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

Yongbo Yu1, Feng Chen1, Yaqiong Jin1, Yeran Yang1, Shengcai Wang2, Jie Zhang2, Chenghao Chen3, Qi Zeng3, Wei Han4, Huanmin Wang4, Yongli Guo1,2 and Xin Ni1,2

1Beijing Key Laboratory for Pediatric Diseases of Otolaryngology, Head and Neck Surgery, MOE Key Laboratory of Major Diseases in Children, Beijing Pediatric Research Institute, Beijing Children’s Hospital, Capital Medical University, National Center for Children’s Health (NCCH), Beijing 100045, China; 2Department of Otolaryngology, Head and Neck Surgery, Beijing Children’s Hospital, Capital Medical University, National Center for Children’s Health (NCCH), Beijing 100045, China; 3Department of Thoracic Surgery, Beijing Children’s Hospital, Capital Medical University, National Center for Children’s Health (NCCH), Beijing 100045, China; 4Department of Surgical Oncology, Beijing Children’s Hospital, Capital Medical University, National Center for Children’s Health (NCCH), Beijing 100045, China

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, 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

Received: 18 August, 2020; revised: 16 October, 2020; accepted: 05 November, 2020; available on-line: 16 December, 2020

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; Matthey 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 (hTERT) 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; Borrello 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 SNHG1 control NB progression through cell proliferation and migration (Pandey et al., 2014; Yu et al., 2019). As molecular sponges, NeD125 and KCNQ1OT1 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 (NOR-4D) 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 NOR-4D expression significantly increased in colorectal cancer (Wang et al., 2018), and promoted cell proliferation and invasion in cervical cancer (Huo et al., 2018). However, NOR-4D 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 NOR-4D 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 NOR-4D 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 NOR-4D function. To study the underlying mechanism of NOR-4D, 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 NOR-4D 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 NOR-4D 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 NOR-4D.

Clinical specimens and cell lines. To verify NOR-4D 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). 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–ΔΔCt method.

Cell transfection. For cell transfection assays, the siRNA oligomers 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: NOR-4D: siRNA1#, 5′-UAGCCCUUUCAGAUUG-GAAAAdTdT-3′ and siRNA2#, 5′-CCACUGGCU-GUGGCCAGAdTdT-3′; negative control (5′-UUUCUG-CGAACGUGUCAGUdTdT-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 × 104 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 NOR-4D in DNA damage, cells were exposed to doxorubicin (0.25 mM, 0.5 mM, 1 mM; Sigma-Aldrich) for 24 h and NOR-4D expression was determined by qRT-PCR. The level of DNA damage sensor, poly (ADP-ribose) polymerase 1 (PARP1), was examined 48 h after NOR-4D 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 × 106 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 determine protein concentration.

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

| Groups     | 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).
termine protein concentration. Each protein sample (20 μg) 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, **p<0.01, ***p<0.001.

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 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).
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 μm. *p<0.05 vs control.
NORAD in NB progression and prognosis prediction

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 knockdown. 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).

**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.

**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.
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 downregulated 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) (Makrantoni 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 (Matsumaga 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 SMC3A, 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 instable, 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
XX 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; YQI 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.

REFERENCES
Barbholomew J, Washington T, Bergeron S, Nielsen D, Saggio J, Quirk I. (2016) Dinutuximab: a novel immunotherapy in the treatment of pediatric patients with high-risk neuroblastoma. J Pediatr Oncol Nurs 34: 5–12. https://doi.org/10.1177/1043454216659448
Bevilacqua V, Gioia U, Di Carlo V, Tortorelli AF, Colombo T, Bozzoni I, Laneve P, Caiffarelli E (2015) Identification of linc-NeD125, a novel long non coding RNA that hosts miR-125B-1 and negatively controls proliferation of human neuroblastoma cells. RNA Biol 12: 1323–1337. https://doi.org/10.1080/15476286.2015.1096488
Borriello L, Seeger RC, Asgharzadeh S, DeClerck YA (2016) More than TERRA: a noncoding RNA connecting telomere biology to genome integrity. Front Genet 6: 143. https://doi.org/10.3389/fgene.2015.00143
Domingo-Fernandez R, Watters K, Piskareva O, Stallings RL, Bray I (2013) The role of genetic and epigenetic alterations in neuroblastoma disease pathogenesis. Pediatr Surg Int 29: 101–119. https://doi.org/10.1007/s00383-012-3299-7
Eichinger CS, Kurze A, Oliveira RA, Nasmyth K (2013) Disengaging the Smc3/kleisin interface releases cohesin from Drosophila chromosomes during interphase and mitosis. EMBO J 32: 686–685. https://doi.org/10.1038/emboj.2012.346
Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144: 646–674. https://doi.org/10.1016/j.cell.2011.02.013
Hemith KO, Bendt D, Sauder M, Drexler D, Guntherhuber M, Shao G, Herrmann C, Wiesentbarg M, Parzonska M, Wehmann L, Fischer M, Duffy DJ, Bell E, Torkov A, Schmezer P, Plass C, Hofer T, Bennet A, Pfister SM, Westermann F (2016) Integrative genome-scale analysis identifies epistatic mechanisms of transcriptional de-regulation in unfavorable neuroblastomas. Cancer Res 76: 5523–5537. https://doi.org/10.1158/0008-5472.CAN-15-2507
Otto T, Sicinski P (2017) Cell cycle proteins as promising targets in cancer therapy. Nat Rev Cancer 17: 93–115. https://doi.org/10.1038/nrc.2016.138

Pandey GK, Kanduri C (2015) Long noncoding RNAs and neuroblastoma. Onco-target 6: 18265–18275. https://doi.org/10.18632/oncotarget.4251

Pandey GK, Misra S, Subhash S, Hertwig F, Kanduri M, Mishra K, Fransson S, Ganesan T, Buchner J, Meckmann J, Ikram F, Subbithotlu AKurek LM, Abrahamsen J, Pfeifer S, Larsson E, Shi L, Peng Z, Fischer M, Martinsson T, Hedberg F, Kogner P, Kanduri C (2014) The risk-associated long noncoding RNA NBAT-1 controls neuroblastoma progression by multiple mechanisms that involve post-transcriptional differentiation. Cancer Cell 26: 722–737. https://doi.org/10.1016/j.ccell.2014.09.014

Peifer M, Hertwig F, Roels F, Dreekax D, Gatenby M, Menon R, Kramer A, Roncadori AJ, Salani F, Schumacker R, Ackermann S, Engesser A, Kahlert Y, Vogel W, Almuller J, Nurnberg P, Thierry-Mieg J, Thierry-Mieg D, Mariappan A, Heynek S, Manzetti E, Henriek HG, Glosecker C, Bosco G, Leuchner I, Schweiger MR, Ibrahimova L, Watkins S, Hofeer T, Acher V, Lang J, Thiessen J, Volland R, Saadati M, Eggert A, de Wilde B, Berthold F, Peng Z, Zhao C, Shi L, Ostmann M, Buttermann R, Soro S, HERmann A, Schild L, Schawe J, HERTzen C, O'Sullivan RJ, Westermann F, Thomas RK, Fischer M (2015) Telomerase activation by genomic rearrangements in high-risk neuroblastoma. Nature 526: 700–704. https://doi.org/10.1038/nature14980

Peters JM, Nishimawa T (2012) Sister chromatid cohesion. Cold Spring Harb Perspect Biol 4: a011130. https://doi.org/10.1101/cshperspect.a011130

Pugh BF, Motozov O, Attiyeh EF, Asgharzadeh S, Wei JS, Ausdail D, Carter SL, Gibusik K, Hanna M, Kiezun A, Kim J, Lawrence MS, Lichtenstein I, McKenna A, Pedamalla CS, Ramos AH, Sheller F, Sivachenko A, Sougnez C, Stewart C, Alay A, Biro I, Chiu R, Corbett RD, Hirst M, Jang Y, Li J, Lister R, Lovat AJ, Mann J, Marshall B, Marshall JF, Ringer K, Rysanuk E, Saba-Ngandja N, Shkumatov Z, Zhukova M, Zhou Q, Zou Q, Zou Y, Ho C, Song J, Guo Z, Ivan C, Barbarotto E, De Vries I, Lahn B, Shoham M, Verma D, Wiltshire T, Zhi B, Zhi S (2015) A comprehensive catalogue of human long noncoding RNAs. Nat Rev Cancer 15: 143–152. https://doi.org/10.1038/nrc3899

Ray Chaudhuri A, Nussenzweig A (2017) The multifaceted roles of PARPi in DNA repair and chromatin remodelling. Nat Rev Mol Cell Biol 18: 610–621. https://doi.org/10.1038/nrm.2017.53

Richman SA, Nunez-Cruz S, Rodriguez M, Li B, Liang J, Gershenson ZT, Mourelatos Z, Barrett DM, Grupp SA, Milone MC (2018) High-affinity GD2-specific CAR T cells induce fatal encephalitis in a preclinical neuroblastoma model. Cancer Immunol Res 6: 36–46. https://doi.org/10.1158/2326-6066.CIR-17-0212

Richter M, Lanza G, Mendell JT (2016) Noncoding RNA NORAD regulates transforming growth factor-beta signaling and epithelial-to-mesenchymal phenotype. Cancer Res 76: 2387–2397. https://doi.org/10.1158/0008-5472.CAN-15-2937

Rossi D, Lisi E, Cesana G, Menghini D, Sciliro C, Maria G, Auffret V, Krombach A, Landi E, Viazzini V, Navasa M, Vazquez-Martin A, Yi HE, Selmi S, Ripoche F, Malekzadeh S, O’Reilly MD, Windler C, Taddei F, Lewis J, Linder R, Villanueva A, Shenas M, Chouaier M, Vanacore N, Zuffa F, Huynh M, Kuehn M, Hendl L, Berger M, Cortez M, Xiao H, Kanzler H, von Kalle C, Horstmann S, Vainchenker W, Soussi T, Ravasi D, Bonini MN, D’Amico M, Garini G, Rosti V, H血液学研究センターの測定目標を包括する非コードレキューラーRNAの発現を設定する細胞周期応答性を促進する。Nat Rev Cancer 17: 93–115. https://doi.org/10.1038/nrc.2016.138

Sahu D, Hsu CL, Lin CY, Wei TW, Hsu WM, Ho SY, Juan HF, Huang HC (2016) Co-expression analysis identifies long noncoding RNA SNHG1 as a novel predictor for event-free survival in neuroblastoma. Oncotarget 7: 58022–58037. https://doi.org/10.18632/oncotarget.11158

Schmitt AM and Chang HY (2016) Long Noncoding RNAs in Cancer Pathways. Cancer Cell 29: 452–463. https://doi.org/10.1016/j.ccell.2016.03.010

Sun Y, Wang J, Pan S, Yang J, Sun X, Wang Y, Shi X, Zhao X, Guo J, Zhang X (2018) LINCC00657 played oncogenic roles in esophageal squamous cell carcinoma by targeting miR-615-3p and JunB. Biochemical Pharmacology 108: 316–324. https://doi.org/10.1016/j.bcp.2018.09.003

Tang W, Dong K, Li K, Dong R, Zheng S (2016) MEG3, HCN3 and line10115 influence the proliferation and apoptosis of neuroblastoma cells via the HIF-1alpha and p53 pathways. Sci Rep 6: 36268. https://doi.org/10.1038/srep36268

Tichon A, Gil N, Lubelsky Y, Havkin Solomon T, Lemze D, Itzko Akyurek LM, Abrahamsson J, Pfeifer S, Larsson E, Shi L, Peng Z, Fischer M, Martinsson T, Hedberg F, Kogner P, Kanduri C (2014) The risk-associated long noncoding RNA NBAT-1 controls neuroblastoma progression by multiple mechanisms that involve post-transcriptional differentiation. Cancer Cell 26: 722–737. https://doi.org/10.1016/j.ccell.2014.09.014

Venkateswaran B (2016) NORAD: Exander of the Genome. Trends Genet 32: 390–392. https://doi.org/10.1016/j.tig.2016.04.002

Wang C, Gong B, Bushel PR, Thierry-Mieg J, Thierry-Mieg D, Xu J, Yang H, Hong H, Shen J, Su Z, Meehan J, Li A, Yang L, Li H, Labaj PP, Kreil DP, Meghebsi D, Gaj S, Cairnie F, van Delt J, Klein J, Schwister J, Scherer A, Devaney V, Wang J, Luo M, Zhang L, Hertwig F, Ratafia J, Altmuller J, Wang Y, Lian J, Lananceh LJ, Bessarrabova M, Nikolsky Y, Furlanello C, Chierici M, Albianese D, Jurman G, Recadoon M, Filioti M, Visitsavtiner F, Li Y, Qu C, Wang N (2018) Long noncoding RNA NORAD regulates transforming growth factor-beta signaling and epithelial-to-mesenchymal phenotype. Cancer Res 76: 2387–2397. https://doi.org/10.1158/0008-5472.CAN-15-2937

Weiss S, Mark M, Saito DF, Lee S, Kopp F, Chang TC, Sataluri A, Chen B, Sivakumar S, Yu H, Kunkele A, Taraseviciute A, Finn LS, Johnson AJ, Berger C, Finney O, Huo H, Tian J, Wang R, Zhao L, Ringer K, Zuffa F, Xiao H, Kanzler H, von Kalle C, Horstmann S, Vainchenker W, Soussi T, Ravasi D, Lewis J, Linder R, Villanueva A, Shenas M, Chouaier M, Vanacore N, Zuffa F, Huynh M, Kuehn M, H血液学研究センターの測定目標を包括する非コードレキューラーRNAの発現を設定する細胞周期応答性を促進する。Nat Rev Cancer 17: 93–115. https://doi.org/10.1038/nrc.2016.138

Xie Y, Mendell JT (2016) Noncoding RNA NORAD regulates transforming growth factor-beta signaling and epithelial-to-mesenchymal phenotype. Cancer Res 76: 2387–2397. https://doi.org/10.1158/0008-5472.CAN-15-2937

Yu Y and others
R, Zhang KK, Li J, Hsieh JH, Svoboda DL, Fuscoe JC, Deng Y, Shi L, Paules RS, Auerbach SS, Tong W (2014) The concordance between RNA-seq and microarray data depends on chemical treatment and transcript abundance. Nat Biotechnol 32: 926–932. https://doi.org/10.1038/nbt.3001

Wang L, Du L, Duan W, Yan S, Xie Y, Wang C (2018) Overexpression of long noncoding RNA NORAD in colorectal cancer associates with tumor progression. Onco Targets Ther 11: 6757–6766. https://doi.org/10.2147/OTT.S176354

Wei H, Yu X (2016) Functions of PARylation in DNA Damage Repair Pathways. Genomics Proteomics Bioinformatics 14: 131–139. https://doi.org/10.1016/j.gpb.2016.05.001

Yu Y, Chen F, Yang Y, Jin Y, Shi J, Han S, Chu P, Lu J, Tai J, Wang S, Yang W, Wang H, Guo Y, Ni X (2019) IncRNA SNHG16 is associated with proliferation and poor prognosis of pediatric neuroblastoma. Int J Oncol 55: 93–102. https://doi.org/10.3892/ijo.2019.4813

Yu Y, Zhang J, Jin Y, Yang Y, Shi J, Chen F, Han S, Chu P, Lu J, Wang H, Guo Y, Ni X (2018) MiR-20a-5p suppresses tumor proliferation by targeting autophagy-related gene 7 in neuroblastoma. Cancer Cell Int 18: 5. https://doi.org/10.1186/s12935-017-0499-2