

Downregulated *NORAD* in neuroblastoma promotes cell proliferation *via* chromosomal instability and predicts poor prognosis

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Increasing evidence suggests that long non-coding RNAs (lncRNAs) are involved in neuroblastoma (NB) pathogenesis. The aim of this study was to elucidate the roles and underlying mechanism of non-coding RNA activated by DNA damage (*NORAD*) in childhood NB. Both public data and clinical specimens were used to determine *NORAD* expression. Colony formation, cell proliferation and wound healing assays were performed to evaluate *NORAD* effects on proliferation and migration of SH-SY5Y and SK-N-BE(2) cells. Flow cytometry was used to examine the cell cycle changes. The expression of genes and proteins involved in chromosomal instability was determined by qRT-PCR and western blotting, respectively. Our results showed that low *NORAD* expression correlated with advanced tumor stage, high risk and *MYCN* amplification in both public data and clinical samples. Kaplan–Meier analysis indicated that patients with low *NORAD* expression had poor survival outcomes. Functional research showed that *NORAD* knockdown promoted cell proliferation and migration, and arrested the cell cycle at the G2/M phase. Moreover, the expression of the DNA damage sensor, PARP1, increased after *NORAD* knockdown, indicating a potential contribution of *NORAD* to DNA damage repair. *NORAD* silencing also affected the expression of genes and proteins related to sister chromatid cohesion and segregation, which are involved in chromosomal instability and consequent aneuploidy. These results suggest that *NORAD* may serve as a tumor suppressor in NB pathogenesis and progression. Thus, *NORAD* is a potential therapeutic target and a promising prognostic marker for NB patients.

Key words: Neuroblastoma, long non-coding RNA, *NORAD*, chromosomal instability, DNA damage

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Abbreviations: *ALK*, anaplastic lymphoma kinase; lncRNAs, long non-coding RNAs; NB, Neuroblastoma; *TERT*, telomerase reverse transcriptase

INTRODUCTION

Neuroblastoma (NB) in children accounts for 13% of all pediatric cancer mortality, and the 5-year overall survival rate for high-risk NB children is less than 40% (Maris *et al.*, 2007; Louis *et al.*, 2015; Matthay *et al.*, 2016). Its clinical impact and unique biology have gained attention in translational research. Although great efforts have been made by researchers and physicians, few gene alterations have been identified, such as *MYCN* amplification, telomerase reverse transcriptase (*TERT*) rearrangement, and anaplastic lymphoma kinase (*ALK*) and *ATRX* mutations (Molenaar *et al.*, 2012; Pugh *et al.*, 2013; Peifer *et al.*, 2015). With regard to drug discovery, only Unituxin (dinutuximab) has been approved as a novel targeted drug for NB (Bartholomew *et al.*, 2016). Recently, chimeric antigen receptor (CAR)-modified T cell therapy has been suggested as a promising method for treating NB, but its utility and safety were still controversial during preclinical research (Kunkele *et al.*, 2017; Richman *et al.*, 2018). Difficulties in NB therapy emphasize the need to clarify the mechanisms underlying NB pathogenesis.

Apart from major genome aberrations, it has been shown that the tumor microenvironment, epigenetic and transcriptional regulation are also involved in NB (Domingo-Fernandez *et al.*, 2013; Pandey *et al.*, 2015; Borriello *et al.*, 2016). Long non-coding RNAs (lncRNAs) are transcripts with no protein-coding ability. Functionally, lncRNAs contribute to epigenetic regulation by interacting with cellular macromolecules such as DNA, RNA and protein (Ulitsky *et al.*, 2013; Schmitt *et al.*, 2016). It has been reported that *MEG3*, *HCN3* and *linc01105* negatively correlate with the NB stage (Tang *et al.*, 2016), while *NBAT1* and *SNHG16* control NB progression through cell proliferation and migration (Pandey *et al.*, 2014; Yu *et al.*, 2019). As molecular sponges, *NeD125* and *KCNQ10T1* regulate NB apoptosis by binding to miR-125b-1 and miR-296-5p, respectively (Bevilacqua *et al.*, 2015; Li *et al.*, 2020). For clinical prognosis, *SNHG1* has been identified as a novel predictor of high-risk NB survival (Sahu *et al.*, 2016). These studies suggest that lncRNAs function as oncogenes and/or tumor suppressors in diverse processes in NB.

Recently, non-coding RNA activated by DNA damage (*NORAD*) has been found as a conserved lncRNA in the cytoplasm (Lee *et al.*, 2016; Tichon *et al.*, 2016; Mun-

schauer *et al.*, 2018). Functional studies have explored its effects in various tumor types. It has been shown that *NORAD* expression significantly increased in colorectal cancer (Wang *et al.*, 2018), and promoted cell proliferation and invasion in cervical cancer (Huo *et al.*, 2018). However, *NORAD* has also been reported to be a tumor suppressor that is involved in hepatocellular carcinoma via the PI3K/AKT pathway (Hu *et al.*, 2017; Lei *et al.*, 2018). Increasing inconsistent evidence has indicated that *NORAD* is involved in tumorigenesis, however, it is still unclear whether it is an oncogene or tumor suppressor in NB tumorigenesis.

To elucidate the precise function and underlying mechanism of *NORAD* in NB, both public data and clinical NB specimens were collected to examine its expression. Cell proliferation, colony formation and wound healing assays were performed in SH-SY5Y and SK-N-BE(2) cells to clarify *NORAD* function. To study the underlying mechanism of *NORAD*, cell cycle changes were evaluated by flow cytometry, and the expression of genes and proteins involved in chromosomal instability was determined by qRT-PCR and western blotting, respectively.

MATERIALS AND METHODS

Data processing and gene expression analysis. The Gene Expression Omnibus (GEO) public database was used to obtain gene chip and sequencing datasets on NB. Formatted family files and Series Matrix files of GSE62564 (Dataset 1: 498 cases) and GSE12460 (Dataset 2: 51 cases) were downloaded to analyze *NORAD* expression in NB. Clinical information of tumor stages, grades and *MYCN* status was picked out from items in these files. Based on the survival data in Dataset 1, the prognostic value of *NORAD* was evaluated by Kaplan-Meier survival analysis (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE62564>). Patients were divided into low-expression (249 cases) and high-expression (249 cases) groups based on the median expression of *NORAD*.

Clinical specimens and cell lines. To verify *NORAD* expression in NB, 40 NB tumor specimens stored in liquid nitrogen were obtained from Beijing Biobank for Diseases in Children, Beijing Children's Hospital. All the samples in this biobank were collected along with signed informed consents. Clinical staging information was provided by the Pathology Department, based on the International Neuroblastoma Staging System (INSS). The Ethics Committees of Beijing Children's Hospital approved this study (2018-143). The SH-SY5Y and SK-N-BE(2) cell lines were purchased from American Type Culture Collection (Manassas, VA, USA). The cells were cultured in DMEM containing 10% fetal bovine serum (FBS) and maintained in a humidified incubator (5% CO₂, 37°C).

RNA extraction and qRT-PCR assays. Tumor RNA was extracted using an RNA isolation kit (Zymo Research, USA) after tissues were homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA, USA). A First Strand cDNA Synthesis Kit (Invitrogen) was used for RNA reverse transcription. SYBR Green Master mix was applied to detect gene expression using ViiA7 Dx machine (Applied Biosystems, USA). The primer sequences for target genes and reference genes were listed in Supplementary Table 1. The relative expression of RNAs was calculated using the 2^{-ΔCt} method.

Cell transfection. For cell transfection assays, the siRNA oligonucleotides for *NORAD* and negative con-

trol were synthesized by Sangon Biotech (Shanghai, China). These siRNAs were termed siRNA1#, siRNA2# and siNC, respectively. siRNA (100 nM) was transfected into cells using Lipofectamine RNAiMAX (Invitrogen). A series of experiments were performed after the medium was replaced with normal culture medium 8 h after the transfection. The following siRNA sequences used were: *NORAD*: siRNA1#, 5'-UAGCCCUUCUAGAUG-GAAAAdTdT-3' and siRNA2#, 5'-CCACUGGCU-GUGCCCAGACdTdT-3'; negative control (5'-UUCUC-CGAAACGUGACGdTdT-3').

Cell proliferation assay. Cell proliferation was monitored by a cell kinetic analyzer (ACEA, Biosciences, USA) as described previously (Yu *et al.*, 2018). Briefly, about 2 × 10³ cells were seeded into an E-plate, incubated at 37°C for 24 h, transfected with siRNA and cultured for 3 days. Data analysis was finally performed by the preinstalled software.

Colony formation assay. A total of 1 × 10³ cells per well were plated into 6-well plates and transfected with siRNA. The medium was replaced every 3 days. Ten days later, 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) and 0.1% crystal violet (Sigma-Aldrich) were used to fix and stain the cells for 10 min, respectively. Cell colonies were observed using a light microscope (IX73; Olympus, Japan).

Wound healing assay. Cells were plated into 6-well plates at 3 × 10⁵ cells per well. A "wound" was created with a micropipette tip when the cells reached about 50% confluence. The detached cells were gently removed with PBS and the attached cells were transfected with siRNA. The wound was observed and photographed by a light microscope at 0, 24, 48 and 72 h. AlphaView SA 3.4.0 (ProteinSimple, USA) was used to measure the wound width.

DNA damage and cell cycle analysis. To verify the role of *NORAD* in DNA damage, cells were exposed to doxorubicin (0.25 mM, 0.5 mM, 1 mM; Sigma-Aldrich) for 24 h and *NORAD* expression was determined by qRT-PCR. The level of DNA damage sensor, poly (ADP-ribose) polymerase 1 (PARP1), was examined 48 h after *NORAD* silencing. For cell cycle analysis, SH-SY5Y cells were transfected with siRNAs, incubated for 72 h, and then collected and resuspended in cold ethanol (70%) overnight at 4°C. The cells were pelleted and then incubated with working solution [2% FBS, 10 μL propidium iodide (1 mg/mL, Sigma-Aldrich) and 2 μL RNase A (10 mg/mL, TIANGEN, China)] for 30 min at 37°C in the dark. The cell cycle was evaluated using a flow cytometer (FACSCalibur, BD, USA). At least 1 × 10⁴ cells were analyzed for each sample.

Western blotting. After transfection with siRNA, the cells were incubated for 72 h, harvested and lysed with cold RIPA buffer for 30 min on ice. Bicinchoninic acid (BCA) protein assay (Pierce, USA) was used to de-

Table 1. Cell cycle arrest of SH-SY5Y cells after siRNA transfection.

Groups	Distribution of cell cycle		
	G0/G1	S	G2/M
siNC	59.7±2.43	27.2±3.42	13.1±1.10
siRNA1#	49.8±1.48*	30.1±0.43	20.1±1.90*
siRNA2#	50.4±2.11*	31.3±0.94	18.3±0.82*

Data are expressed as means ± S.D. from three independent experiments (**p*<0.05).

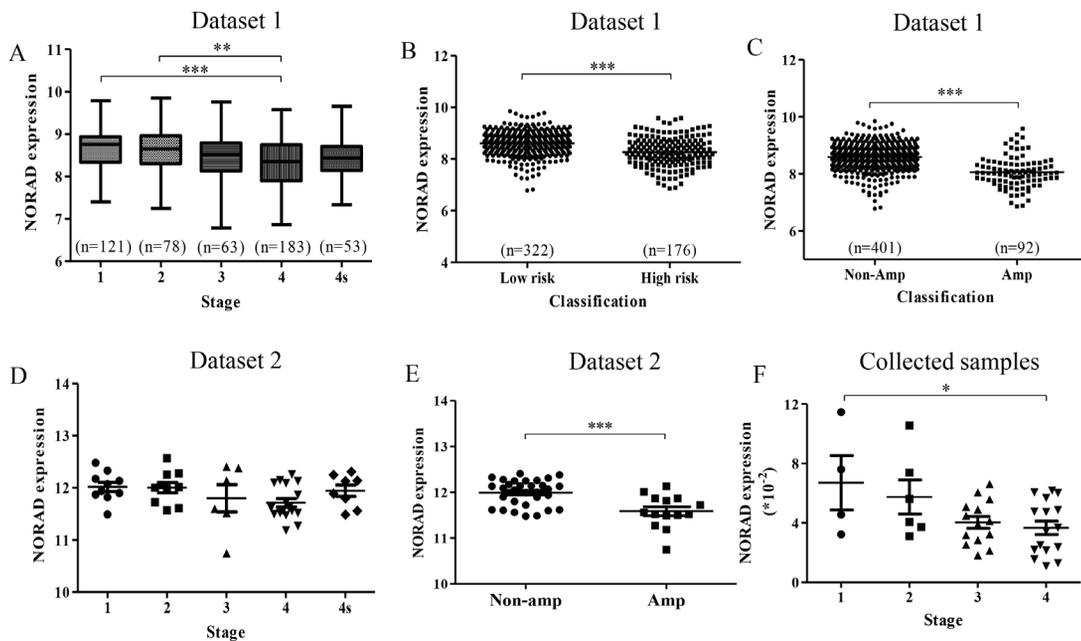


Figure 1. NORAD expression negatively correlates with clinical characteristics of NB tumor.

(A–C) NORAD expression in different NB risk groups, stages and *MYCN* status in Dataset 1. (D and E) NORAD expression in different stages and *MYCN* status in Dataset 2. (F) NORAD expression was downregulated in higher clinical stages of the collected NB tumors. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

termine protein concentration. Each protein sample (20 μ g) was separated by SDS-PAGE and electrophoretically transferred to a nitrocellulose filter membrane (PALL, USA). Non-fat milk (5%) was used to block the membranes, which were subsequently incubated with the following primary antibodies overnight at 4°C: CDK1 (Abcam, Cambridge, UK; 1:5000), SMC3 (Abcam; 1:1000), Aurora B (Abcam; 1:10000), CENPA (Abcam; 1:1000) and GAPDH (Abcam; 1:1000). After incubation with secondary antibodies (IR800-IRDye®800, Abcam; 1:5000), the membranes were scanned using the Odyssey CLx system, and relative densitometric analysis was performed using ImageJ software.

Statistical analysis. All graphs were generated using GraphPad Prism 5.0. The *t*-test was performed to analyze the differences between two groups. Multiple comparisons were assessed by one-way ANOVA. Kaplan–Meier survival analysis was used to evaluate prognosis. $p < 0.05$ was set as statistically significant. All the results were expressed as means \pm standard deviations (S.D.).

RESULTS

NORAD expression negatively correlates with the clinical characteristics of NB

Based on stratification of tumor stage, risk group and *MYCN* status in public GEO Dataset 1 (GSE62564: 498 cases), we found that *NORAD* expression gradually decreased with the increase in tumor stage (Fig. 1A). Additionally, *NORAD* was downregulated in the high-risk NB (Fig. 1B) and *MYCN* amplification groups (Fig. 1C). Independent Dataset 2 (GSE12460: 51 cases) further confirmed the downregulation tendency of *NORAD* (Fig. 1D and E). To further validate the results, we measured the *NORAD* expression level in 40 NB tissues and found that it was downregulated with the

increase in tumor stage (Fig. 1F). These findings firmly indicated that *NORAD* expression negatively correlates with NB progression.

NORAD silencing promotes cell proliferation

The effects of *NORAD* on cell proliferation were assessed in SH-SY5Y and SK-N-BE(2) cells. We first confirmed that siRNAs could effectively silence *NORAD* expression (Fig. 2A). Furthermore, cell growth was significantly elevated when *NORAD* was silenced (Fig. 2B and C). Consistent with this finding, the colony formation assay showed that *NORAD* knockdown promoted colony formation both in number and in size (Fig. 2D). Therefore, we speculated that *NORAD* downregulation promotes cell proliferation of NB cells.

NORAD silencing promotes cell migration

The *NORAD* effects on cell migration were examined by the wound healing assay. The micropipette tip-made “Gap” represents a “wound”, and a decrease in the width of the “Gap” indicates cell migration. As shown in Fig. 3A and B, the “Gap” width in the *NORAD* silencing group decreased faster compared to the control group, suggesting that *NORAD* knockdown significantly increased the cell migration ability. Quantification analysis further confirmed that the difference in the “Gap” width between the *NORAD* silencing groups and the control group was statistically significant (Fig. 3C and D).

NORAD silencing induces cell cycle arrest

NORAD is a non-coding RNA activated by DNA damage and we confirmed that *NORAD* was activated by doxorubicin-induced DNA damage (Fig. 4A). Moreover, the expression of PARP1, which is a DNA damage sensor, increased after *NORAD* silencing, suggesting that *NORAD* contributes to DNA damage repair (Fig. 4B). DNA damage is closely associated with

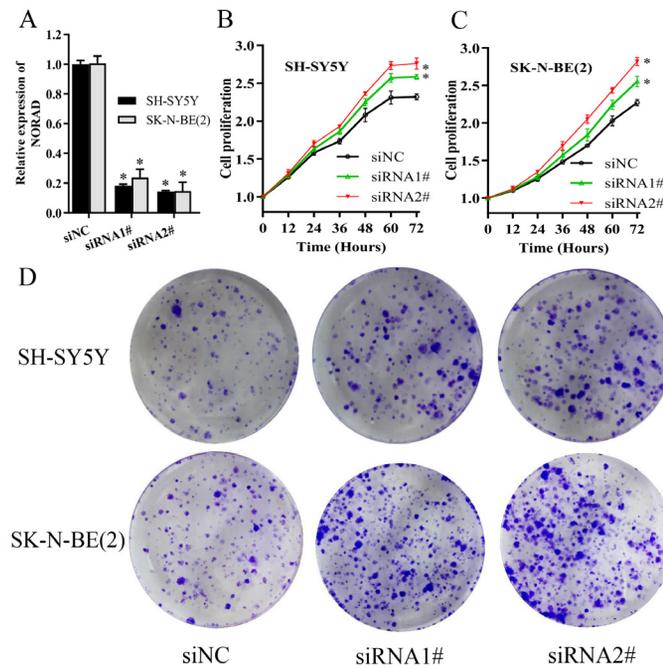


Figure 2. NORAD silencing promotes cell proliferation of SH-SY5Y and SK-N-BE(2) cells. **(A)** Validation of *NORAD* knockdown by siRNAs. **(B, C)** Cell proliferation of SH-SY5Y **(B)** and SK-N-BE(2) cells **(C)**. **(D)** Representative photographs of SH-SY5Y and SK-N-BE(2) colonies. * $p < 0.05$ vs control.

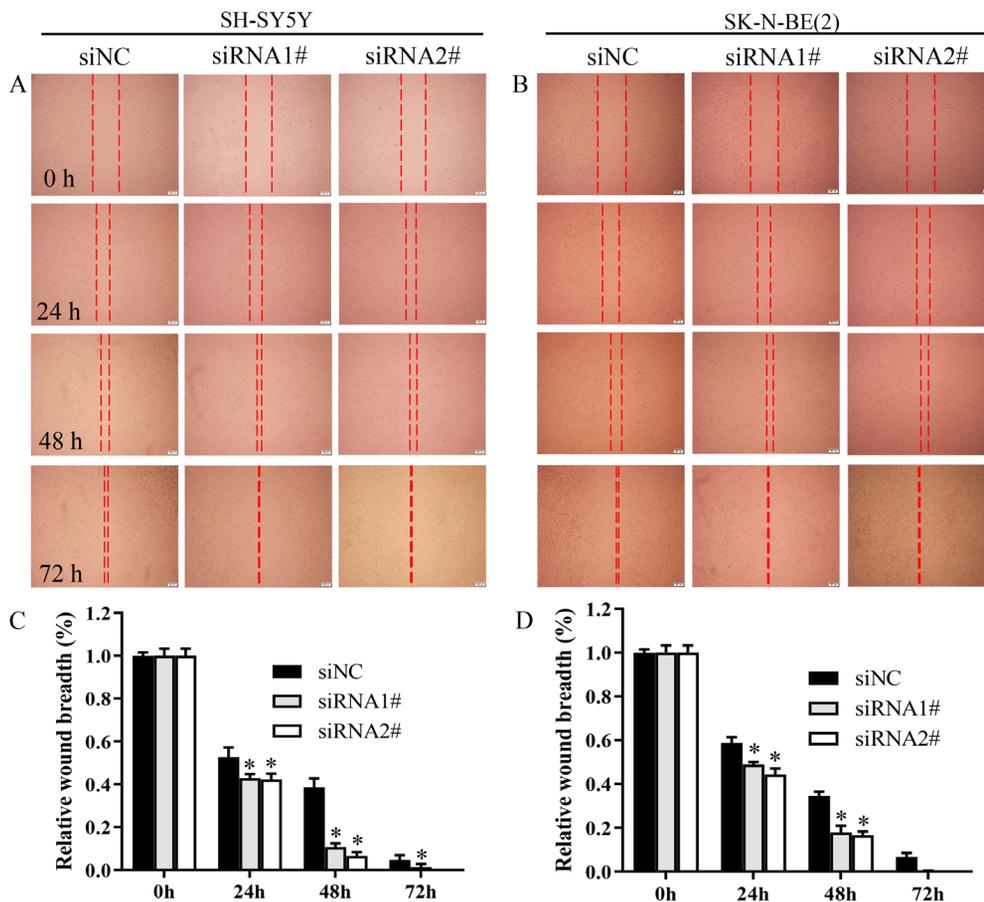


Figure 3. NORAD silencing promotes cell migration of SH-SY5Y and SK-N-BE(2) cells. **(A, B)** Representative wound healing images of SH-SY5Y **(A)** and SK-N-BE(2) cells **(B)** captured at 0, 24, 48 and 72 h. **(C, D)** Quantification of the wound width at the indicated time points with SH-SY5Y **(C)** and SK-N-BE(2) **(D)** cells. Scale bar: 200 μ m. * $p < 0.05$ vs control.

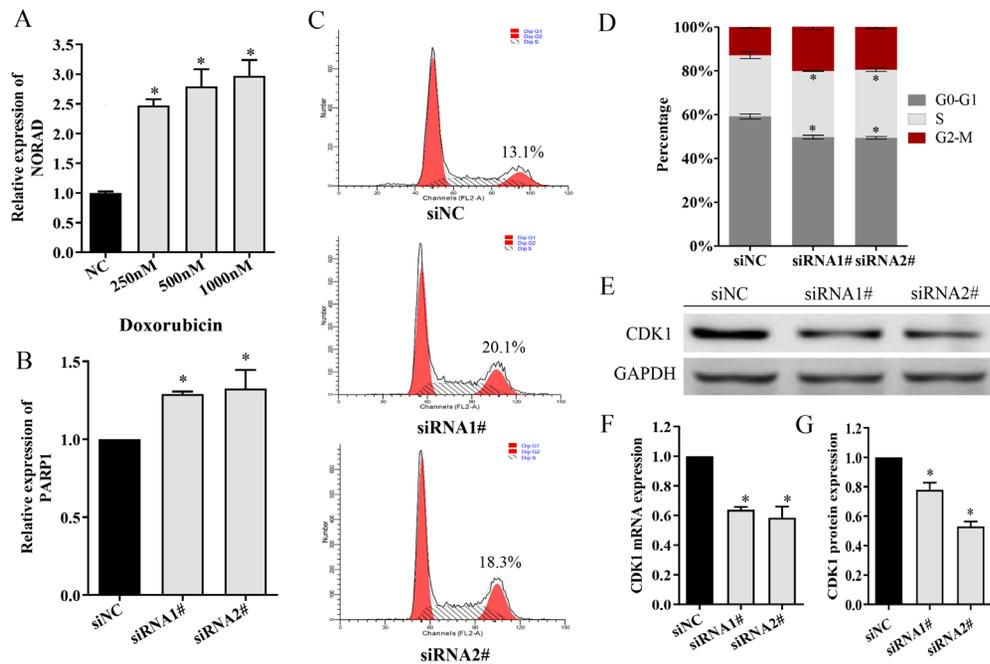


Figure 4. DNA damage and cell cycle delay after NORAD silencing in SH-SY5Y cells.

(A) Doxorubicin (0.25, 0.5 and 1 mM) stimulated *NORAD* expression in a dose-dependent manner. (B) The DNA damage sensor, PARP1, was elevated after *NORAD* silencing. (C) Flow cytometry was used to determine cell cycle changes, which were quantified (D), showing that the percentage of cells in the G2/M phase increased after *NORAD* silencing. (E–G) The mRNA and protein expression level of CDK1 was downregulated after *NORAD* silencing in SH-SY5Y cells. * $p < 0.05$ vs control.

the cell cycle, thus we examined the effect of *NORAD* on the cell cycle by flow cytometry (Fig. 4C). As shown in Fig. 4D and Table 1, the number of cells in the G0/G1 phase declined, while the number of cells in the G2/M phase increased after *NORAD* knock-down. Cyclin-dependent kinase 1 (CDK1) is a critical player in promoting G2/M transition. We found that *NORAD* knockdown downregulated CDK1 at the mRNA and protein level, further confirming cell cycle arrest at G2/M in SH-SY5Y cells (Fig. 4E–G).

***NORAD* silencing impairs sister chromatid cohesion and segregation**

Sister chromatid cohesion and segregation are critical events for maintaining chromosomal stability in response to DNA damage. We examined the genes and proteins involved in these processes. As shown in Fig. 5A, the sister chromatid cohesion-related genes: structural maintenance of chromosomes 1A (*SMC1A*), cohesin complex component (*RAD21*), extra spindle pole bodies like 1 (*ESPL1*) and polo-like kinase 1 (*PLK1*),

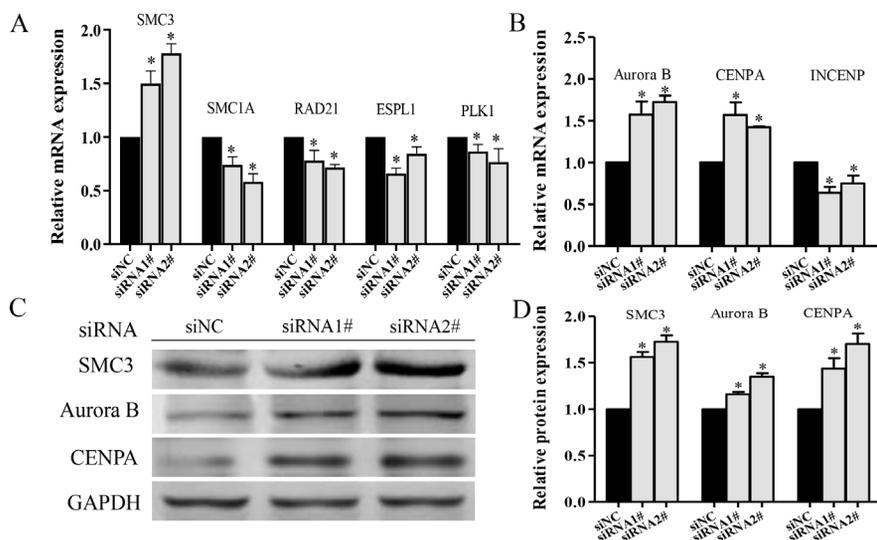


Figure 5. Impairment of sister chromatid cohesion and segregation is induced by NORAD silencing.

(A) mRNA expression of sister chromatid cohesion-related genes and (B) sister chromatid segregation-related genes in cells transfected with siRNAs. (C) Protein expression of SMC3, Aurora B and CENPA measured by western blotting and (D) relative densitometric analysis of the protein bands. * $p < 0.05$ vs control.

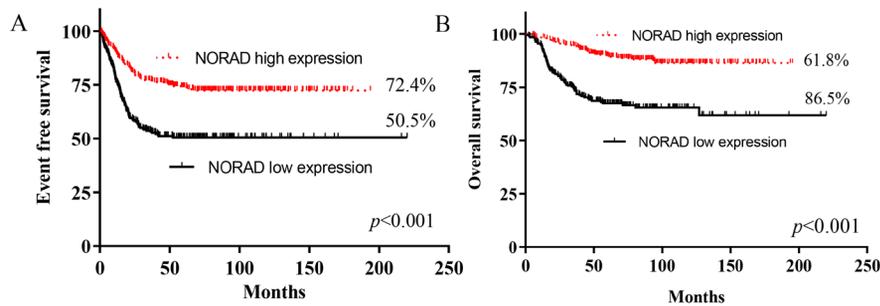


Figure 6. Kaplan–Meier survival analysis for NB patients in GSE62564 dataset.

The Kaplan–Meier curves for (A) event-free survival (EFS) and (B) overall survival (OS) of NB patients with low *NORAD* expression versus high *NORAD* expression. The *p*-values were obtained by the log-rank test. $p < 0.001$.

were suppressed after *NORAD* silencing, while *SMC3* was significantly upregulated. Chromosomal passenger complex (CPC) is related to chromosome separation; our results demonstrated that the CPC component genes, *Aurora B* and centromere protein A (*CENPA*), were upregulated upon *NORAD* silencing, while inner centromere protein (*INCENP*) was downregulated (Fig. 5B). The protein expression for the key genes was examined to confirm the deregulation of sister chromatid cohesion and segregation after *NORAD* knockdown (Fig. 5C and D).

High *NORAD* expression is critical for patient survival

To explore the prognostic value of *NORAD*, patients in Dataset 1 were divided into two groups according to the median expression of *NORAD*: high expression group (249 cases) and low expression group (249 cases). Kaplan–Meier survival analysis was performed to analyze event-free survival (EFS) and overall survival (OS). The results showed that the EFS was 50.5% and 72.4% in low- and high-expression group, respectively, and the OS was 61.8% and 86.5%, respectively (Fig. 6A and B), indicating that patients with low *NORAD* expression had poorer EFS and OS than patients with high *NORAD* expression. These results suggest that *NORAD* is a potential prognostic marker for NB patients.

DISCUSSION

In children, NB is the most common solid tumor, but NB pathogenesis still has not been comprehensively understood after decades of research efforts. Genome instability is the most marked characteristic of cancer and recently lncRNAs, such as *CCAT2*, *TERRA* and *NORAD*, have been demonstrated to be involved in genome regulation (Ling *et al.*, 2013; Cusanelli *et al.*, 2015; Lee *et al.*, 2016). As a novel cytoplasmic non-coding RNA, *NORAD* has been reported to preserve genome stability in human cells, but its effects and molecular mechanism in NB are unknown (Lee *et al.*, 2016; Munschauer, Nguyen *et al.* 2018). In the present study, we found that *NORAD* was downregulated in NB; this downregulation promoted cell proliferation of cell lines *via* chromosomal instability and predicted poor prognosis.

Deregulated gene expression is involved in tumorigenesis. Previous studies have explored genome-scale transcriptional alterations in NB by microarray and RNA-sequencing (Wang *et al.*, 2014; Henrich *et al.*, 2016). Herein, we found that *NORAD* expression negatively correlated with the NB stage and was downreg-

ulated in both high-risk and *MYCN*-amplified NB (Fig. 1A–C). Independent Dataset 2 (GSE12460) and clinical samples further validated our results (Fig. 1D–F). Consistent with our findings, low *NORAD* expression has been also observed in colon cancer and hepatocellular carcinoma (Hu *et al.*, 2017; Lei *et al.*, 2018). Decreased *NORAD* expression in tumors suggests it may be a tumor suppressor (Ventura 2016), but this speculation has not been confirmed in detail. Additionally, it has been reported that *NORAD* was highly expressed and played oncogenic roles in esophageal squamous cell carcinoma (Huo *et al.*, 2018; Sun *et al.*, 2018; Wang *et al.*, 2018). Tumor type-dependent expression of *NORAD* makes its function and mechanism more complex to elucidate, thus it is still unclear whether *NORAD* is involved in NB tumorigenesis.

Previous studies have demonstrated that *NORAD* promoted tumor cell proliferation and invasion, and epithelial-mesenchymal transition (EMT) (Huo *et al.*, 2018; Kawasaki *et al.*, 2018). To clarify the effects of *NORAD* in NB, functional studies were performed. *NORAD* silencing significantly stimulated cell proliferation and migration of SH-SY5Y and SK-N-BE(2) cells (Figs. 2 and 3), which is in line with the finding that NB with downregulated *NORAD* showed high tumor stage and risk. Taken together, the results from the public datasets, clinical validation and *in vitro* study verified that *NORAD* acts as a tumor suppressor in NB progression.

Tumor cell proliferation is associated with various biological processes, such as cell death, apoptosis, cell cycle and DNA damage (Hanahan *et al.*, 2011). Our results showed that *NORAD* expression was significantly increased in a dose-dependent manner upon DNA damage by doxorubicin in SH-SY5Y cells (Fig. 4A). However, whether *NORAD* plays a role in DNA damage promotion or DNA damage repair has not been reported. With regard to this issue, the DNA damage sensor, PARP1, was examined. As PARP1 can recognize DNA breaks and serves as a scaffold for early recruitment of repair proteins to facilitate DNA repair (Wei *et al.*, 2016; Ray Chaudhuri *et al.*, 2017), the elevation in PARP1 expression after *NORAD* knockdown suggests that *NORAD* contributes to DNA damage repair (Fig. 4B). Generally, cell cycle checkpoints arrest the cell cycle to maintain genome stability in response to cellular DNA damage (Malumbres *et al.*, 2009; Uryga *et al.*, 2016). Our data showed that the cell number in the G0/G1 phase declined, whereas it increased in the G2/M phase after *NORAD* knockdown (Fig. 4C and D). Moreover, CDK1, which is a critical player in promoting G2/M transition (Otto *et al.*, 2017), was significantly downregulated at the mRNA and protein level

(Fig. 4E and F). Cell cycle delay provides more time for DNA damage repair and reduces gene alterations. Our results indicated that *NORAD* silencing induced DNA damage and cell cycle arrest at the G2/M transition. These findings establish the functional relevance of *NORAD* in DNA damage and G2/M phase arrest, however, the underlying molecular mechanism remains unclear.

During the G2/M phase transition, sister chromatid cohesion and accurate chromosome segregation are pivotal mechanisms safeguarding genome stability and mitotic processes (M phase) (Makrantonis *et al.*, 2018). Recently, *NORAD* has been reported to assemble a topoisomerase complex by interacting with RNA-binding motif protein X-linked (RBMX), and to function as a potent molecular decoy for PUMILIO proteins. Both RBMX and PUMILIO proteins are cohesion regulators that maintain proper cohesion of sister chromatids (Matsunaga *et al.*, 2012; Lee *et al.*, 2016, Munschauer *et al.*, 2018). Herein, we demonstrated that *NORAD* silencing triggered CIN by interfering with sister chromatid cohesion and chromosome segregation during mitosis. We found that the expression of *SMC1A*, *RAD21*, *ESPL1* and *PLK1* was remarkably suppressed after *NORAD* silencing, while *SMC3* was significantly increased (Fig. 5A, C and D). These genes are components of cohesin and associated regulators. Cohesin complex forms a ring-shaped molecule to entrap sister DNA molecules together until the onset of anaphase (Peters *et al.*, 2012). The core of this ring is composed of SMC1, SMC3 and the kleisin subunit, RAD21 (Eichinger *et al.*, 2013). The connection is cleaved and released by *ESPL1* and *PLK1*, thereby initiating further chromosome segregation (Losada, 2014). Herein, the downregulation of cohesion-related proteins may render the cohesin ring less condensed and the cohesion process unstable, which may cause subsequent dysfunction of chromosome segregation.

Chromosome segregation involves the partitioning of genomic material during metaphase and anaphase, which is dependent on highly conserved CPC. Our results showed that the level of CPC-related genes, *Aurora B* and *CENPA*, was increased, while for *INCENP* it was decreased after *NORAD* silencing (Fig. 5B–D). Although it is unclear how *NORAD* regulates CPC-related genes, the fact that it controls centromere components by sequestering PUMILIO may account for this regulation to some extent (Lee *et al.*, 2016). CPC cellular localization is dynamic during the cell cycle and regulates the mitotic process (Carmena *et al.*, 2012). It has a central role in the regulation of kinetochore-microtubule attachments, and it controls the correct alignment of chromosomes on the spindle equator. The CPC translocates to the spindle midzone in anaphase and facilitates the movement of sister chromatids to the opposite poles (Ruchaud *et al.*, 2007). In the present study, deregulated expression of the CPC component proteins suggested potential defects in chromosome segregation. A previous study has reported that *NORAD* inactivation induced anaphase bridges and mitotic slippage (Lee *et al.*, 2016), which further supports our hypothesis.

Defects in sister chromatid cohesion and deregulated separation are involved in CIN and consequent aneuploidy. These events are characteristic features of human malignancies and have been proposed as important drivers of tumor formation and progression (Hanahan & Weinberg, 2011). *NORAD* inactivation has been previously reported to cause CIN and aneuploidy (Lee

et al., 2016). Accordingly, *NORAD* downregulation-induced genetic instability may account for NB tumor initiation, progression and poor outcome.

In conclusion, we demonstrated that *NORAD* is significantly downregulated in NB with high risk or high tumor stage. Clinically, patients with low *NORAD* expression had a worse survival outcome than patients with high *NORAD* expression. Our functional study showed that *NORAD* silencing promoted cell proliferation, migration and G2/M cell cycle arrest. The underlying mechanism studies verified that *NORAD* silencing caused defects in both sister chromatid cohesion and accurate separation, which are involved in DNA damage. Our results suggest that *NORAD* contributes to NB pathogenesis and progression by modulating sister chromatid cohesion and separation. *NORAD* is thus a potential therapeutic target and a promising prognostic marker for NB patients. This study demonstrated that lncRNA-mediated genome instability is involved in the regulation of NB development.

Authors' contributions

XN and YLG designed the study and critically revised the paper; YBY performed the experiments and wrote the manuscript; SCW, JZ, CHC, WH, HMW and QZ contributed to patient recruitment and tumor tissue collection; YQJ and YRY performed the bioinformatic analysis; FC cultured cells and conducted the statistical analysis. All authors read and approved the final manuscript.

Declaration of conflict of interests

The authors declare they have no actual or potential competing financial interests.

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