136-1). The written informed consents were signed by the family members of the tissues provider. The experiments were strictly in accordance with the provisions of the Declaration of Helsinki.

2.3. Cell culture and transfection

Human umbilical vein endothelial cells (HUVECs) and human NB cell lines (SHEP, IMR32 and SMS-KAN) were purchased from ATCC (Manassas, VA, USA). HUVECs were cultivated in MCDB 131 medium (Gibco, The Netherlands) with 1% Gibco antibiotics and 10% v/v Gibco fetal bovine serum. SHEP, IMR32 and SMS-KAN were cultured in RPMI 1640 complete. All cells were cultured at 37 °C in an incubator with 5% CO₂. Transfection was performed according to the instructions of Lipofectamine 3000 (Invitrogen, USA) according to the previous study, and transfection efficiency was measured by qRT-PCR after transfection [25]. All primer sequences were designed and synthesized by GenePharma (Shanghai, China) and listed in Table 1.

2.4. RNA extraction and real-time quantitative PCR (qRT-PCR)

Total RNA was extracted from NB tissues or cells using Trizol according to the instructions. 500 ng of RNA was reverse transcribed into cDNA using PrimeScript™ RT Reagent Kit. 2 µl of cDNA was used as template, 1 µl of each upstream and downstream primers were taken, and PCR was performed using SYBR Green qPCR Mix kit. The reaction

Table 2

The sequences of primers for qRT-PCR.

Name		Sequence
LINC00200	Forward	5'-GGTTTACCACATGTAGTAAG-3'
	Reverse	5'-GTTGTGCCAATTCCTGTGCT-3'
IGF2BP3	Forward	5'-TATATCGGAAACCTCAGCGAGA-3'
	Reverse	5'-GGACCGAGTGCTCAACTTCT-3'
ZIC2	Forward	5'-AGCCCCACTTCTACCAGATG-3'
	Reverse	5'-TGAGAACTGTTATTTCCCATGC-3'
ZIC5	Forward	5'-GCGCAACTCCACAACCAAGTA-3'
	Reverse	5'-TGCCGCATATAGCGGAAAAAG-3'
TECRL	Forward	5'-CACAAAGTCCCTCGCTTCGG-3'
	Reverse	5'-CCCCTGAGAGTACAAGTTT-3'
DDX1	Forward	5'-TCTCCGAGATGGGTGTAATGC-3'
	Reverse	5'-ACCTCCTCCTAAGATCAATGGG-3'
NKX2-5	Forward	5'-CAAAGGACCCTAGAGCCGAA-3'
	Reverse	5'-ATAGCGGGGTAGGCGTTAT-3'
NBAS	Forward	5'-AGTACAGCCTAGAGGCAACCA-3'
	Reverse	5'-ACACACTGATCTTGAACAGCAG-3'
MNX1	Forward	5'-CTCCTACTCGTACCCGAG-3'
	Reverse	5'-TTGAAGTCGGCATCTTATGGC-3'
NPW	Forward	5'-CGTCGCTCACCTATCTGTG-3'
	Reverse	5'-GCCTCTCCCTCTCACGTA-3'
GAPDH	Forward	5'-GGAGCGAGATCCCTCCAAAT-3'
	Reverse	5'-GGCTGTTGTCATCTTCTCATGG-3'
U6	Forward	5'-ACTTCAGCAGCATATACTAAAAA-3'
	Reverse	5'-CGCTTCACGAATTGTCATGCAT-3'

conditions were: pre-denaturation at 95 °C for 10 min, 40 cycles, denaturation at 95 °C for 30 s, annealing at 60.0 °C for 30 s, extension at 72 °C for 30 s, and extension at 72 °C for 10 min. QRT-PCR was performed on a Roche 480II fluorescent PCR instrument. U6 and GAPDH were used as internal reference, and the relative concentration of samples to be tested = $2^{-\Delta\Delta CT}$ [26]. The primer sequences are listed in Table 2.

2.5. EdU experiment

We used the EdU kit (RiboBio, China) to detect the effect of LINC00200 on the proliferative capacity of NB cells according to the previous study [27]. Briefly, we inoculated treated NB cells into 96-well plates at a density of 5000 cells/well, followed by the addition of 50 mM of EdU solution and continued to culture the cells. After approximately 24 h of incubation, the cells were fixed with 4% paraformaldehyde, treated with 0.1% TritonX-100 for 5 min, closed with bovine serum albumin for 30 min, removed from the cell crawl, washed, added rhodamine, and incubated with EdU reaction mixture for 20 min, and the nuclei were restained with Hoechst. Five randomly selected fields of view were photographed under the microscope, and the ratio of EdU fused cell numbers was calculated.

2.6. Transwell assay

We performed transwell assay based on a previous study [28]. Briefly, each group of cells was transfected for 24 h. The cells were then washed twice with PBS, digested with trypsin, terminated with culture medium, centrifuged, discarded, washed twice with PBS, resuspended, and adjusted to a cell density of 5×10^5 /ml. 200 μ l of single cell suspension was added to the upper chamber of each well, and 600 μ l of culture medium containing 10% FBS was added to the lower chamber. The chambers were incubated for 48 h at 37 °C in a 5% CO₂ incubator. Then the chambers were removed, washed twice with PBS, fixed in pre-cooled formaldehyde at 4 °C for 30 min, air-dried, stained with 0.1% crystal violet for 15 min, and the upper layer of immigrated cells was wiped off with a cotton swab. For the invasion assay, the pre-gelled transwell chambers were removed from the -20 °C refrigerator, 500 μ l of baseplate was added to the upper and lower chambers, and the chambers were hydrated in a 37 °C, 5% CO₂ incubator for 2 h. The cell suspension was added and incubated in a 37 °C, 5% CO₂ incubator for

30 h. The rest of the experiments were performed as above.

2.7. Nucleoplasm separation experiments

Nucleoplasm separation experiments were performed as reported in a previous study [29]. Briefly, NB cells were transferred into 1.5 ml EP tubes, followed by the addition of cytoplasmic lysis solution (RLA), incubation on ice for 20 min, and centrifugation for 15 min (3000 r/min) using a centrifuge. For the extraction of nucleoproteins, the cells were washed and precipitated by RLA, and nuclear lysis solution (RIPA) was added after three iterations. Then the treated cells were incubated on ice for 20 min with vortex shaking for 30 s at 5 min intervals, and centrifuged for 15 min (3000 r/min) using a centrifuge for 15 min. Subsequently, the expression levels of LINC00200 in the nucleus and cytoplasm were detected by qRT-PCR, respectively.

2.8. RNA binding protein immunoprecipitation (RIP) assay

RIP experiments were performed according to a previous study [27]. Briefly, we conducted RIP experiments by utilizing the Magna RIP RNA Binding Protein Immunoprecipitation Kit (Millipore). Lysed IMR32 and SMS-KAN cell lysates were incubated with protein A+G magnetic beads that were incubated overnight at 4 °C using IGF2BP3 antibody (Abcam, ab26271) or control IgG. The eluted solutions were analyzed by protein blotting.

2.9. RNA pull-down experiment

RNA pull-down experiments were performed according to a previous study [30]. Briefly, 100 μ g of total RNA from NB cells was extracted by the Trizol kit. Biotinylated IGF2BP3 was incubated with cellular proteins extracted from IMR32 or SMS-KAN cells, followed by the introduction of streptavidin beads at room temperature. The mixture of streptavidin magnetic beads and probe solution is lightly mixed with total RNA and incubated for 30 min. Elution buffer is added and the bound RNA complexes are collected. Purify and quantify the immunoprecipitated RNA by qRT-PCR.

2.10. Western blot assay

Western blot experiments were performed according to a previous study [25]. Cells were first lysed using RIPA (Beyotime, Shanghai, China) and cellular proteins were extracted. Protein concentrations were quantified and leveled using the BCA kit (Beyotime, Shanghai, China). Equal amounts of protein (30 mg) were taken, separated by 10% SDS-PAGE, transferred to PVDF membrane by semi-dry transfer method, placed on 5% skimmed milk powder and closed at room temperature for 2 h. Appropriate concentrations of IGF2BP3 antibody (1:2000, ab177477, abcam), MYCN antibody (1:2000, ab16898, abcam) and ZIC2 antibody (1:2000, ab150404, abcam) were added overnight at 4 °C. Then the cells were washed and incubated for 1 h at 37 °C with secondary antibody and exposed for color development. GAPDH was used as the internal reference.

2.11. Actinomycin D assay

Actinomycin D assay was performed according to the steps reported in a previous study [31]. Briefly, we exposed treated IMR32 with SMS-KAN cells to 2 μ g/ml of actinomycin D (Sigma, St. Louis, MO, USA) at 0, 24, 48, 72, 96 h. Cells were collected and total RNA was extracted. QRT-PCR was used to analyze the expression levels of ZIC2.

2.12. Xenograft model assays

The animal studies were conducted according to the institutional ethics guidelines for animal assays approved by the animal management

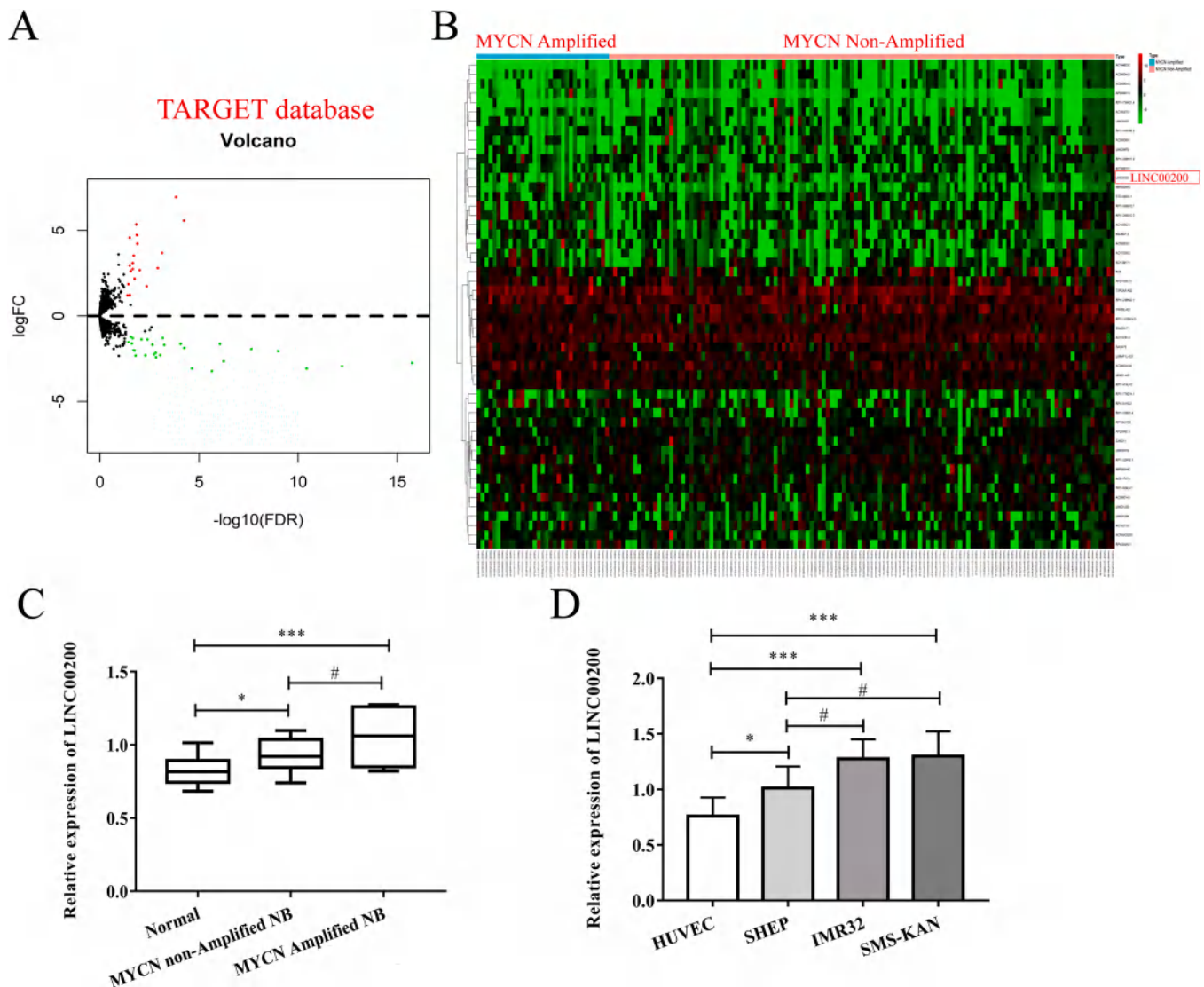


Fig. 1. Increased expression of LINC00200 in MYCN-Amplified NB. A. Volcano plot showing differentially expressed lncRNAs in MYCN Amplified NB tissues and MYCN Non-Amplified NB tissues. B. Heat map showing differentially expressed lncRNAs in MYCN Amplified NB tissues and MYCN Non-Amplified NB tissues, including LINC00200. C. Expression levels of LINC00200 in normal tissue, MYCN Amplified NB tissue and MYCN Non-Amplified NB tissue by qRT-PCR. NB cells by qRT-PCR. * $p < 0.05$, *** $p < 0.001$ and # $p < 0.05$.

committee of Children's Hospital of Nanjing Medical University (approve number: IACUC-2207035). About 4×10^6 cells were injected subcutaneously into the axilla of the female athymic BALB/C nude mice. The mice were then randomized into two groups and the tumor volume was calculated using the following formula: volume (mm^3) = length \times width²/2 [32].

2.13. Statistical analysis

All data were processed with SPSS 22.0 statistical software (SPSS Inc., USA). Measured data were displayed as mean \pm standard deviation and differences were regarded as statistically significant at $P < 0.05$. NB tissues were compared with normal control tissues using unpaired t-test. All results were subjected to three independent experimental replications.

3. Results

In this study, we identified the differentially expressed lncRNAs in MYCN amplified NB tissues by analyzing the TARGET database and

selected LINC00200 for further study. The expression level of LINC00200 was significantly increased in NB tissues by conducting qRT-PCR assay. While LINC00200 expression level in MYCN amplified NB tissues and cells was higher than that in MYCN non-amplified NB tissues and cells. Overall, we identified that LINC00200/IGF2BP3/ZIC2 regulatory axis might be the potential pathogenic mechanism and therapeutic target of NB.

3.1. LINC00200 expression was increased in MYCN-amplified NB

To investigate the role and potential mechanism of lncRNAs in NB, we analyzed the differentially expressed lncRNAs in MYCN amplified and MYCN non-amplified NB tissues by analyzing the TARGET database. The results showed that 17 lncRNAs were significantly upregulated and 17 lncRNAs were downregulated in MYCN amplified NB tissues (Fig. 1A, B). Among them, LINC00200 expression was significantly abnormal expressed and closely associated with tumor initiation and progression, therefore we selected it for further study. Subsequently, we verified the expression of LINC00200 in the collected NB tissues. As shown in Fig. 1C, the expression of LINC00200 in NB tissues was remarkably

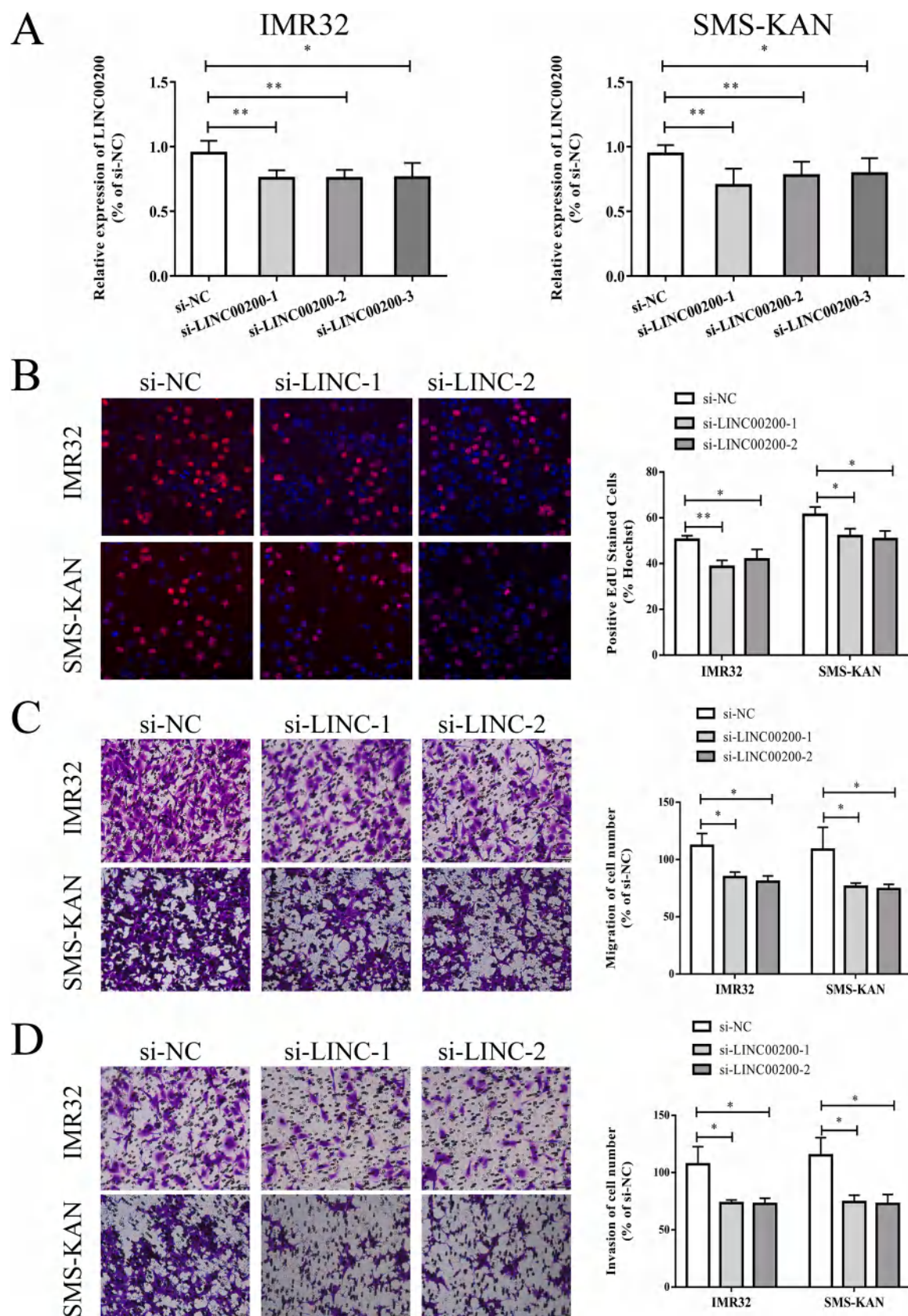


Fig. 2. Inhibition of LINC00200 significantly inhibited the proliferation, migration and invasion of NB cells. A. Transfection efficiency was measured by qRT-PCR after transfection of LINC00200 siRNAs in IMR32 and SMS-KAN cells. B. The effect of inhibition of LINC00200 on the proliferation of IMR32 and SMS-KAN cells was examined by performing EdU assay. LINC00200 on the migration and invasion of IMR32 cells as well as SMS-KAN cells. * $p < 0.05$, ** $p < 0.01$.

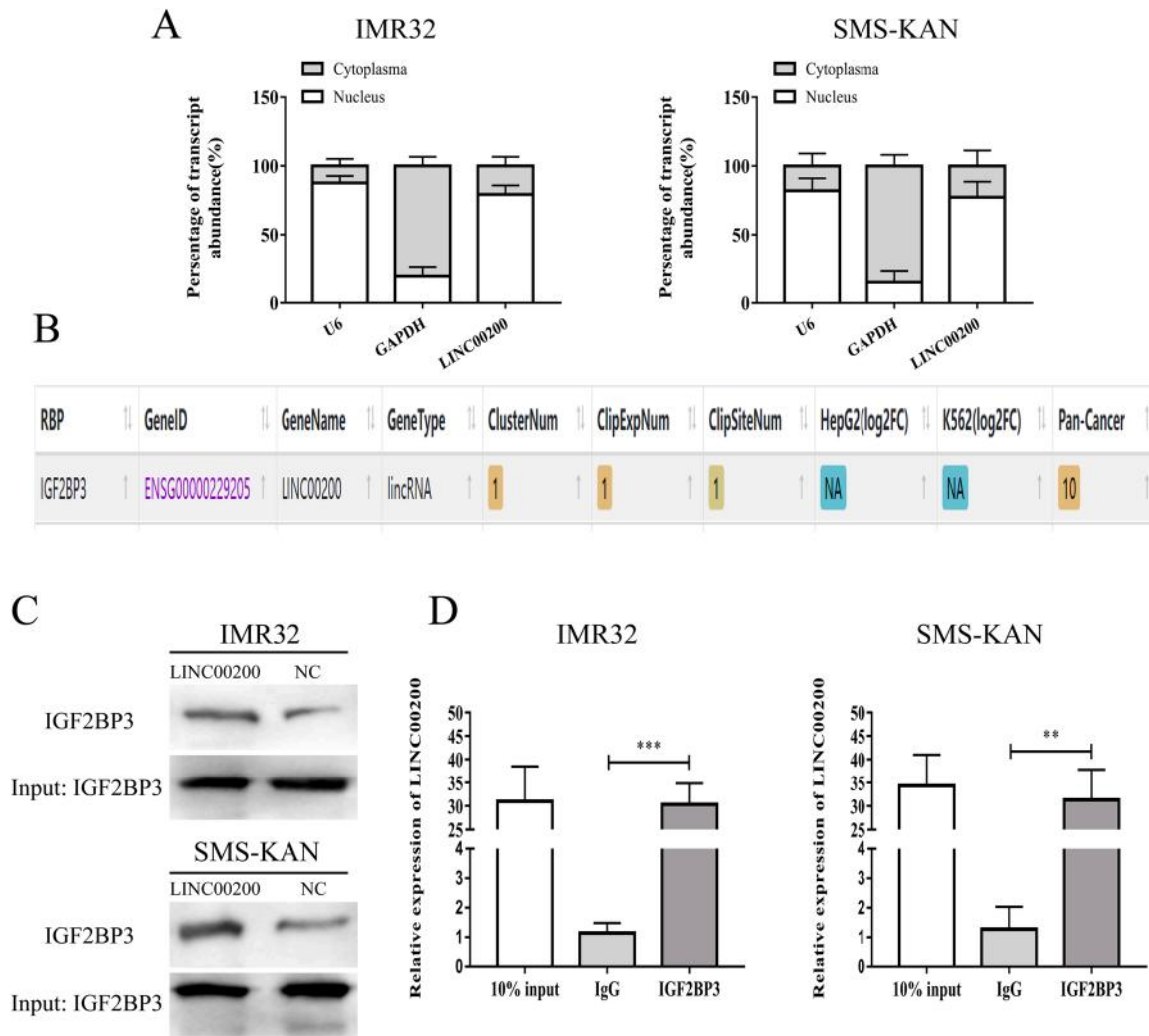


Fig. 3. The LINC00200 was capable of incorporating the IGF2BP3. A. Localization of LINC00200 in IMR32 cells and SMS-KAN cells by nucleoplasmic isolation assay. B. Detection of RBPs capable of binding to LINC00200 by starbase database (<https://starbase.sysu.edu.cn/>). C. Detection of the binding relationship between LINC00200 and IGF2BP3 by RIP assay. D. Binding of IGF2BP3 to LINC00200 was detected by RNA pull-down assay. **p < 0.01, ***p < 0.001.

higher than that in normal control tissues, and the expression in MYCN amplified NB tissues was obviously higher than that in MYCN non-amplified NB tissues. Moreover, we also found that the expression of LINC00200 in NB cell lines was higher than that in normal control cells and was higher in MYCN amplified NB cells than in MYCN non-amplified NB cells (Fig. 1D). The above results suggested that LINC00200 might have a pro-carcinogenic role in the development of NB. To explore the correlation between LINC00200 and MYCN, we conducted qRT-PCR and western blot assays. The results showed that MYCN expression was increased after overexpression of LINC00200, while decreased after inhibiting LINC00200 (Fig. S1). However, the regulatory mechanism between LINC00200 and MYCN remained to be further explored.

3.2. Inhibition of LINC00200 obviously suppressed the proliferation, migration and invasion of NB cells

We selected MYCN amplified NB cells (IMR32 and SMS-KAN cells) for biological role and functional studies. We transfected IMR32 and SMS-KAN cells with siRNAs and examined the transfection efficiency by qRT-PCR. The results showed that all LINC00200 siRNAs could significantly inhibit LINC00200 expression in NB cells, among which siRNA-1 and siRNA-2 had higher transfection efficiency, so we selected them for the follow-up study (Fig. 2A). After inhibiting LINC00200 expression in IMR32 and SMS-KAN cells, we examined the effects of cell proliferation,

migration and invasion ability by EdU and transwell assays. The results indicated that inhibition of LINC00200 obviously inhibited the proliferative, migrative and invasive ability of IMR32 and SMS-KAN cells.

3.3. The LINC00200 could bind to IGF2BP3

The studies indicate that the subcellular localization of lncRNAs determines the potential biological functions. To investigate the possible biological function of LINC00200, we examined the localization of LINC00200 in IMR32 and SMS-KAN cells by nucleoplasm separation assay. The results showed that LINC00200 was mainly located in the nucleus of IMR32 and SMS-KAN cells (Fig. 3A), suggesting that LINC00200 might regulate downstream gene expression at the transcriptional level. We detected the RBPs that could bind to LINC00200 through the Starbase database (<https://starbase.sysu.edu.cn/>), and the results showed a high binding fraction of IGF2BP3 (Fig. 3B). The RIP results showed that IGF2BP3 could be pulled down by LINC00200 (Fig. 3C), while the RNA pull-down results showed that LINC00200 was enriched by anti-IGF2BP3 antibody both in IMR32 and SMS-KAN cells (Fig. 3D). We also examined the expression of IGF2BP3 after inhibition of LINC00200 in IMR32 and SMS-KAN cells and showed no significant difference (data not shown). These results suggested that LINC00200 could bind to IGF2BP3 and had no regulatory effect on IGF2BP3 expression.

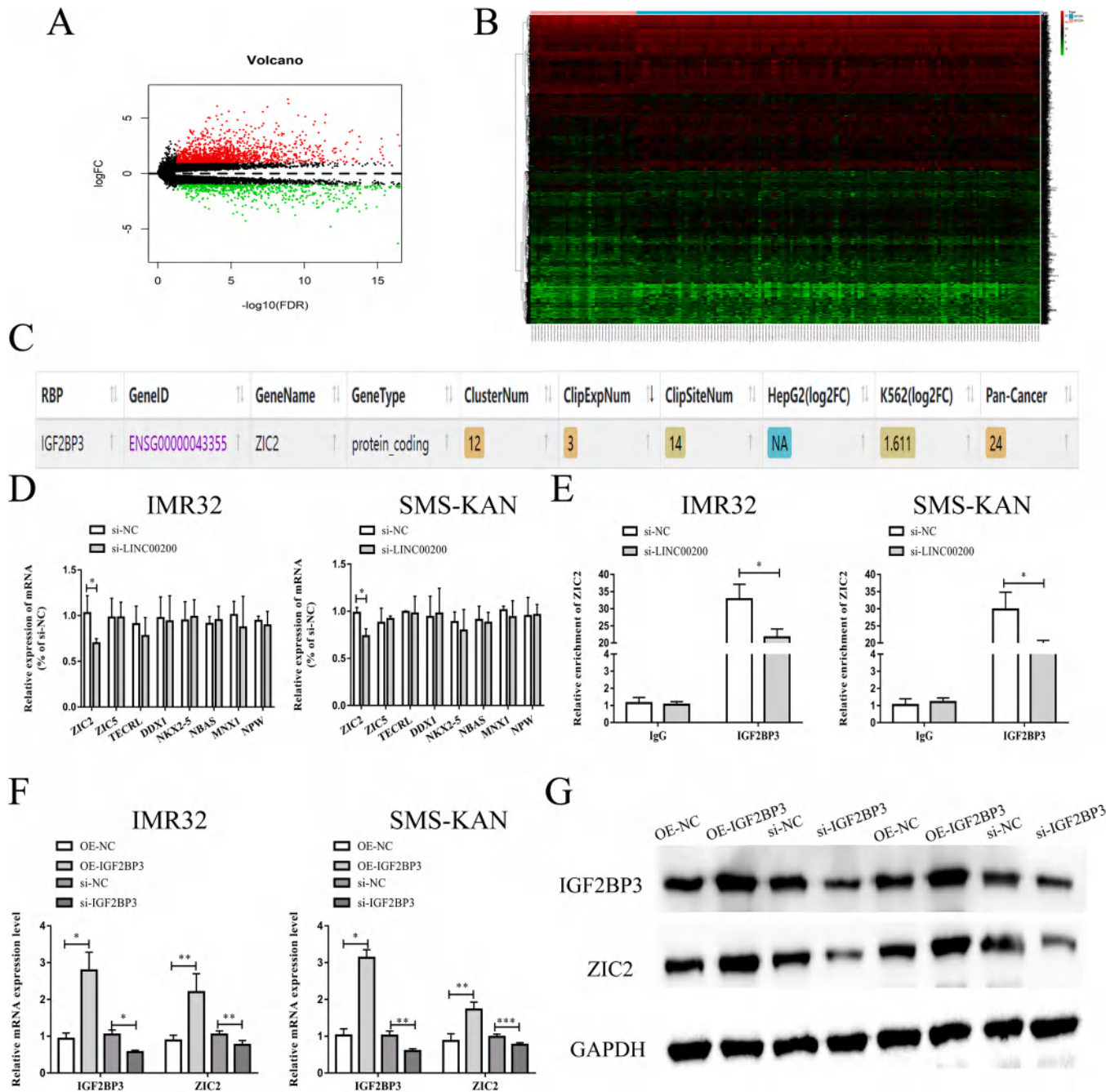


Fig. 4. IGF2BP3 could promote the stability of ZIC2 mRNA. **A.** Volcano plot showing differentially expressed mRNAs in MYCN Amplified NB tissues and MYCN Non-Amplified NB tissues. **B.** Heat map showing differentially expressed mRNAs in MYCN Amplified NB tissues and MYCN Non-Amplified NB tissues. mRNAs in MYCN Amplified NB tissues as well as in MYCN Non-Amplified NB tissues. **C.** Binding of mRNAs highly expressed in MYCN Amplified NB tissues (top 20 mRNAs with significantly higher expression) to IGF2BP3 was detected by starbase database (<https://starbase.sysu.edu.cn/>). **D.** Inhibition of LINC00 in IMR32 cells as well as in SMS-KAN cells. **E.** Expression of ZIC2 by RNA pull down assay after inhibition of LINC00200 in IMR32 cells and SMS-KAN cells. ZIC2 expression was detected by qRT-PCR and western blot assay after inhibition of IGF2BP3. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.4. IGF2BP3 could promote the stability of ZIC2 mRNA

IGF2BP3 can promote gene expression by promoting the stability of mRNAs of genes. To investigate the potential downstream of IGF2BP3, we analyzed the genes significantly highly expressed in MYCN-amplified NB tissues through the TARGET database and screened the top 20 genes with significant differences (Fig. 4A, B). Eight of these genes (ZIC2, ZIC5, TECRL, DDX1, NKX2-5, NBAS, MNX1 and NPW) were identified to bind to IGF2BP3 by Starbase database (Fig. 4C). Subsequently, we examined the expression of these eight genes by qRT-PCR after

inhibition of LINC00200 in IMR32 and SMS-KAN cells, and the results showed that si-LINC00200 could inhibit the expression of ZIC2 but had no significant effect on the expression of other genes (Fig. 4D). This suggested that ZIC2 may be a potential downstream. The expression level of ZIC2 was detected by RNA pull-down assay after inhibition of LINC00200 in IMR32 cells and SMS-KAN cells. The results verified that inhibition of LINC00200 could suppress the binding of IGF2BP3 to ZIC2 (Fig. 4E). Furthermore, we detected the expression of ZIC2 in NB cells after overexpression or inhibition of IGF2BP3. The results demonstrated that overexpression of IGF2BP3 could promote the expression level of

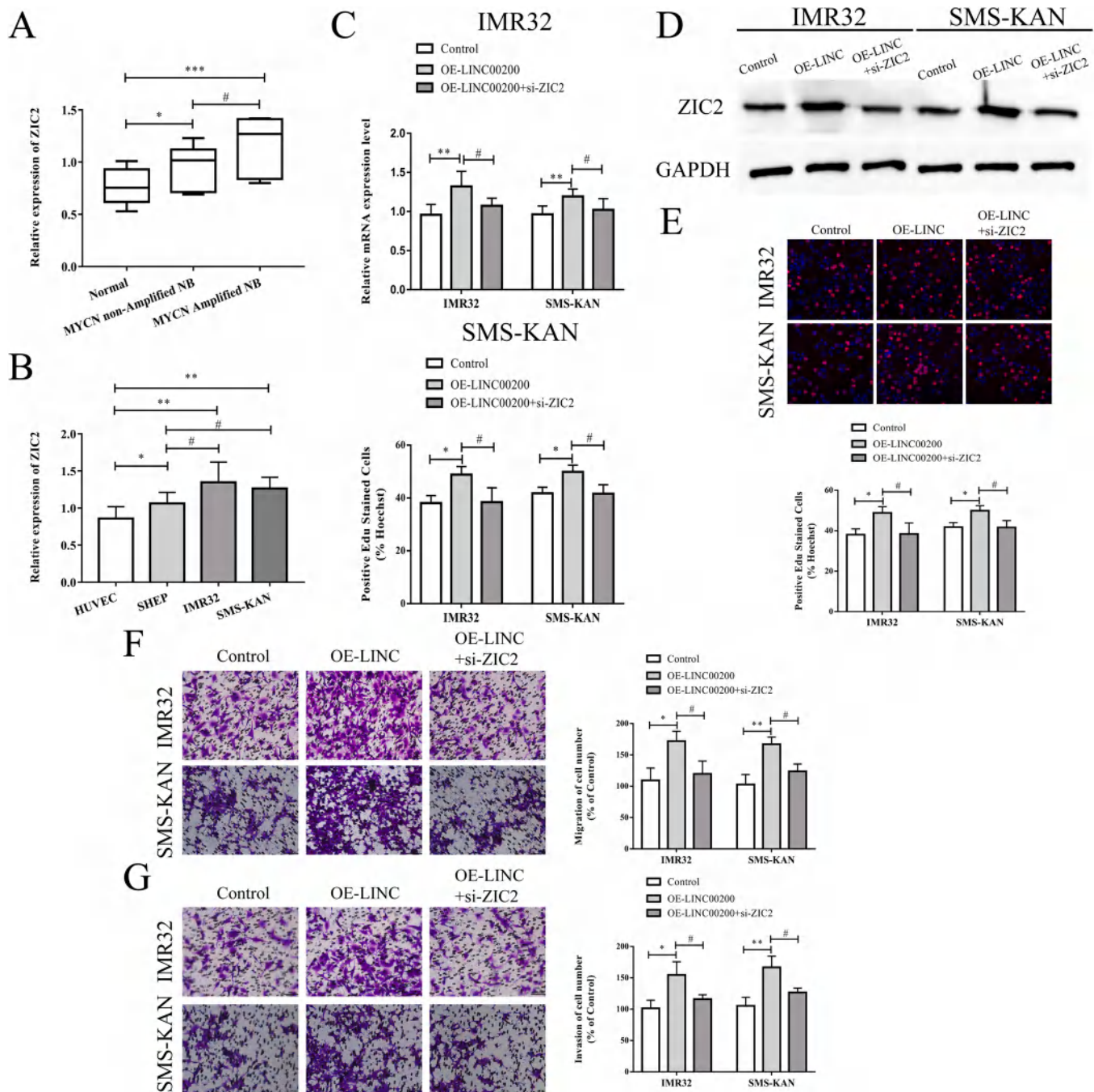


Fig. 5. LINC00200 could promote the malignant progression of NB by promoting the expression of ZIC2. A. ZIC2 expression in normal, MYCN Amplified NB and MYCN Non-Amplified NB tissues by qRT-PCR. B. ZIC2 expression in HUVEC cells and NB cells by qRT-PCR. C-D. ZIC2 expression in IMR32 cells and SMS-KAN cells after simultaneous overexpression of LINC00200 and inhibition of ZIC2 by qRT-PCR and western blot. cells by qRT-PCR and western blot assay. E. Proliferation of IMR32 and SMS-KAN cells by EdU assay after simultaneous overexpression of LINC00200 and inhibition of ZIC2. The migration and invasion ability of the cells were measured by transwell assay after overexpression of LINC00200 and inhibition of ZIC2 in both IMR32 and SMS-KAN cells. *p < 0.05, **p < 0.01, ***p < 0.001 and #p < 0.05.

ZIC2, while inhibition of IGF2BP3 decreased the expression of ZIC2 (Fig. 4F, G). The results indicated that overexpression of IGF2BP3 promoted the stability of ZIC2 mRNA, while inhibition of IGF2BP3 had the opposite biological effect (Fig. S2A, B). These results suggested that IGF2BP3 might promote the mRNA stability of ZIC2 and thus promote its expression.

3.5. LINC00200 could promote malignant progression of NB by promoting the expression of ZIC2

Subsequently, we further verified the expression of ZIC2 in the collected NB tissues. As shown in Fig. 5A, the expression of ZIC2 in NB tissues was higher than that in normal control tissues, and in MYCN amplified NB tissues was significantly higher than that in MYCN non-amplified NB tissues. Moreover, we also found that the expression of ZIC2 in NB cell lines was significantly higher than that in normal control cells and in MYCN amplified NB cells than that in MYCN non-amplified

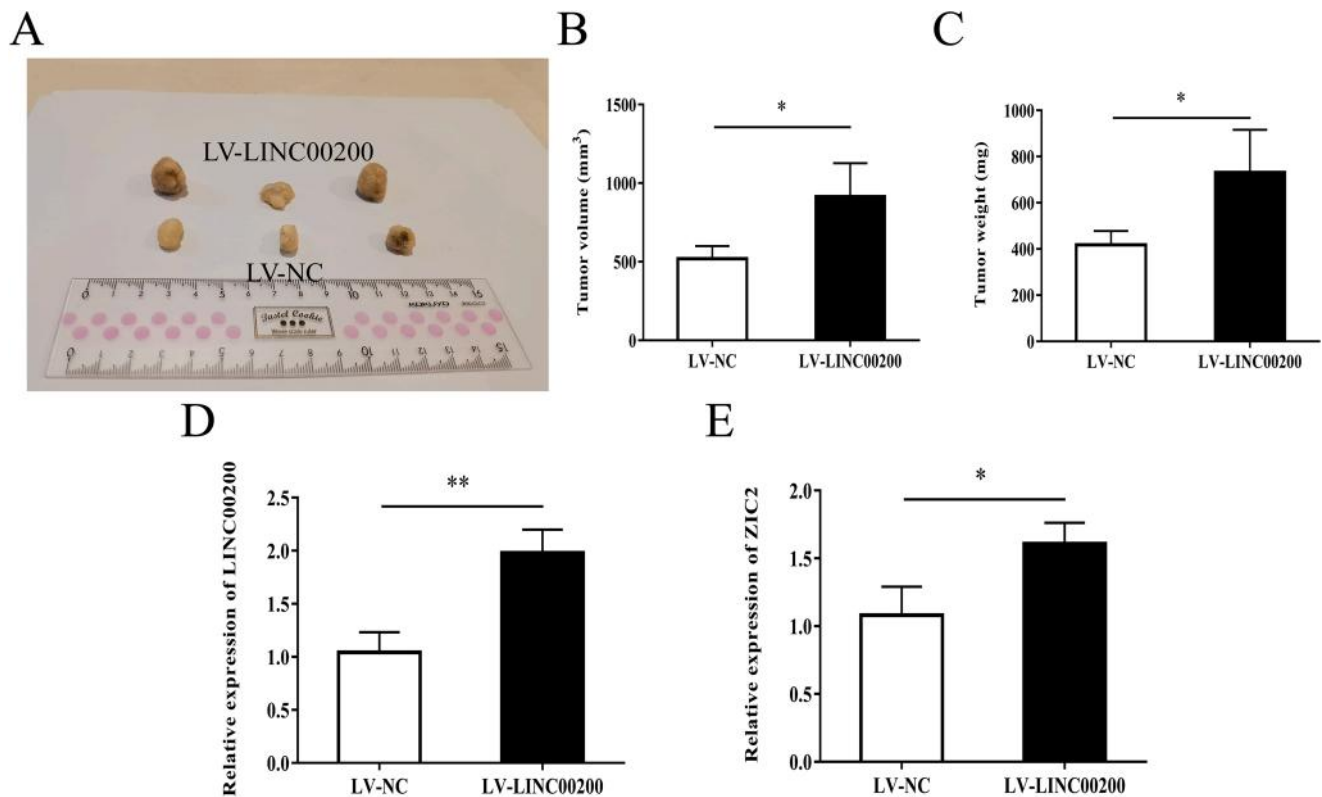


Fig. 6. LINC00200 could promote tumor growth. The effect of overexpression of LINC00200 on the growth ability of NB tumors was examined by in vivo experiments. a. Representative pictures of the excised tumors. b. the average volume of the excised tumors. c. the average weight of the excised tumors. D. The expression level of LINC00200 in tumor tissues by qRT-PCR. E. The expression level of ZIC2 in tumor tissues by qRT-PCR. * $p < 0.05$, ** $p < 0.01$.

NB cells (Fig. 5B). The above results suggest that ZIC2 might have a pro-oncogenic role in NB development and progression. The expression of ZIC2 was detected by qRT-PCR and western blot assay after overexpression of LINC00200 and inhibition of ZIC2 in IMR32 cells and SMS-KAN cells simultaneously. The results showed that overexpression of LINC00200 significantly promoted ZIC2 expression, while ZIC2 expression was significantly decreased after simultaneous transfection with ZIC2 siRNA (Fig. 5C, D). The degradation rate of ZIC2 mRNA in NB cells after simultaneous overexpression of LINC00200 and inhibition of IGF2BP3 was measured by qRT-PCR. The results showed that overexpression of LINC00200 promoted the stability of ZIC2, while the degradation rate of ZIC2 mRNA was significantly higher after simultaneous inhibition of IGF2BP3 (Fig. 51C). Moreover, we examined the proliferation, migration and invasion ability of the cells by EdU and transwell assays after overexpression of LINC00200 and inhibition of ZIC2 in both IMR32 and SMS-KAN cells. The results showed that overexpression of LINC00200 significantly promoted the proliferation, migration and invasion of NB cells, while simultaneous inhibition of ZIC2 inhibited the malignant progression of NB cells (Fig. 5E-G). These results suggested that LINC00200 might promote the malignant progression of NB cells by promoting the expression of ZIC2.

3.6. LINC00200 could promote tumor growth

To further verify the biological effect of LINC00200 on NB, we constructed an IMR32 cell line stably expressing LINC00200 and performed in vivo experiments. The results showed that the tumor growth ability was significantly enhanced in the LV-LINC00200 group compared with the LV-NC group (Fig. 6A-C). Besides, we found that LV-LINC00200 also significantly promoted the expression of LINC00200 and ZIC2 in tumor tissues by assay (Fig. 6D, E).

4. Discussion

The development of NB is very complex and the exact cause is still unclear. It is currently believed that the development of NB is a multifactorial and multi-step process, which is caused by a combination of environmental and genetic factors [33]. MYCN gene amplification is widespread in neuroblastoma and has been found to be present in approximately 20% of neuroblastoma patients, and is strongly associated with the degree of malignancy, rate of progression and poor prognosis of NB [34]. Recent studies have shown that lncRNAs may participate in tumor development and play an integral role in NB. LINC00839 promotes NB by regulating the miR-338-3p/GLUT1 axis cell proliferation, migration, invasion and glycolysis and promotes apoptosis [35]. LINC01410 promotes tumorigenesis and increases radiosensitivity by regulating the miR-545-3p/HK2 axis [36]. LncRNA HCP5 promotes NB proliferation by regulating the miR-186-5p/MAP3K2 signaling axis [37].

In this study, we identified that LINC00200 was upregulated in MYCN amplified NB tissues by analyzing TARGET database. Besides, we also showed that LINC00200 might act as an oncogene in the malignant progression of NB. However, the correlation between the expression level of LINC00200 and the clinical characteristics of NB patients did not analyze because of the small number of specimens we collected so far. In future studies we will collect more specimens and explore the potential of LINC00200 as the clinical diagnostic marker for NB.

LncRNAs have long sequences and complex secondary structures and can bind to proteins or interact with other DNA or RNA and play an important role in the regulation of cellular functions [38]. RBPs are a class of proteins that can bind to double- or single-stranded RNAs in cells and RNA-protein complexes. RBPs can bind to many RNAs, including mRNA and lncRNA, and plays an important regulatory role at the post-transcriptional level [39,40]. We verified that LINC00200 was

mainly located in the nucleus, and speculated that LINC00200 might play an important biological role by binding RBP. Through bioinformatic analysis and in vitro cellular assays, we demonstrated that LINC00200 might play a regulatory role by recruiting IGF2BP3, which has been previously reported to promote tumor progression by stabilizing mRNA. Lnc-CERS6-AS1 enhances the stability of CERS6 mRNA by binding to IGF2BP3, which leads to malignant progression of breast cancer [41]. RBM15 can promote the stability of TMBIM6 by binding to IGF2BP3, leading to malignant progression of laryngeal squamous cell carcinoma [42]. Linc01305 can promote metastasis and proliferation of esophageal squamous cell carcinoma by binding to IGF2BP2 and IGF2BP3, thereby stabilizing HTR3A mRNA [43].

There are five members of the ZIC gene family, ZIC1, ZIC2, ZIC3, ZIC4 and ZIC5, each of which contains three exonic structures and encodes a C-terminal zinc finger structure with C2H2 [44]. The ZIC2 gene is located on the long arm of human chromosome 13, region 13q32.3, and encodes 532 amino acid residues with a relative molecular weight of 55 kDa [45]. Several studies have confirmed that abnormal ZIC family functions can lead to the development of various neurological disorders, while abnormal expression of ZIC genes, such as mutations and promoter methylation, can often lead to the development of various malignancies [45]. We found that IGF2BP3 could bind to ZIC2 and promote its mRNA stability through bioinformatic analysis and in vitro cellular assays. Through reversal experiments, we found that LINC00200 might promote the proliferation, migration and invasion of NB cells by promoting the stability of ZIC2 mRNA, which in turn led to the malignant progression of NB.

5. Conclusion

Overall, our study indicated that LINC00200 was upregulated in MYCN amplified NB tissues. Besides, LINC00200 could promote the proliferation, migration and invasion of NB cells by enhancing the stability of ZIC2 mRNA by binding to IGF2BP3. The LINC00200/IGF2BP3/ZIC2 regulatory axis might be the potential pathogenic mechanism and therapeutic target of NB.

CRedit authorship contribution statement

Lei Huang and Yongjun Fang: Conceptualization, Methodology, Writing – review & editing. **Ji Chen and Mengjiao Sun:** Investigation, Data curation, Writing – original draft. **Ji Chen and Lei Huang:** Visualization, Validation, Supervision, Software.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Data availability

All data included in this study are available upon request from the corresponding author.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.prp.2022.154059](https://doi.org/10.1016/j.prp.2022.154059).

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