

A Cornelia de Lange syndrome NIPBL 5'-UTR mutation reduces cell proliferation in an *in vitro* model by downregulating RAD21 and β -catenin

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Received April 25, 2025; Accepted August 19, 2025

DOI: 10.3892/br.2025.2050

Abstract. Cornelia de Lange Syndrome (CdLS) is a prevalent chromatinopathy, frequently caused by mutations in genes encoding cohesin complex components, with *NIPBL* being the most commonly affected. The present study aimed to investigate the effects of a 5'-untranslated region (UTR) mutation (c.-467C>T) in the *NIPBL* gene on gene expression and cohesin complex integrity. Using CRISPR/Cas9 technology, a heterozygous cell line harboring the *NIPBL* 5'-UTR mutation was generated. A combination of molecular biology techniques, including RNA secondary structure prediction and luciferase reporter assays, was employed to evaluate the effect of the mutation. These findings indicated that the 5'-UTR mutation introduces an additional upstream open reading frame, leading to diminished expression levels of NIPBL protein. This decrease was associated with downregulation of RAD21, a pivotal component of the cohesin complex, and reduced β -catenin levels, thereby affecting cell proliferation. The present study elucidates the significance of 5'-UTR elements in regulating gene expression and the potential consequences of sequence variations within this region, demonstrating that a 5'-UTR mutation in *NIPBL* contributes to CdLS by disrupting gene expression and cellular processes. These results advance

the understanding of the molecular mechanisms underlying CdLS.

Introduction

Cornelia de Lange Syndrome (CdLS; OMIM 122470, 300590, 610759, 614701, 300882, 617116, 618736), also referred to as Brachmann-de Lange syndrome, is the most common disorder among the chromatinopathies, with a prevalence estimated between 1 in 10,000 to 1 in 30,000 live births (1). This condition is characterized by a variety of anomalies that affect the craniofacial region, musculoskeletal system, gastrointestinal tract and neurodevelopment (2). CdLS belongs to the group of disorders known as chromatinopathies, disorders caused by variants in proteins involved in chromatin remodeling and transcriptional regulation, resulting in global dysregulation of gene expression (3). CdLS results from mutations in any of the seven genes that constitute the cohesin complex: *NIPBL*, *SMC1A*, *SMC3*, *RAD21*, *HDAC8*, *ANKRD11*, *MAU2*, *AFF2* and *BRD4*, each playing a role in the construction or regulation of the complex (4,5). The *NIPBL* gene mutation is most frequently implicated in CdLS cases, particularly those exhibiting the typical phenotype, accounting for ~60-70% of instances (6). Most *NIPBL* mutations associated with CdLS are located in the coding region, while a few are in the 5'-UTR (untranslated region) (7-9). Previously, a novel mutation c.-467C>T was identified in the *NIPBL* gene, which is associated with CdLS. Cs (10). Although the 5'-UTR mutation in *NIPBL* linked to CdLS is recognized for introducing a novel upstream open reading frame (uORF) (7), the underlying pathogenic mechanisms remain unclear.

The 5'-UTR includes structural elements and uORFs, which are essential for regulating translation. The extended length and intricacy of 5'-UTRs underscore the significance of post-transcriptional and translational mechanisms in modulating the expression levels of the proteins they encode, particularly in the context of dose-sensitive genes (11). The NIPBL protein, with >2,500 amino acids, is crucial for the function of the cohesin complex and its role in chromatin organization, gene regulation and developmental processes (12,13). In total, ~10% of CdLS cases involve mutations in cohesin regulators and components, indicating that *NIPBL* mutations

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Key words: Cornelia de Lange Syndrome, *NIPBL*, RAD21, β -catenin

may contribute to CdLS pathogenesis by disrupting cohesin function (14). The expression levels of the *NIPBL* gene are critical, as even minor decreases in them can lead to CdLS, indicating its dosage sensitivity (14,15). Cells derived from patients with CdLS with a heterozygous pathogenic variant in the *NIPBL* gene, as well as mice heterozygous for a *Nipbl* mutation, exhibit decreased overall or regional cohesin association and impaired three-dimensional interactions within the chromatin structure, which is consistent with the role of *NIPBL* as a component that assists in the attachment of cohesion (16-19).

The present study established a heterozygous cell line with a point mutation to investigate the impact of the 5'-UTR mutation on *NIPBL* gene expression, cohesin complexes and the transcription of genes involved in cellular development. The present study aimed to further elucidate the preliminary mechanisms by which the 5'-UTR mutation of the *NIPBL* gene contributes to CdLS development.

Materials and methods

RNA secondary structure prediction. The RNA secondary structure was computed, and the folding energy for RNA sequences was estimated using the RNAfold tool, which is part of the ViennaRNA package (version 2.6.4), as previously described (20).

Cell culture and knock-in cell line construction. The 293t cell line was sourced from the American Type Culture Collection repository of the Chinese Academy of Sciences. These cells were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (Shanghai ExCell Biology, Inc.) and cultured at 37°C in a humidified atmosphere with 5% CO₂. A variant of the 293t cell line, designated as *NIPBL*-mut, was engineered to harbor a 5'-UTR mutation in the *NIPBL* gene using the CRISPR/Cas9 system. The sgRNA targeting the *NIPBL* 5'-UTR was designed with CRISPOR: web-based design tool (<https://crispor.tefor.net>; Kircher Lab, Max Delbrück Center for Molecular Medicine, Berlin, Germany). The *NIPBL*-specific sgRNA sequence (5'-TTT GTTCTGAGAGGGAGAGA-3'), designed to target the first exon of the *NIPBL* gene, was cloned into the PX459 vector, which was acquired from Addgene, Inc. The sgRNA-PX459 construct (2 µg in 6 well plate) and the homology repair template (500 ng; 5'-GTCGGCATTGTTGTTCTGAGAGGGA GAGATGGAACGAGA-3') were co-transfected into 293t cells using Lipofectamine 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C for 36 h. Following transfection, the cells were subjected to selection with 2 µg/ml puromycin for 48 h, after which the antibiotic-resistant cells were single-cell cloned in the same medium. Genomic DNA was extracted from the edited cells (Tissue DNA Kit; Omega Bio-Tek, Inc.) and a 517-bp fragment spanning the entire sgRNA insertion site (chr5: 36,876,519-36,877,036, hg38) was amplified with PrimeSTAR® HS DNA Polymerase (Takara Biotechnology Co., Ltd.) under standard high-fidelity cycling conditions (98°C for 30 sec; 35 cycle x [98°C for 10 sec, 63°C for 20 sec, 72°C for 30 sec]; 72°C for 2 min). The purified 517-bp product was then sent to Tsingke Biological Technology for Sanger sequencing with the forward PCR

primer (5'-GCAAACGTCGCTCTGAAGCAT-3') and a reverse primer (5'-CGAGAAGACAAATCGTTGCT-3'). Chromatograms were aligned to the reference gene sequence with SnapGene to confirm 100% identity and absence of indels. The template was verified on three separate occasions to confirm sequence identity.

Luciferase reporter assay. The procedures for plasmid construction and dual-luciferase reporter analysis were conducted as previously described by the authors (10). The data were standardized to account for transfection efficiency by quantifying the activities of firefly and *Renilla* luciferases, with the results presented as a ratio of these two luciferase activities. Most control samples were set to a value of 100% for normalization purposes. The results are depicted as the mean values ± standard deviations, derived from triplicate experiments.

Immunofluorescence. WT and mut 293t cells were seeded onto glass coverslips in their respective growth media. The cells were cultured for the specified number of days until they reached ~80% confluence. The cell layers were then fixed with a 4% paraformaldehyde solution in phosphate-buffered saline (PBS, pH 7.5) at room temperature (RT, 25°C) for 20 min. Following fixation, the coverslips were permeabilized with a 0.2% Triton X-100 solution for 15 min and then were blocked with 3% bovine serum albumin (BSA; Sangon Biotech Co., Ltd.) for 1 h at RT. Primary antibodies targeting *NIPBL* (cat. no. sc-374625; Santa Cruz Biotechnology, Inc.), diluted to a ratio of 1:100 in 1% BSA, were then applied, and the solution was incubated overnight (for >18 h) at 4°C in a light-protected environment. Subsequently, the Alexa Fluor 488 Goat Anti-Mouse IgG (H+L) secondary antibodies (cat. no. ab150113; Abcam) were incubated for 1 h at RT. The cells were then stained at RT with 0.2 mg/ml DAPI for 7 min to visualize the nuclei. After staining, the slides were air-dried and mounted with anti-fade reagents to preserve the fluorescence. Finally, the samples were analyzed using a fluorescence microscope.

Cell fractionation. This process started with the collection of cells and their subsequent incubation in lysis buffer I, which is composed of 20 mM HEPES at a pH of 8.0, 2 mM MgCl₂, 10 mM KCl, 0.5% NP-40 and protease inhibitors, for 10 min, under refrigeration at 4°C and with the inclusion of protease inhibitors in all solutions. The mixture was centrifuged at 1,500 x g for 5 min after incubation. The supernatant, which was rich in cytosolic components, was then carefully separated and set aside. The remaining pellet was rinsed twice with PBS and then subjected to lysis using nucleus lysis buffer II, which is prepared by incorporating 0.5 M NaCl into lysis buffer I. After lysis, the nuclear lysate was allowed to chill on ice for 5 min before centrifugation at 15,000 x g for 10 min. The supernatant, containing the nuclear components, was subsequently collected and preserved as the nuclear fraction.

Western blot analysis. Cells were subjected to lysis using RIPA buffer (cat. no. P0013C; Beyotime Institute of Biotechnology) supplemented with phenylmethylsulfonyl fluoride. The concentration of total proteins in the lysates was quantified

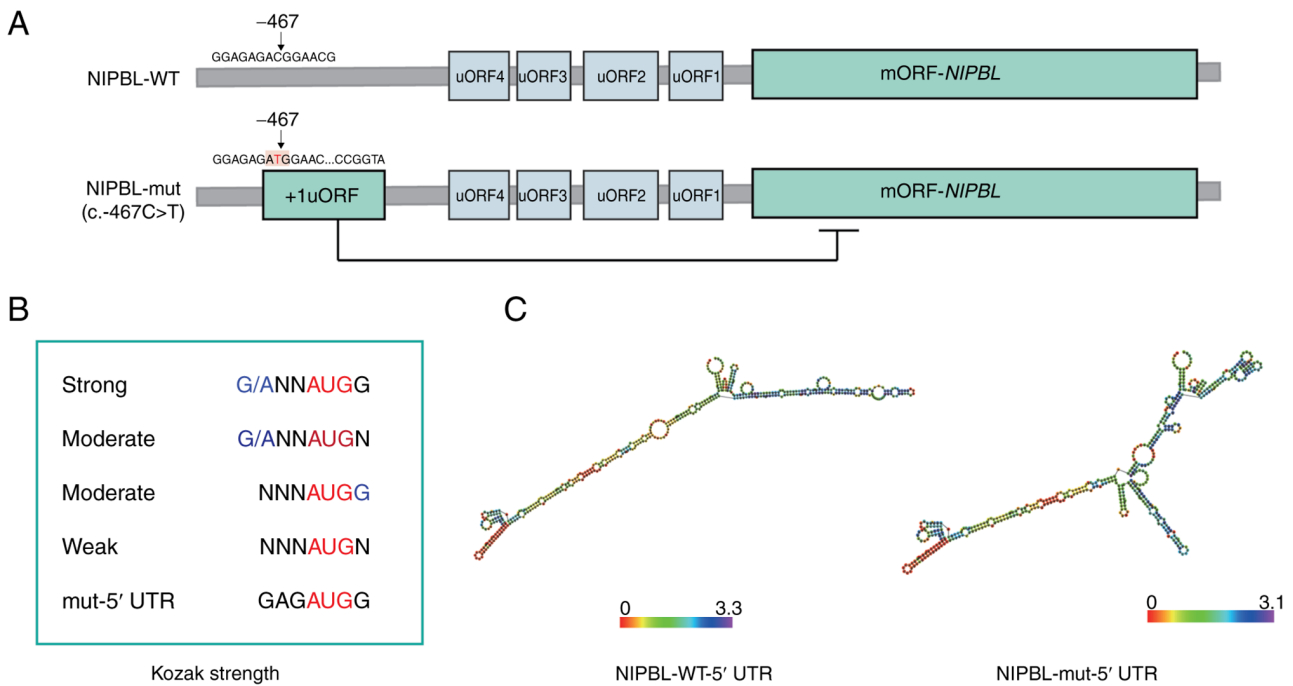


Figure 1. Impact of 5'-UTR mutation on uORFs. (A) Identification and comparison of uORFs between the WT and mut variants of the 5'-UTR of the *NIPBL* gene. (B) Kozak sequence within the 5'-UTR of the mut variant of the *NIPBL* gene. (C) Predicted secondary structures of the RNA for the mutated 5'-UTR of the *NIPBL* gene, and the WT RNA predicted secondary structure is shown on the left and the mut structure on the right. UTR, untranslated region; uORF, upstream open reading frame; WT, wild-type; mut, mutant.

using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology). Aliquots of 20 μ g of protein samples were resolved using sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 10% acrylamide gel and then transferred onto polyvinylidene fluoride membranes. The membranes were then blocked with a 5% skim milk in TBS-T at RT for 1 h. After blocking, the membranes were incubated overnight at 4°C with a panel of primary antibodies targeting NIPBL (cat. no. sc-374625; Santa Cruz Biotechnology, Inc.), α -Tubulin (cat. no. ab7291; Abcam), RAD21 (cat. no. DF7520; Abcam), SMC1A (cat. no. AF6439; Affinity Biosciences), CTNNB1 (cat. no. 8480; Cell Signaling Technology, Inc.) and GAPDH (cat. no. ab8245; Abcam). Subsequently, the membranes were treated with HRP-Goat Anti-rabbit IgG (H+L) or HRP-Goat Anti-mouse IgG (H+L) secondary antibodies (BK-R050-50 μ l or BK-M050-50 μ l; Hangzhou Baoke Biotech Co., Ltd.), diluted at 1:5,000, for 1 h. The immunoreactive bands were visualized using an enhanced chemiluminescence detection system (Hangzhou Fude Biological Technology Co., Ltd.) and their intensities were quantified through densitometric analysis using ImageJ 1.54f (National Institutes of Health). The following primary antibodies were used: NIPBL (1:500; Santa Cruz Biotechnology, Inc.); α -Tubulin and GAPDH (1:1,000; both from Abcam); RAD21 and SMC1A (1:1,000; both from Affinity Biosciences) and CTNNB1 (1:1,000; Cell Signaling Technology, Inc.).

EDU staining. The DNA replication in 293t cells was detected using an EdU staining kit (Beyotime Institute of Biotechnology), following the manufacturer's instructions. Initially, 293t cells were plated in 24-well plates and cultured for 12 h until they reached a confluency of ~50%. Subsequently,

the cells were treated with a 10 μ M concentration of EdU solution, which was incorporated into the cell medium and left for 2 h. After this incubation period, the cells were fixed with 4% paraformaldehyde in PBS (pH 7.4) at room temperature for 15 min and then rinsed three times (5 min each) with PBS to remove any unincorporated EdU. A click reaction solution containing Azide 488 was then added to the cells and incubated for 30 min to facilitate the detection of EdU incorporation. To visualize the cell nuclei, 0.2 mg/ml DAPI staining was performed at RT for 7 min, and the stained cells were subsequently examined using a fluorescence microscope.

Statistical analyses. The experiments were repeated at least three times unless otherwise indicated. Statistical analyses were performed using unpaired two-tailed t-tests in GraphPad Prism 10.1.2 (GraphPad Software; Dotmatics). Most control groups were normalized to 100%. The data are presented as the mean $\pm$ standard error of the mean. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Impact of a 5'-UTR mutation on uORFs. To assess the impact of the c.-467C>T variant on post-transcriptional regulation, the 5'-UTR of *NIPBL* was analyzed for uORFs using NCBI ORFfinder. A total of Four uORFs were identified in the wild-type (WT) sequence, whereas the mutant harbored an additional uORF, yielding a total of five (Fig. 1A). The *de novo* uORF initiates at a strong Kozak sequence (GAGAUGG) (21) and spans 53 codons, terminating upstream of the *NIPBL* start codon (Fig. 1B). Secondary structure prediction with ViennaRNA revealed a complex secondary RNA structure for

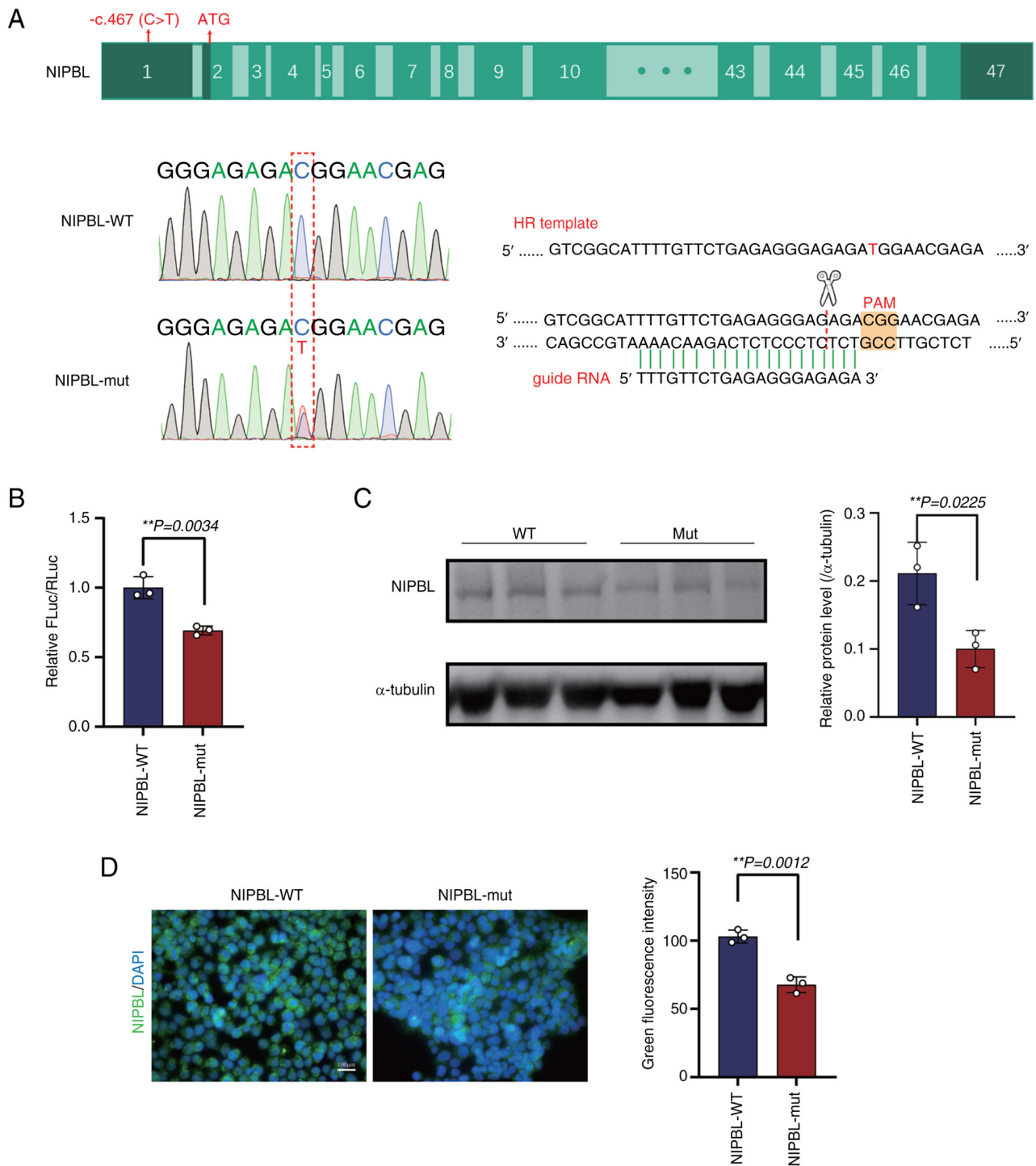


Figure 2. 5'-untranslated region mutation of the *NIPBL* gene affects expression. (A) Schematic illustration depicting the generation of *NIPBL*-mut cells using the CRISPR/Cas9 System. (B) Luciferase reporter assay measuring the transcriptional activity of the *NIPBL* gene in WT vs. mutant groups. (C) Western blot comparing NIPBL protein levels in WT and mut cells. (D) Immunofluorescence staining of NIPBL in wild-type and mutant cells; nuclei were counterstained with DAPI (blue). Fluorescence intensity was quantified and normalized to the WT level. Scale bar, 100 μ m. Data are presented as the mean $\pm$ SEM and the value of NIPBL-WT was normalized as 100%. ** $P < 0.01$. WT, wild-type; mut, mutant.

the mutated 5'-UTR, characterized by numerous stem-loops (Fig. 1C), with no significant difference in the lowest free energy between the WT (-2548.10 kcal/mol) and the mutant (-2546.70 kcal/mol) for the initial 10,000 bases.

Impact of uORF variants on *NIPBL* expression. The CRISPR/Cas9 system-mediated editing introduced the c.-467C>T variant into the endogenous *NIPBL* locus, yielding

heterozygous *NIPBL*-mut cells (Fig. 2A). A dual-luciferase reporter assay was used to quantify the transcriptional impact of the variant. Compared with the WT, the mutant (mut) 5'-UTR produced a modest but significant reduction in reporter activity (1 ± 0.046 vs. 0.693 ± 0.019 ; $P = 0.0034$; Fig. 2B). Consistently, immunoblot analysis revealed a ~50% decrease in endogenous NIPBL protein levels in mutant cells relative to isogenic controls (0.211 ± 0.027 vs. 0.100 ± 0.016 ; $P = 0.0225$;

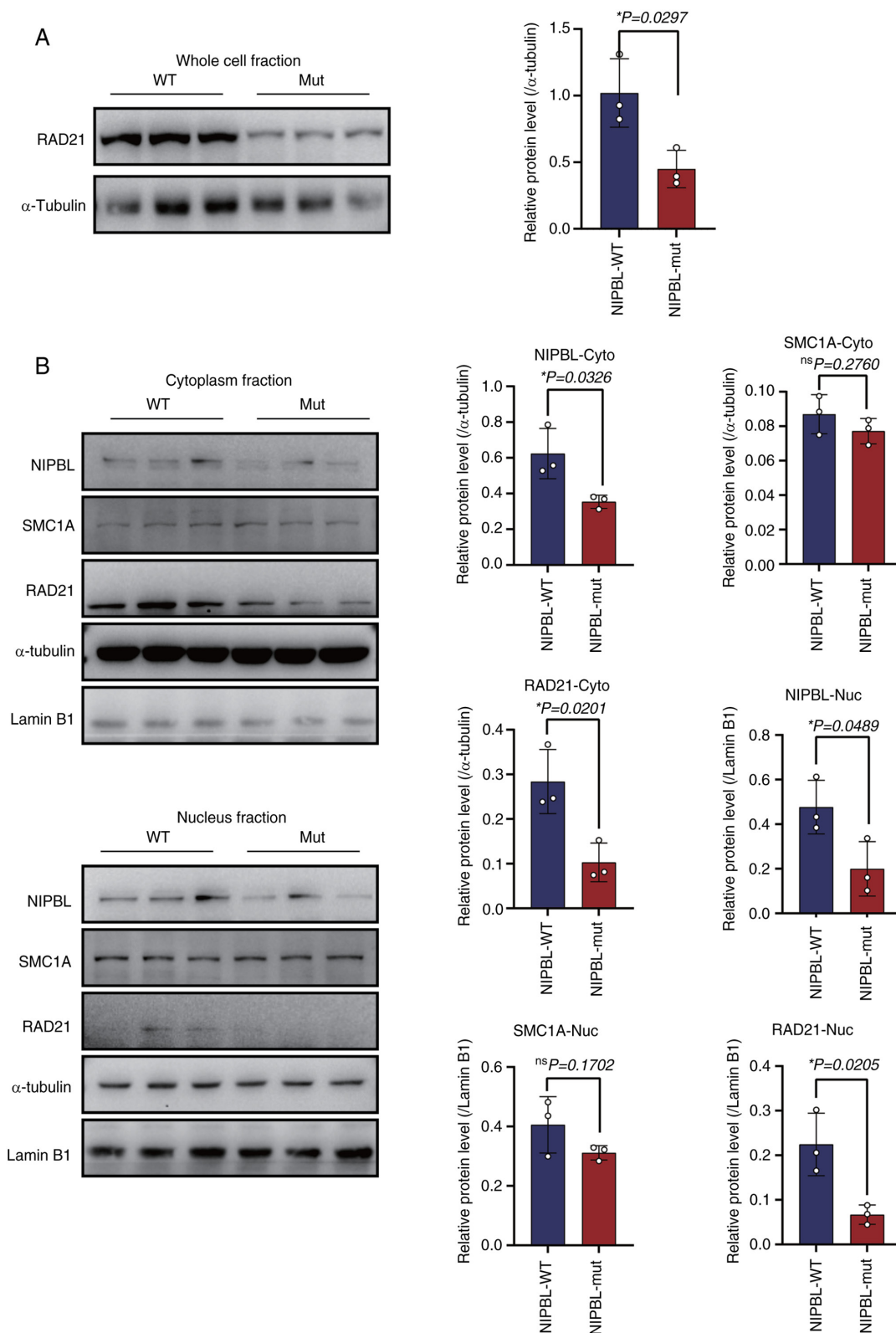


Figure 3. Cohesin complex integrity. (A) RAD21 protein levels in WT and mut cells. (B) Western blot analysis of cohesin complex proteins (NIPBL, RAD21 and SMC1A) in cytoplasmic (Cyto) and nuclear (Nuc) fractions of wild-type and mutant cells. Data are presented as the mean $\pm$ SEM and the value of NIPBL-WT was normalized as 100%. $*P<0.05$. WT, wild-type; mut, mutant; ns, not significant.

Fig. 2C). Immunofluorescence staining further showed that the subcellular distribution of NIPBL was unaltered by the mutation. However, the fluorescence intensity in the mutant cells was significantly reduced by 40% compared with that in the WT (103.000 ± 2.767 vs. 67.680 ± 3.323 ; $P=0.0012$; Fig. 2D).

NIPBL haploinsufficiency downregulates *RAD21*. It was next examined whether the *NIPBL* mutation affects the steady-state abundance of core cohesin subunits. Whole-cell lysates revealed a significant reduction in *RAD21* protein (1.020 ± 0.257 vs. 0.449 ± 0.141 ; $P=0.0297$; Fig. 3A). To determine

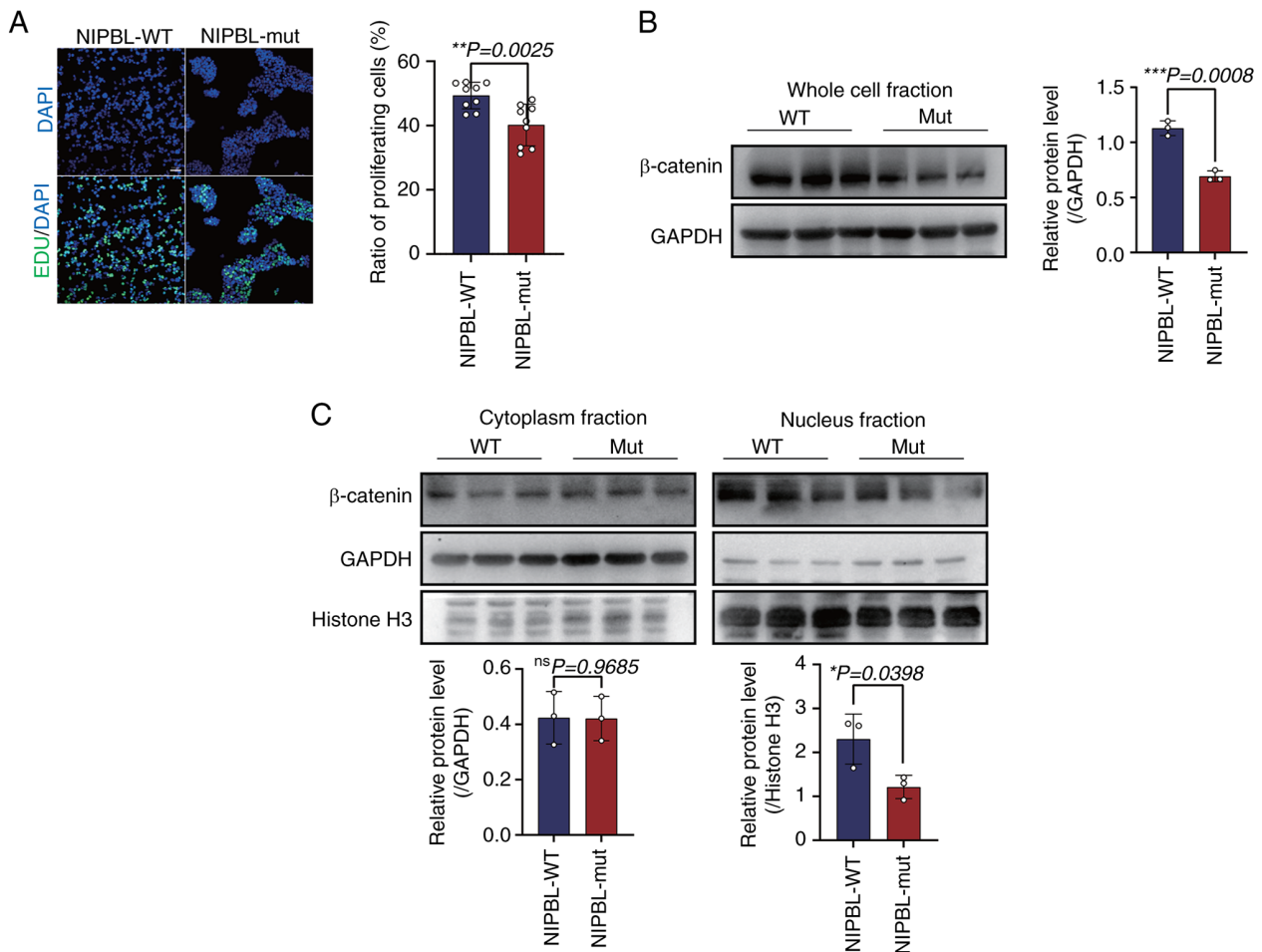


Figure 4. NIPBL downregulation reduces β -catenin levels, thereby affecting cell proliferation. (A) EdU assays assessing proliferation in WT and *NIPBL*-mut cells; EdU-positive nuclei were quantified and normalized to total DAPI counts. Scale bar, 100 μ m. (B) Total β -catenin protein levels in WT and mut cells. (C) Subcellular β -catenin levels in WT and mut cells (cytoplasmic vs. nuclear fractions). Data are presented as the mean $\pm$ SEM and the value of NIPBL-WT was normalized as 100%. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$. WT, wild-type; mut, mutant; ns, not significant.

compartment-specific changes, nuclei and cytoplasm were fractionated. Consistent with our previous findings, NIPBL protein levels decreased in both the nucleus (0.476 ± 0.070 vs. 0.199 ± 0.070 , $P=0.0489$ Fig. 3B) and cytoplasm (0.623 ± 0.081 vs. 0.354 ± 0.021 , $P=0.0326$, Fig. 3B). Similarly, RAD21 expression in cellular fractions decreased in the mut compared with the WT (Cytoplasm, 0.283 ± 0.042 vs. 0.103 ± 0.025 ; $P=0.0201$; nucleus 0.224 ± 0.041 vs. 0.067 ± 0.013 ; $P=0.0205$; Fig. 3B). By contrast, SMC1A levels were comparable between genotypes in both compartments (Fig. 3B).

Reduction in NIPBL expression leads to decreased β -catenin and impacts cell proliferation. EdU staining assays were conducted to quantify the proliferative capacity of *NIPBL*-mutant cells. Compared with WT controls, the fraction of EdU-positive cells were significantly lower in the mutant population (49.360 ± 1.384 vs. 40.200 ± 2.154 ; $P=0.0025$; Fig. 4A). Western blot analysis revealed that total β -catenin protein levels were significantly reduced in mut cells compared with WT controls (1.129 ± 0.039 vs. 0.692 ± 0.028 , $P=0.0008$; Fig. 4B). Since nuclear accumulation of β -catenin is required for canonical Wnt signaling, β -catenin was further resolved into nuclear and cytoplasmic fractions. Nuclear β -catenin was significantly decreased in the mut group (2.301 ± 0.328 vs.

1.213 ± 0.152 ; $P=0.0398$; Fig. 4C); cytoplasmic β -catenin levels remained unaffected in both the WT and mutant groups.

Discussion

In our previous publication, a novel *NIPBL* variant (c.-467 C>T) was identified in a Chinese boy affected by CdLS (10). In the present study, it was demonstrated that this mutation creates a strong uORF that represses *NIPBL* translation without affecting mRNA stability. Mechanistically, the uORF stalls ribosome scanning, preventing initiation at the downstream *NIPBL* start codon (22). Critically, reduced NIPBL diminishes cohesin loading onto chromatin, disrupting long-range chromatin interactions, a core defect in CdLS pathogenesis (23,24). These data establish uORF-mediated translational inhibition as a recurrent mechanism underlying NIPBL haploinsufficiency in CdLS.

Reduced NIPBL dosage, in turn, perturbs the cohesin complex. Western blotting and subcellular-fractionation analyses revealed a decrease in RAD21 protein. This finding contrasts with some studies in which *NIPBL* mutations altered cohesin chromosomal occupancy without affecting total cohesin subunit abundance (25,26). However, it aligns with recent evidence from small-cell lung cancer showing that NIPBL protein promoted *RAD21* gene transcription by

enhancing H3K27 demethylation via recruiting lysine demethylase 6B to the *RAD21* gene promoter (27), reconciling our findings with the emerging view that *NIPBL* dosage can exert both occupancy and abundance-dependent effects on cohesin. Because cohesin dysfunction underlies the group of disorders termed chromatinopathies, CdLS being the archetype, the observed *RAD21* downregulation provides a direct mechanistic link between the *NIPBL* 5'-UTR mutation and CdLS pathogenesis (28,29). These findings suggest that downregulation of *RAD21* may be caused by pathogenic mutations in the *NIPBL* 5'-UTR. This interpretation is further supported by clinical genetics: Heterozygous *RAD21* variants can themselves cause CdLS, but the resulting phenotype is typically mild (30-32), mirroring the attenuated presentation associated with *NIPBL* 5'-UTR mutations. Thus, converging molecular and clinical evidence indicates that reduced *NIPBL* dosage elicits *RAD21*-mediated disruption of cohesin that contributes to the milder end of the CdLS phenotypic spectrum.

To determine how *NIPBL*-*RAD21* deficiency translates into cellular pathology, the Wnt/ β -catenin pathway was interrogated. Functional assays revealed modest yet consistent defects in cell proliferation that were accompanied by a marked reduction in nuclear β -catenin. This result is consistent with the downregulation of the classical Wnt signaling pathway observed in the CdLS zebrafish model (33,34), and extends it to human cells. Importantly, this finding also supports the notion that *NIPBL* or *RAD21* knockdown leads to a decrease in cell proliferation ability, suggesting a causal rather than correlative relationship (15,33,35). Supporting this notion, transcriptomic profiling of CdLS-associated cardiac dysplasia demonstrates that *RAD21* depletion alone is sufficient to derail Wnt signaling (8). Consequently, it was hypothesized that *NIPBL* mutations trigger the downregulation of *RAD21*, leading to the dysregulation of β -catenin and ultimately to defects in cell proliferation.

In conclusion, it was demonstrated that a single 5'-UTR variant (c.-467C>T) creates a *de novo* uORF, quantitatively reduces *NIPBL* and cohesin levels, and thereby disrupts Wnt signalling and proliferation. These findings establish a direct molecular link between a non-coding mutation and mild Cornelia de Lange phenotypes.

Acknowledgements

Not applicable.

Funding

The present study was supported by the National Natural Science Foundation of China (grant no. 82270837).

Availability of data and materials

The data generated in the present study are included in the figures and/or tables of this article.

Authors' contributions

CW and CZ conceived and designed the research. QC and YC performed the experiments and analyzed the data. QC, YC

and CW wrote the manuscript. All authors read and approved the final version of the manuscript. QC and YC confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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