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CircATRNL1 Facilitates Malignant Progression of Non-Small Cell Lung Cancer via the miR-103a-3p/IGFBP3/PI3K-AKT Axis

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Received: 11 May 2026; Accepted: 12 August 2026; Published: 19 October 2026

ABSTRACT: Background: Circular RNAs have emerged as important regulators of non-small cell lung cancer progression through competing endogenous RNA networks, but the specific role and mechanism of CircATRNL1 in NSCLC remain unclear. This study investigates the mechanistic role of CircATRNL1 in the malignant progression of non-small cell lung cancer (NSCLC). **Methods:** Gene expression levels in NSCLC cell lines were quantified using reverse transcription quantitative PCR (RT-qPCR). Functional assays, including Western blot analysis of epithelial-mesenchymal transition (EMT)-related proteins, wound-healing assays, and Transwell migration and invasion assays, were performed to assess the effects of CircATRNL1 on cellular motility and invasive potential. Potential RNA-RNA interactions were predicted using the ENCORI and CircBank databases, followed by luciferase reporter assays to validate these interactions experimentally. To determine whether the phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) signaling pathway mediated the biological effects of insulin-like growth factor-binding protein 3 (IGFBP3), rescue experiments were performed using the PI3K inhibitor LY294002. **Results:** CircATRNL1 was significantly upregulated in NSCLC cell lines and showed typical circular RNA characteristics, including RNase R resistance and enhanced transcript stability ($***p < 0.001$). CircATRNL1 knockdown inhibited NSCLC cell migration and invasion, whereas CircATRNL1 overexpression exerted the opposite effects. Mechanistically, CircATRNL1 functioned as a sponge for miR-103a-3p and relieved miR-103a-3p mediated repression of IGFBP3, as validated by luciferase reporter and rescue assays ($**p < 0.01$). IGFBP3 further activated PI3K/AKT signaling by increasing p-PI3K/PI3K and p-AKT/AKT ratios, while LY294002 attenuated IGFBP3-induced migration and invasion. **Conclusions:** CircATRNL1 promotes NSCLC cell migration and invasion by acting as a ceRNA for miR-103a-3p, thereby regulating IGFBP3 and activating PI3K/AKT signalling. These findings highlight the CircATRNL1/miR-103a-3p/IGFBP3/PI3K-AKT axis as a potential regulatory mechanism contributing to *in vitro* migration and invasion in NSCLC.

KEYWORDS: Non-small cell lung cancer (NSCLC); CircATRNL1; insulin-like growth factor binding protein 3 (IGFBP3); phosphatidylinositol 3-kinase/protein kinase B signaling pathway (PI3K/AKT); epithelial-mesenchymal transition (EMT)

1 Introduction

Lung cancer continues to impose a substantial burden on public health and remains among the most commonly diagnosed malignancies and a principal cause of cancer-related death worldwide, consistently ranking among the top cancers in both incidence and mortality, posing a significant public health threat [1]. Even though significant advances have been achieved in the detection and treatment of lung cancer, long-term clinical outcomes for affected patients remain poor, particularly for patients diagnosed with advanced-stage disease [2], reflecting its poor clinical prognosis. Nearly 85% of all lung cancer cases are classified as non-small cell lung cancer (NSCLC) [3]. Among these, the incidence of lung adenocarcinoma has been steadily increasing, particularly in women, non-smokers, and younger individuals [4,5]. Notably, over half of patients with NSCLC are diagnosed at locally advanced stages or with distant metastasis, making curative surgery no longer an option, with systemic therapy becoming the primary treatment approach [6]. While the advent of targeted therapies and immune checkpoint inhibitors has provided clinical benefits to certain patient groups, therapeutic responses remain highly variable, and the development of drug resistance is almost inevitable, limiting long-term efficacy [7]. Furthermore, current biomarkers for early detection and prognostic assessment are insufficient, contributing to low early diagnosis rates and hindering clinical decision-making [8]. Elucidating the molecular events that drive the development and progression of NSCLC is therefore essential for identifying novel diagnostic biomarkers and therapeutic targets.

Non-coding RNAs are increasingly recognised as important regulators of NSCLC biology and therapeutic response. For example, lncRNA CRYBG3 has been reported to regulate adaptive radioresistance in NSCLC cells through the p53/HSF1/TRAP1 axis [9]. Among the diverse classes of non-coding RNAs, circular RNAs (circRNAs) have emerged as a major research focus owing to their unique molecular architecture and broad regulatory potential. Circular RNAs (circRNAs) represent a distinct group of non-coding transcripts generated through the back-splicing of precursor messenger RNAs, resulting in a covalently closed loop without free 5' or 3' ends. Owing to their covalently closed-loop structure, circRNAs are generally more stable and more resistant to exonuclease-mediated degradation than their linear RNA counterparts. In addition, many circRNAs exhibit a high degree of evolutionary conservation [3]. CircRNAs exhibit tissue- and developmental stage-specific expression patterns and are closely associated with cellular states and pathological conditions. Increasing evidence indicates that circRNAs regulate cancer progression through diverse mechanisms. For instance, hsa_circ_0136666 facilitates gastric cancer progression and immune evasion by modulating the miR-375/PRKDC pathway and enhancing PD-L1 phosphorylation [10]. Among the best-characterized modes of circRNA action is their participation in competitive endogenous RNA (ceRNA) networks. By binding specific microRNAs (miRNAs), circRNAs can reduce the availability of these miRNAs for their target transcripts, thereby attenuating post-transcriptional repression and altering the expression of downstream genes [11,12]. Additionally, certain circRNAs interact with RNA-binding proteins (RBPs), influencing alternative splicing, transcriptional regulation, or the assembly of protein complexes [13]. Moreover, recent studies suggest that some circRNAs possess coding potential and can be translated into functional peptides [14,15]. These mechanistic features highlight the involvement of circRNAs in critical aspects of tumour biology, including cancer initiation, proliferation, invasion, and therapeutic response.

Accumulating evidence has highlighted the functional significance of circRNAs in a variety of malignancies. For instance, in gastric cancer cells, CircPVT1 enhances proliferative capacity by binding and functionally sequestering miR-125 family members. CircPVT1 has been shown to drive gastric cancer cell proliferation by regulating miR-125, positioning it as a central node in tumour progression [16]. In lung cancer, several circRNAs have been identified as potent miRNA sponges, modulating oncogenic signalling

networks, such as hsa_circ_0012673, which promotes lung adenocarcinoma malignancy by sequestering miR-22 [17]. More broadly, miRNA-mediated regulation of downstream targets can influence diverse tumour-associated processes; miR-122-5p, for example, promotes ferroptosis in hepatocellular carcinoma cells by suppressing the glutamine transporter SLC1A5 [18]. Collectively, current evidence identifies circRNAs as important components of tumor-associated regulatory networks and suggests their potential value as diagnostic biomarkers and therapeutic targets. Nevertheless, the dysregulation and functional contribution of circRNAs in NSCLC have not yet been fully characterized. In particular, it remains unclear how circRNA-mediated interactions between miRNAs and their downstream transcripts influence NSCLC cell migration, invasion, and other aggressive phenotypes.

Insulin-like growth factor-binding protein 3 (IGFBP3) is the predominant IGF-binding protein in circulation and regulates IGF signaling by controlling the availability of IGF-1 and IGF-2 for interaction with the insulin-like growth factor 1 receptor (IGF1R), thereby influencing cell proliferation, survival, and apoptosis [19–21]. The biological effects of IGFBP3 are not restricted to modulation of the IGF axis, as this protein can also regulate cellular processes through mechanisms that do not require IGF signalling, participating in intracellular signalling, transcriptional regulation, and a wide range of tumour-associated biological processes. These diverse functions highlight the complexity and multifaceted nature of IGFBP3 during tumour initiation and subsequent malignant progression. Notably, the contribution of IGFBP3 varies across tumour types and microenvironmental contexts. In ovarian cancer, Ye et al. demonstrated that circ_0044362 enhances tumour cell growth and invasiveness by activating the HOXB4–RUNX1–IGFBP3 axis, suggesting an oncogenic role for IGFBP3 in certain cancer contexts [22]. Additionally, Long et al. identified IGFBP3 as a critical target within the circRNA–miRNA regulatory network in osteosarcoma, where its expression is controlled by the circ_0000285/miR-409-3p axis, contributing to malignant cellular phenotypes [23]. These findings further emphasise that IGFBP3 expression is finely regulated by noncoding RNA networks and may exhibit functional plasticity depending on the tumour context. Current evidence suggests that IGFBP3 can function either as a tumour suppressor or as an oncogenic factor, with its biological role being dictated by tissue-specific microenvironments and upstream regulatory mechanisms [24]. However, the upstream regulation of IGFBP3 in NSCLC, particularly whether it is regulated through a circRNA–miRNA axis and contributes to NSCLC progression, remains insufficiently understood. This knowledge gap represents a key scientific question and the rationale for the current investigation.

This work examined how CircATRNL1 influences the migratory and invasive properties of non-small cell lung cancer cells under *in vitro* conditions. We further examined its effects on epithelial–mesenchymal transition-associated protein expression and explored the regulatory relationship among CircATRNL1, miR-103a-3p, and IGFBP3. We hypothesized that CircATRNL1 may regulate the motility and invasiveness of phenotypes in NSCLC cells through the miR-103a-3p/IGFBP3 axis.

2 Methods

2.1 Cell Culture and Transfection

Human bronchial epithelial (HBE) cells and four non-small cell lung cancer (NSCLC) cell lines, including A549, H1299, H2126, and PC-9, were obtained from Immocell (Xiamen, China). Before experimental use, all cell lines underwent short tandem repeat authentication and were confirmed to be mycoplasma-free. Routine culture was carried out in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) supplemented with fetal bovine serum at 10% and penicillin–streptomycin at 1% (both from BDBIO, Shanghai, China). Cells were routinely incubated under humidified conditions at 37°C in an atmosphere containing 5% CO₂. For transient transfection, NSCLC cells were transfected with miR-103a-3p mimics, a miR-103a-3p

inhibitor, small interfering RNAs targeting CircATRNL1 or IGFBP3, the corresponding negative-control siRNA, or plasmids expressing CircATRNL1 or IGFBP3. Transfection was conducted with GenMute™ siRNA/DNA Transfection Kit (SignaGen, Frederick, MD, USA; catalog no. SL100568) or PolyJet (SignaGen; catalog no. SL100688), in accordance with the relevant manufacturer's protocol. Phenotypic and functional analyses were subsequently carried out between 24 and 72 h after transfection. The IGFBP3 overexpression plasmid and corresponding empty vector control were purchased from General Biol (Anhui, China). The IGFBP3 overexpression plasmid contained the full-length human IGFBP3 coding sequence. The remaining sequences used in this study are listed as follows: si-circATRNL1-1: AAUACAGACAGCCUUUUGAUUdTdT; si-circATRNL1-2: GGCAAGUCAUCUCAUAGUUAUdTdT; si-circATRNL1-3: AGUUGGCAAGGUUAACA-GAAdTdT; miR-103a-3p mimic sense: 5'-AGCAGCAUUGUACAGGGCUAUGA-3'; miR-103a-3p inhibitor: 5'-UCAUAGCCCUGUACAAUGCUGCU-3'; The target sequence of si-IGFBP3, presented in DNA notation, was 5'-GCACAGATACCCAGAACTT-3'.

2.2 RNA Extraction and Reverse Transcription Quantitative PCR (RT-qPCR) Analysis

Total RNA was extracted from the collected HBE, A549, H1299, H2126, and PC-9 cells using the Total RNA Extraction Kit (Shanghai Promega Biological Products, Shanghai, China, LS1040) according to the manufacturer's protocol. RNA concentration and purity were determined with a NanoDrop 2000 spectrophotometer. (Thermo Fisher Scientific, Wilmington, DE, USA). Equal amounts of total RNA were reverse-transcribed into complementary DNA (cDNA) using the reverse transcription reagents supplied with the GENESEED® circRNA qRT-PCR Kit (Guangzhou Geneseed Biotech Co., Ltd., Guangzhou, Guangdong, China; GS0201) according to the manufacturer's instructions. Reverse transcription quantitative PCR (RT-qPCR) was subsequently performed using GoTaq® qPCR Master Mix (Promega Corporation, Madison, WI, USA; A6001) on a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). All samples were analyzed in triplicate to ensure technical reproducibility. The primer sequences used for RT-qPCR are listed in Table 1.

Table 1: Primers used for RT-qPCR.

Gene	Primer (5'–3')
CircATRNL1	Forward: TCAGCAACTTTTCTGGCCTAT Reverse: TTCGACTGTTCTGGGTTTGCTA
IGFBP3	Forward: CGCTACAAAGTTGACTACGAGTC Reverse: GTCTTCCATTTCTCTACGGCAGG
β-actin	Forward: CCTTCCTGGGCATGGAGTCCT Reverse: GGAGCAATGATCTTGATCTTC

2.3 Specificity Verification for CircATRNL1

To validate the circular configuration of CircATRNL1, cDNA reverse-transcribed from total RNA and genomic DNA (gDNA) isolated from the same cells were used as PCR templates. Divergent primers crossing the back-splice junction and convergent primers targeting the corresponding linear ATRNL1 sequence were employed for amplification. The resulting amplicons were separated on a 2% agarose gel, and the amplification patterns obtained from cDNA and gDNA were compared to confirm junction-specific amplification, product size, and the back-splicing structure of CircATRNL1.

2.4 RNase R Treatment Assay

Total RNA was isolated from A549 cells with the RNAsimple Total RNA Kit (TIANGEN Biotech, Beijing, China; DP419) following the protocol supplied by the manufacturer. The isolated total RNA was subsequently separated into two aliquots and incubated either with RNase R (Beyotime Bio, Shanghai, China; R7092S) or without RNase R as a mock-treated control. RNA samples were treated with RNase R at 37°C for half an hour, after which the enzyme was heat-inactivated at 70°C for 10 min in accordance with the manufacturer's instructions. The treated RNA was then purified and converted into complementary DNA using the reverse transcription components included in the GENESEED® circRNA qRT-PCR Kit. (Guangzhou Geneseed Biotech Co., Ltd., Guangzhou, Guangdong, China; GS0201). The relative levels of CircATRNL1 and β -actin mRNA were detected by RT-qPCR. β -actin mRNA served as an RNase R-sensitive linear RNA control to evaluate the efficiency of RNase R digestion. The expression levels of CircATRNL1 and β -actin mRNA in RNase R-treated samples were calculated relative to their corresponding mock-treated controls.

2.5 Immunoblot Analysis

Total protein was prepared from treated A549 and H1299 cells using a protein lysis buffer (Sigma-Aldrich, St. Louis, MO, USA; catalog no. S3401). After protein quantification, equal amounts of each lysate were separated by SDS–polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Pall Corporation, Port Washington, NY, USA; catalog no. 66485). The membranes were blocked with rapid blocking buffer (LiJi Bio, Shanghai, China; catalog no. AP36L106) for 15 min at room temperature and then incubated with the indicated primary antibodies at 4°C overnight. Following washing, the membranes were exposed to HRP-conjugated goat anti-rabbit IgG or goat anti-mouse IgG secondary antibodies (ZhongHe, Beijing, China; catalog nos. ZH1103 and ZH1104, respectively), both diluted 1:20,000. Protein bands were developed using an enhanced chemiluminescence reagent (Tanon, Shanghai, China; catalog no. 180-501) and recorded with a ChemiDoc Touch Imaging System (Bio-Rad Laboratories, Hercules, CA, USA). The concentration and treatment duration were selected based on previous studies [25]. Cells were exposed to 20 μ M LY294002 (MedChemExpress, Monmouth Junction, NJ, USA; catalog no. HY-10108) for 24 h. An equivalent volume of DMSO was added to the vehicle-control group. The treatment conditions were determined from previous studies using LY294002 in lung cancer cells and preliminary optimization in our experimental system. After treatment, the cells were used for functional assays or immunoblotting. Pathway inhibition was verified by examining phosphorylated AKT and total AKT levels. The antibodies used were as follows: E-cadherin (HUABIO, Hangzhou, China; catalog no. ET1607-75; 1:5000), N-cadherin (HUABIO; catalog no. ET1607-37; 1:5000), vimentin (HUABIO; catalog no. ET1610-39; 1:20,000), Snail (ABclonal, Wuhan, China; catalog no. A5243; 1:1000), IGFBP3 (ABclonal; catalog no. A16052; 1:1000), pan-AKT (ABclonal; catalog no. A18675; 1:2000), phospho-AKT (ABclonal; catalog no. AP0637; 1:2000), PI3K (ABclonal; catalog no. A18355; 1:2000), phospho-PI3K (ABclonal; catalog no. AP0427; 1:2000), and β -actin (ABclonal; catalog no. AC004; 1:10,000).

2.6 Scratch-Wound Migration Assay

A549 and H1299 cells were plated in 6-well culture plates at 2×10^5 cells per well and maintained until the monolayers reached approximately 90%–100% confluence. A straight scratch was made in each cell monolayer using a sterile 200- μ L pipette tip. Detached cells were removed by gently washing with PBS (0.01 M, pH 7.2–7.5 at 25°C; LiJi Bio, Shanghai, China; AC08L011). The cultures were then supplied with serum-free medium to reduce the potential influence of cell proliferation. Scratch images were obtained immediately after wound generation and again at 48 h using an ECLIPSE Ts2 microscope fitted with a 10 $\times$

objective lens (Nikon Corporation, Tokyo, Japan). Changes in the wound area were measured with ImageJ software and used to determine the migratory ability of the cells.

2.7 Transwell Invasion Assays

For the Transwell invasion assay, phenol red-free basement membrane matrix (Wuhan Servicebio Technology, Wuhan, Hubei, China; catalog no. G4131-5ML) was thawed at 4°C overnight and kept on ice during use. Transwell inserts were coated with the matrix at 50 µL/cm² and maintained at 37°C for 30 min. A549 and H1299 cells were resuspended in serum-free medium, and 2×10^4 cells in 200 µL were placed in each upper chamber. The lower chamber was filled with 500 µL of complete medium containing 10% fetal bovine serum. After 24 h of incubation at 37°C with 5% CO₂, cells and matrix remaining on the upper surface were removed with a cotton swab. Cells on the lower side of the membrane were fixed with 4% paraformaldehyde for 10 min and stained with 0.1% crystal violet (Solarbio, Beijing, China; catalog no. G1059) for 15 min. Following PBS rinsing, the invaded cells were imaged under an ECLIPSE Ts2 inverted microscope with a ×10 objective (Nikon Corporation, Tokyo, Japan). Cell numbers were determined in three randomly chosen fields per insert.

2.8 Dual-Luciferase Reporter Analysis

The wild-type and mutant firefly luciferase reporter plasmids containing the predicted miR-103a-3p binding sites in CircATRNL1 or the IGFBP3 3'UTR were commercially synthesized by GenePharma (Shanghai, China). The mutant reporter plasmids were generated by mutating the predicted miR-103a-3p seed-matching sequences. All reporter plasmids were sequence-verified by the provider before delivery. For the dual-luciferase reporter assay, for each experimental condition, 24-well culture plates were prepared by plating 1×10^5 A549 or H1299 cells in each well. Each well was co-transfected with 400 ng of the indicated wild-type or mutant pmirGLO firefly luciferase reporter plasmid and miR-103a-3p mimics (Monmouth Junction, NJ, USA, MCE, HY-R00040) or mimics-NC at a final concentration of 50 nM. The canonical mature hsa-miR-103a-3p sequence was 5'-AGCAGCAUUGUACAGGGCUAUGA-3' (MIMAT0000101). After 48 h of transfection, cells were harvested and lysed for luciferase activity measurement. Firefly and Renilla luciferase activities were sequentially detected using the Dual-Luciferase Reporter Assay Kit (YEASEN, Shanghai, China; Cat. No. 11402ES60). Firefly luciferase activity was normalized to the corresponding Renilla luciferase activity to control for transfection efficiency. All experiments were performed in at least three independent biological replicates, and data are presented as the mean ± standard deviation (SD).

2.9 Actinomycin D-Mediated Transcriptional Blockade Assay

A549 cells were plated in 6-well plates at 2×10^5 cells per well and allowed to grow to approximately 70%–80% confluence. The cells were then treated with actinomycin D (MedChemExpress, Monmouth Junction, NJ, USA; HY-17559) at a final concentration of 2 µg/mL to block RNA transcription. Cells were harvested at 0, 4, 8, and 12 h after treatment, and total RNA was isolated. The expression levels of CircATRNL1 and its linear counterpart, ATRNL1 mRNA, were quantified by RT-qPCR using their respective transcript-specific primers, with β-actin serving as the internal reference gene. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method, and the expression level at 0 h was set to 1 to evaluate RNA stability over time.

2.10 Gene Set Enrichment Analysis

Gene set enrichment analysis (GSEA) was conducted with the GSEA software package (version 4.4.0; Broad Institute, Cambridge, MA, USA). Based on the median abundance of CircATRNL1, the RNA-sequencing cohort was divided into two subsets representing relatively elevated and reduced expression. All genes were ranked based on the differential expression metric between the two groups and subjected to enrichment analysis.

2.11 RNA-Sequencing Analysis

Total RNA was isolated from the indicated cell samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA integrity and quality were evaluated with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and RNase-free agarose gel electrophoresis. Poly (A)-containing mRNA was captured from the total RNA using Oligo (dT)-coupled beads and subsequently fragmented. The resulting RNA fragments were reverse-transcribed into cDNA, followed by strand-specific library preparation using the NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina® (New England Biolabs, Ipswich, MA, USA; Cat. No. 7760). After purification, the double-stranded cDNA products underwent end repair, 3'-end adenylation, and ligation with Illumina-compatible adapters. The adapter-ligated fragments were purified with AMPure XP beads and enriched through PCR amplification. The completed libraries were sequenced on an Illumina NovaSeq X Plus platform by Hangzhou Astrocyte Technology Co., Ltd. (Hangzhou, China).

2.12 Bioinformatics Analysis

Potential miRNAs associated with CircATRNL1 were initially predicted using ENCORI (<https://rnasysu.com/encori>) and circBank (<https://www.circbank.cn/>), and candidates shared by both databases were retained. Concurrently, the prediction results from ENCORI and miRNet (<https://www.mirnet.ca/>) were integrated to identify miRNAs with potential regulatory effects on IGFBP3. The two resulting candidate groups were subsequently compared to determine the miRNAs common to both regulatory networks.

2.13 Statistical Analysis

All experimental data were obtained from at least three independent biological replicates. Quantitative data are presented as the mean $\pm$ standard deviation (SD). Statistical analyses were performed using GraphPad Prism software (version 6.0; GraphPad Software, Inc., San Diego, CA, USA). For comparisons between two groups, statistical significance was evaluated using an unpaired Student's *t*-test. When three or more groups were compared, one-way analysis of variance was applied, with Tukey's post hoc test applied for Tukey's post hoc procedure was used to evaluate differences between individual groups, with *p* values below 0.05 regarded as statistically significant.

3 Results

3.1 High Expression of CircATRNL1 in NSCLC and Characterization of Its Circular Structure

To explore the potential role of CircATRNL1 in non-small cell lung cancer (NSCLC), we first assessed its expression in normal human bronchial epithelial cells (HBE) and a panel of NSCLC cell lines. RT-qPCR analysis showed that CircATRNL1 expression was markedly elevated in A549, H1299, PC-9, and H2126 cells compared with HBE cells (Fig. 1A), suggesting a possible association between CircATRNL1 and NSCLC pathogenesis. To confirm the circular nature of CircATRNL1, a series of validation assays was subsequently performed. Three independent small interfering RNAs targeting CircATRNL1 were transfected into A549 and H1299 cells, and RT-qPCR analysis demonstrated efficient silencing of CircATRNL1. All three siRNAs significantly reduced

CircATRNL1 expression in A549 cells, whereas only si-1 achieved significant knockdown in H1299 cells. Therefore, si-1, which showed consistent silencing efficiency in both cell lines, was selected for subsequent functional assays (Fig. 1B,C). RNase R digestion assays revealed that linear β -actin mRNA was substantially degraded following RNase R treatment, whereas CircATRNL1 remained largely resistant to exonuclease digestion, supporting its circular configuration (Fig. 1D). Consistently, transcriptional inhibition assays using Actinomycin D further demonstrated that CircATRNL1 displayed significantly greater stability than its linear counterpart, ATRNL1 mRNA, over the indicated time course (Fig. 1E), highlighting the enhanced stability conferred by its circular structure. PCR amplification using convergent and divergent primers was further performed to verify the back-splicing structure of CircATRNL1. Divergent primers amplified CircATRNL1 from cDNA but not from gDNA, whereas convergent primers generated PCR products from both cDNA and gDNA. Collectively, these results support that CircATRNL1 is generated through back-splicing and exists as a bona fide circular RNA. (Fig. 1F).

Collectively, these findings demonstrate that CircATRNL1 is highly expressed in NSCLC cells and possesses the typical molecular features of circular RNAs, laying a solid foundation for subsequent functional and mechanistic investigations.

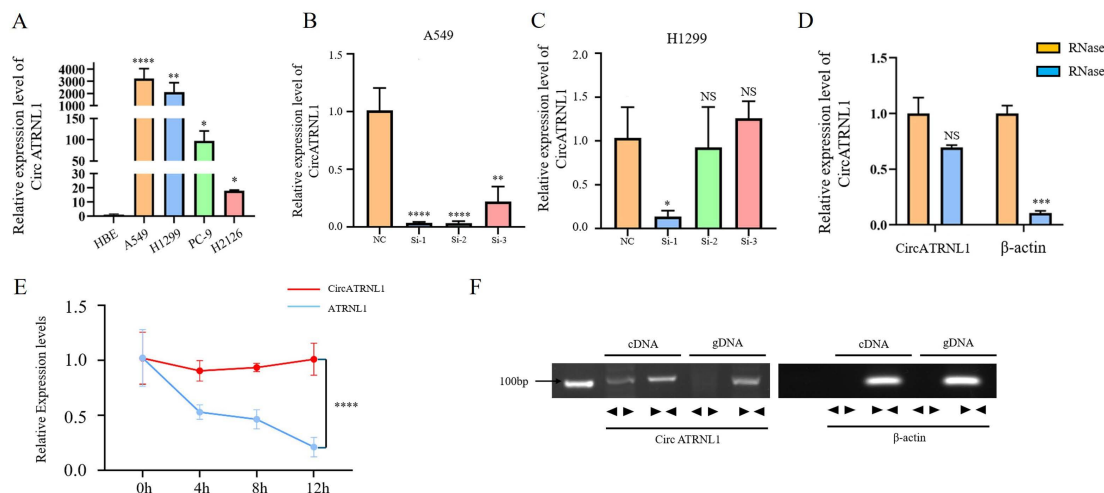


Figure 1: High expression of CircATRNL1 in NSCLC and characterization of its circular structure. (A) Relative expression levels of CircATRNL1 in normal human bronchial epithelial cells (HBE) and NSCLC cell lines (A549, H1299, PC-9, and H2126) were determined by RT-qPCR.; (B,C) RT-qPCR analysis of CircATRNL1 expression in A549 (B) and H1299 (C) cells following transfection with si-CircATRNL1 (si-1, si-2, si-3) or negative control (NC), confirming the knockdown efficiency of CircATRNL1; (D) RNase R digestion assay showing that CircATRNL1 is resistant to RNase R treatment, whereas linear β -actin mRNA is markedly degraded, indicating the circular structure of CircATRNL1; (E) Actinomycin D transcriptional inhibition assay demonstrating that CircATRNL1 is more stable than linear ATRNL1 mRNA over time in NSCLC cells. (F) PCR amplification using convergent and divergent primers was performed with cDNA and gDNA as templates. β -actin was amplified using convergent primers as a linear control. Data are presented as the mean $\pm$ SD from at least three independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001; NS, not significant.

3.2 CircATRNL1 Promotes the Invasion and Migration of NSCLC Cells

To evaluate the impact of CircATRNL1 on cell motility, wound-healing assays were performed. CircATRNL1 knockdown significantly delayed wound closure, whereas CircATRNL1 overexpression accelerated wound healing, indicating its positive regulatory role in NSCLC cell migration (Fig. 2A,B). Consistently,

basement membrane matrix-coated Transwell assays showed that CircATRNL1 silencing significantly reduced cell invasion, whereas CircATRNL1 overexpression markedly enhanced cell invasion (Fig. 2C–F). Next, the influence of CircATRNL1 on EMT was examined. Western blot analysis revealed that CircATRNL1 knockdown increased E-cadherin expression and reduced N-cadherin, Vimentin, and Snail levels in both A549 and H1299 cells, suggesting a reversal of the EMT phenotype (Fig. 2G). Conversely, CircATRNL1 overexpression decreased E-cadherin expression while elevating N-cadherin, Vimentin, and Snail levels, indicating EMT activation (Fig. 2H).

These results demonstrate that CircATRNL1 promotes the transition from an epithelial to a mesenchymal phenotype, thereby enhancing the invasive and migratory abilities of NSCLC cells.

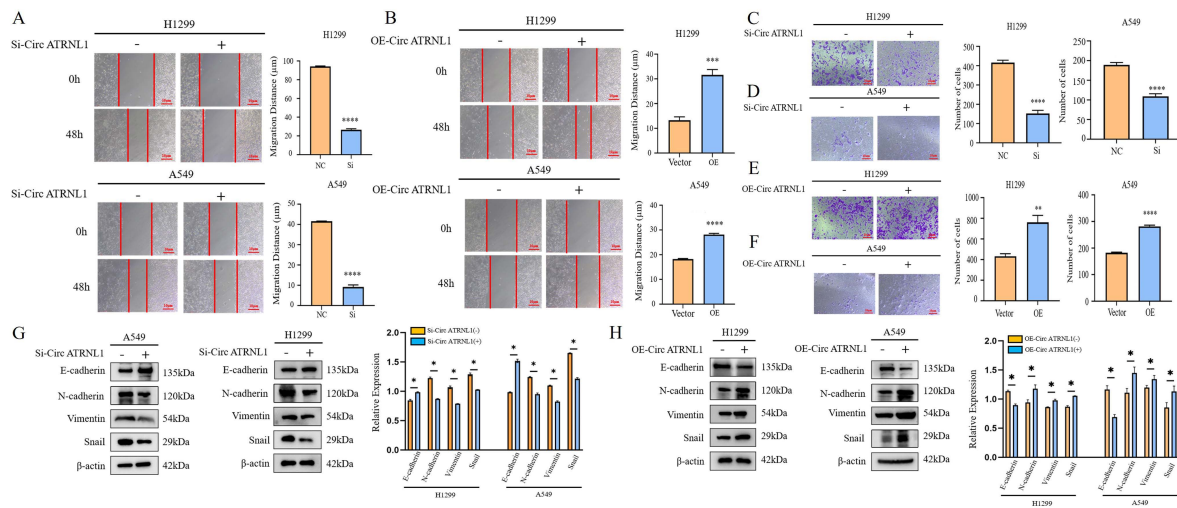


Figure 2: CircATRNL1 promotes the invasion and migration of NSCLC cells. (A,B) Wound-healing assays showing the effects of CircATRNL1 on cell migratory ability (Scale bars, 500 μ m). (C–F) Assessment of cell invasion following CircATRNL1 knockdown or overexpression using Transwell assays (0.1% crystal violet staining; Scale bars, 500 μ m). (G) Effects of CircATRNL1 knockdown on the expression of the indicated proteins in H1299 cells. (H) Effects of CircATRNL1 overexpression on the expression of the indicated proteins in A549 cells. (G) All experiments were performed at least three times independently, and data are presented as the mean $\pm$ standard deviation (SD). Statistical significance is indicated as follows: * $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$, **** $p < 0.0001$. NC, negative control; si-NC, negative control siRNA; OE, overexpression plasmid.

3.3 IGFBP3 Promotes the Invasion and Migration of NSCLC Cells

To explore potential genes associated with CircATRNL1 expression in NSCLC, we analyzed RNA-sequencing data. Samples were divided into CircATRNL1-high and CircATRNL1-low groups according to the median CircATRNL1 expression level. Differential expression analysis identified a set of genes associated with CircATRNL1 expression, among which IGFBP3 was selected for further validation because of its differential expression and reported involvement in tumor-related biological processes (Fig. 3A). Subsequent RT-qPCR analysis showed that IGFBP3 expression was markedly elevated in NSCLC cell lines (H1299 and A549) compared with normal bronchial epithelial cells (HBE) (Fig. 3B). Western blotting further demonstrated a consistent association between CircATRNL1 expression and IGFBP3 protein levels in both A549 and H1299 cells. CircATRNL1 knockdown decreased IGFBP3 protein expression, whereas CircATRNL1 overexpression increased IGFBP3 protein expression (Fig. 3C), suggesting that IGFBP3 may serve as a downstream target of CircATRNL1. To further investigate the functional role of IGFBP3 in NSCLC

migration and invasion, we manipulated IGFBP3 expression by knockdown and overexpression approaches. Wound-healing assays demonstrated that IGFBP3 knockdown significantly impaired wound closure in both H1299 and A549 cells, whereas IGFBP3 overexpression enhanced migratory capacity (Fig. 3D,E). Consistent with these findings, Transwell assays showed that loss of IGFBP3 reduced the number of migrated and invaded cells, while IGFBP3 overexpression markedly increased these numbers (Fig. 3F,G), indicating a critical role for IGFBP3 in regulating NSCLC cell motility. Given the importance of epithelial–mesenchymal transition (EMT) in migration and invasion, we next examined EMT-related markers. IGFBP3 silencing resulted in increased E-cadherin expression and decreased Vimentin and Snail levels, whereas IGFBP3 overexpression produced the opposite effect—downregulating E-cadherin while upregulating N-cadherin, Vimentin, and Snail (Fig. 3H,I), thereby implicating IGFBP3 in EMT regulation.

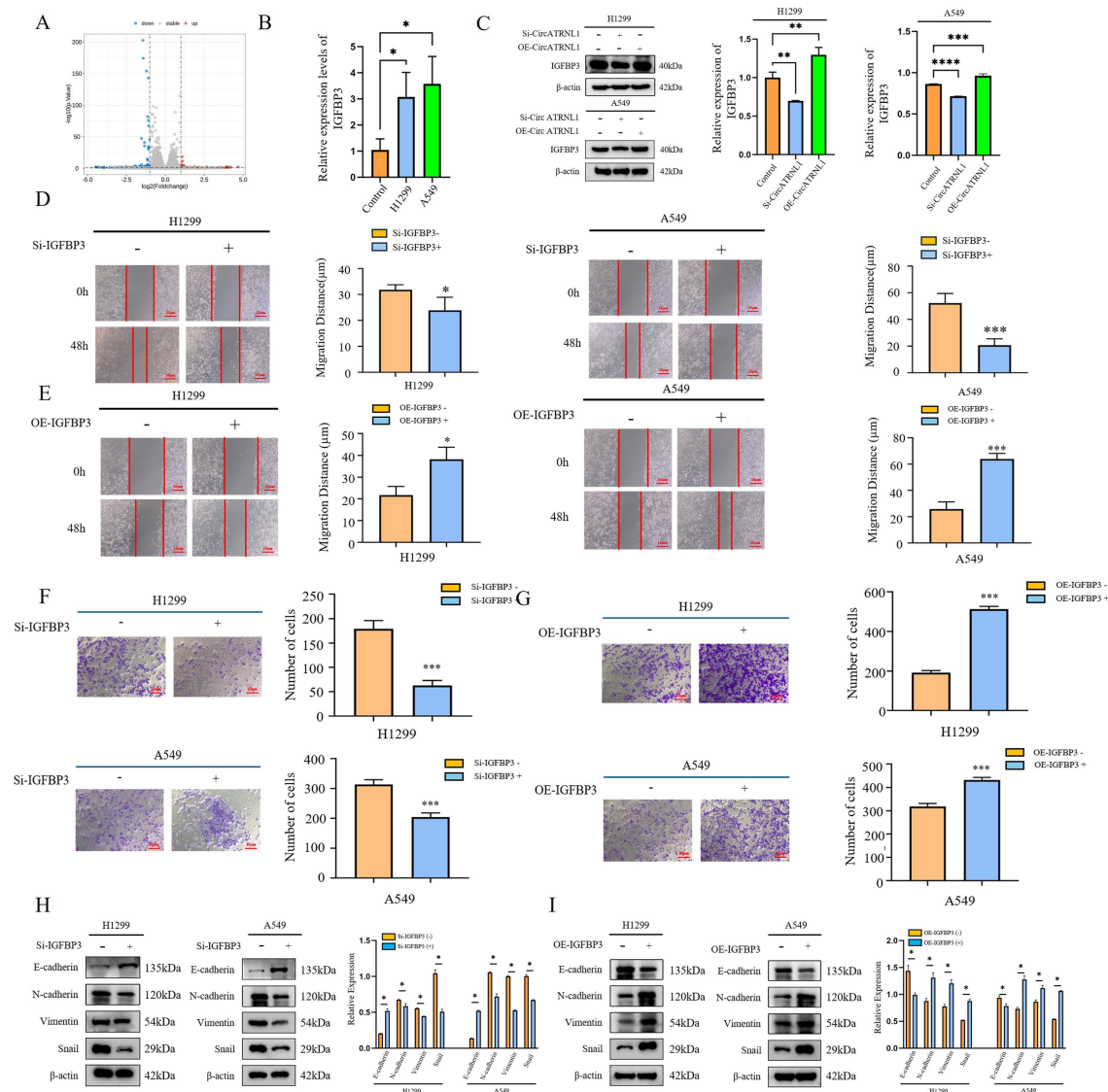


Figure 3: IGFBP3 Promotes the Invasion and Migration of NSCLC Cells. **(A)** Volcano plot showing differentially expressed genes (DEGs) between CircATRNL1 high-expression and low-expression NSCLC samples. **(B)** RT-qPCR analysis of IGFBP3 mRNA levels in human bronchial epithelial cells (HBE) and NSCLC cell lines (H1299 and A549). **(C)** Representative Western blot images and quantitative analysis of IGFBP3 protein expression in A549 and H1299 cells following CircATRNL1 knockdown (si-CircATRNL1) or overexpression (OE-CircATRNL1). **(D)** Wound-healing

assays in H1299 and A549 cells with or without IGFBP3 knockdown (Si-IGFBP3). Representative images at 0 h and 48 h and quantification of migration distance are shown (Scale bars, 500 μ m). (E) Wound-healing assays in H1299 and A549 cells with or without IGFBP3 overexpression (OE-IGFBP3). Representative images at 0 h and 48 h and corresponding migration quantification are provided (scale bar, 500 μ m). (F) Transwell invasion assays in H1299 and A549 cells following IGFBP3 knockdown (Si-IGFBP3). Representative stained images and quantification of invaded cells are shown (0.1% crystal violet staining, scale bar, 500 μ m). (G) Transwell invasion assays in H1299 and A549 cells following IGFBP3 overexpression (OE-IGFBP3). Representative images and invaded-cell quantification are displayed (0.1% crystal violet staining, scale bar, 500 μ m). (H) Western blot analysis of EMT-related protein levels (E-cadherin, Vimentin, Snail) in H1299 and A549 cells after IGFBP3 knockdown (Si-IGFBP3), with β -actin as the internal control. (I) Western blot analysis of EMT markers in H1299 and A549 cells following IGFBP3 overexpression (OE-IGFBP3). Statistical significance is indicated as follows: * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001. NC, negative control; si-NC, negative control siRNA; OE, overexpression plasmid. RT-qPCR, quantitative real-time PCR.

3.4 IGFBP3 Serves as a Key Downstream Effector of CircATRNL1 in Promoting NSCLC Cell Migration and Invasion

To determine whether IGFBP3 mediates the pro-migratory and pro-invasive effects of CircATRNL1, we conducted dual-gene manipulation experiments. Overexpression of IGFBP3 partially restored the reduced migration, invasion, and EMT marker expression caused by CircATRNL1 knockdown (Fig. 4A,C,E). Conversely, IGFBP3 knockdown attenuated the enhanced migration, invasion, and EMT activation induced by CircATRNL1 overexpression (Fig. 4B,D,F).

Collectively, these results demonstrate that IGFBP3 functions as a key downstream effector through which CircATRNL1 enhances migration, invasion, and EMT progression in NSCLC cells.

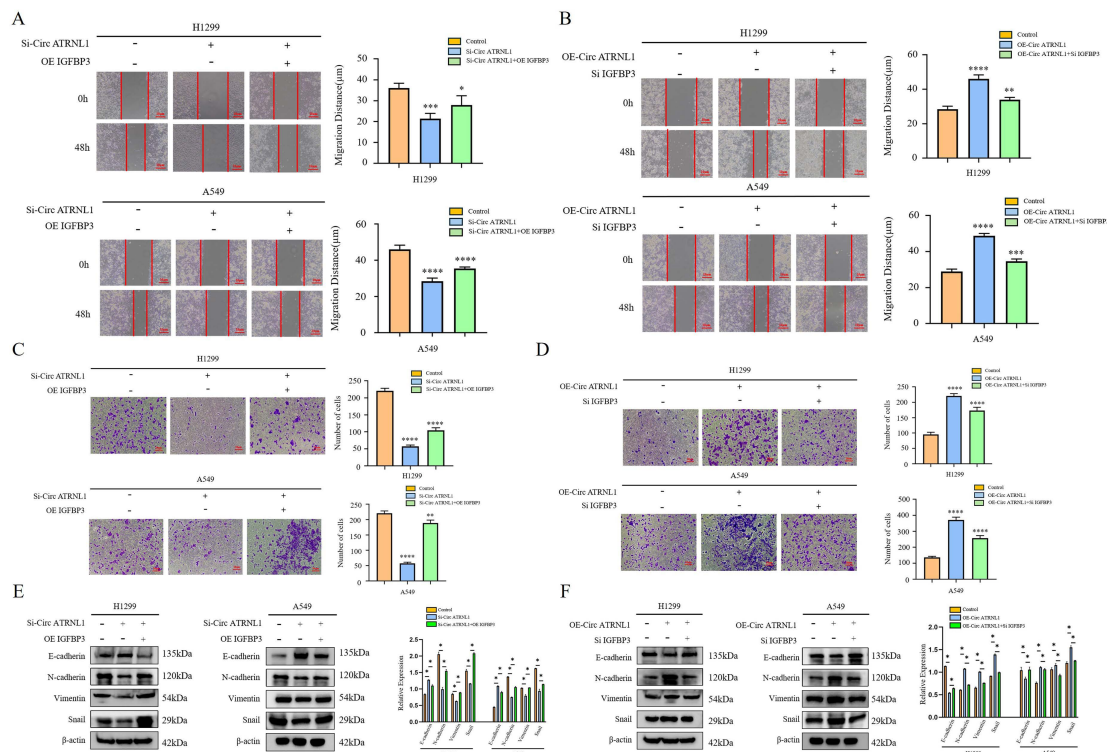


Figure 4: IGFBP3 Serves as a Key Downstream Effector of CircATRNL1 in Promoting NSCLC Cell Migration and Invasion. (A) Wound-healing assays in H1299 and A549 cells co-treated with CircATRNL1 knockdown (Si-CircATRNL1)

and IGFBP3 overexpression (OE-IGFBP3). Representative images at 0 h and 48 h and quantitative analysis are shown (scale bar, 500 μ m). (B) Wound-healing assays in H1299 and A549 cells with CircATRNL1 overexpression (OE-CircATRNL1) combined with IGFBP3 knockdown (Si-IGFBP3). Representative images and migration quantification are provided (scale bar, 500 μ m). (C) Transwell invasion assays in H1299 and A549 cells with CircATRNL1 knockdown (Si-CircATRNL1) combined with IGFBP3 overexpression (OE-IGFBP3). Representative crystal violet-stained images and quantification of invaded cells are shown (scale bar, 500 μ m). (D) Transwell invasion assays in H1299 and A549 cells with CircATRNL1 overexpression (OE-CircATRNL1) combined with IGFBP3 knockdown (Si-IGFBP3). Representative images and invaded-cell quantification are provided (scale bar, 500 μ m). (E) Western blot analysis of EMT markers (E-cadherin, N-cadherin, Vimentin, Snail) in H1299 and A549 cells following Si-CircATRNL1 + OE-IGFBP3 treatment. (F) Western blot analysis of EMT markers in H1299 and A549 cells treated with OE-CircATRNL1 + Si-IGFBP3. Statistical significance is indicated as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. NC, negative control; si-NC, negative control siRNA; OE, overexpression plasmid.

3.5 CircATRNL1 May Function as a Potential Regulator of miR-103a-3p

To identify potential miRNAs interacting with CircATRNL1, candidate binding miRNAs were first predicted using the ENCORI and circBank databases. Simultaneously, ENCORI and miRNet were employed to screen for upstream miRNAs potentially regulating IGFBP3. Integrated analysis revealed miR-103a-3p as the overlapping molecule between the two datasets, suggesting its critical role as a regulatory link between CircATRNL1 and IGFBP3 (Fig. 5A). Sequence alignment further demonstrated that miR-103a-3p forms a highly complementary pairing with a specific site on CircATRNL1, and mutations at this site disrupted the predicted interaction (Fig. 5B). Consistently, dual-luciferase reporter assays confirmed that miR-103a-3p significantly reduced the luciferase activity of the CircATRNL1-WT construct, but did not affect the MUT construct, validating the specific binding between miR-103a-3p and CircATRNL1 (Fig. 5C). MiR-103a-3p mimics were used in the luciferase reporter assay to directly determine whether increased miR-103a-3p could bind to and suppress the wild-type reporter containing the predicted binding sequence. miR-103a-3p inhibitor was not included in this assay because its effect would depend on the endogenous basal level of miR-103a-3p in the tested cells, whereas the mimic-based assay provides a more direct gain-of-function approach for validating the predicted miRNA-binding site. To explore the functional role of miR-103a-3p in NSCLC EMT progression, its effects on EMT markers and cellular motility were examined. Wound-healing assays in A549 and H1299 cells revealed consistent results: inhibition of miR-103a-3p markedly accelerated wound closure and enhanced cell migration, whereas miR-103a-3p mimics significantly suppressed migratory capability (Fig. 5D,E). Transwell invasion assays further supported these findings, showing that miR-103a-3p inhibition substantially increased the number of invading cells, while miR-103a-3p overexpression significantly reduced invasion (Fig. 5F,G). Western blot analysis showed that miR-103a-3p inhibition decreased E-cadherin and increased Vimentin and Snail expression, indicative of EMT activation. In contrast, miR-103a-3p mimics led to elevated E-cadherin levels and reduced N-cadherin, Vimentin and Snail expression, suggesting suppression of EMT (Fig. 5H,I).

These results demonstrate that miR-103a-3p functions as a key negative regulator of migration and invasion in NSCLC cells, highlighting its potential role as a tumour-suppressive miRNA within the CircATRNL1-mediated regulatory network.

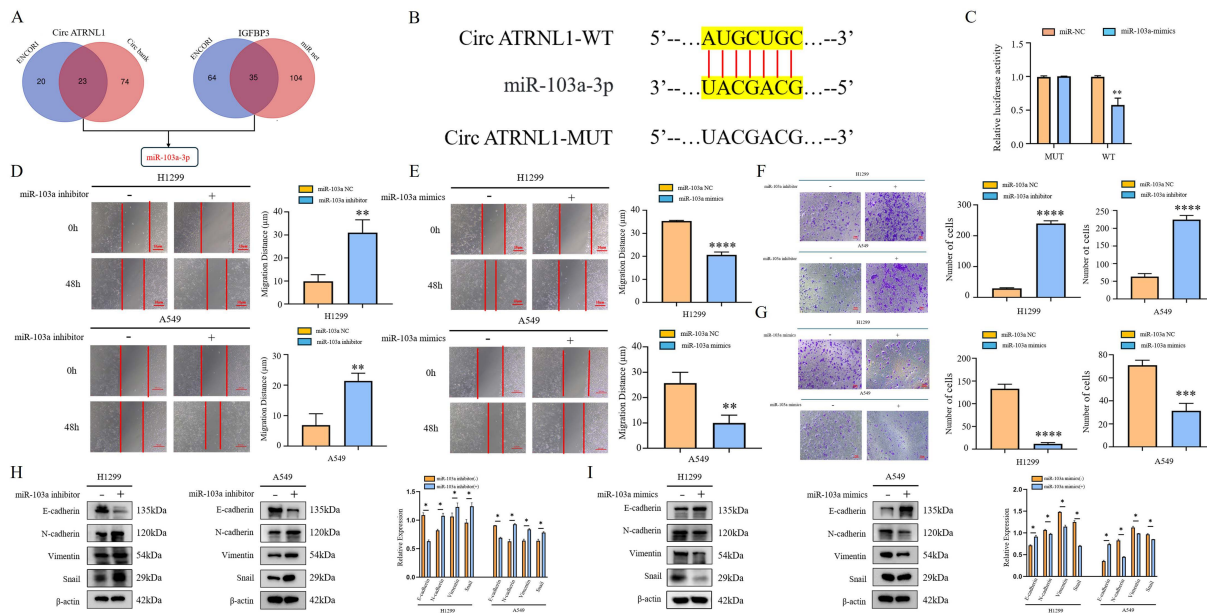


Figure 5: CircATRNL1 may function as a potential regulator of miR-103a-3p. (A) Venn diagrams showing the prediction of candidate miRNAs potentially involved in the CircATRNL1/IGFBP3 regulatory axis. Candidate miRNAs interacting with CircATRNL1 were predicted using ENCORI and circBank, with 23 overlapping miRNAs identified. Candidate miRNAs targeting IGFBP3 were predicted using ENCORI and miRNet, with 35 overlapping miRNAs identified. miR-103a-3p was selected for further validation as a shared candidate miRNA potentially linking CircATRNL1 and IGFBP3. (B) Predicted binding sites between miR-103a-3p and the wild-type CircATRNL1 sequence (CircATRNL1-WT) and schematic representation of mutations introduced into the corresponding binding region to generate the mutant construct (CircATRNL1-MUT). (C) A dual-luciferase reporter assay was performed in cells co-transfected with CircATRNL1-WT luciferase reporter plasmid and miR-103a-3p mimics. (D) Representative images and quantitative analysis of wound-healing assays in H1299 and A549 cells transfected with miR-103a-3p inhibitor or inhibitor negative control (inhibitor NC). Cell migration was quantified by measuring the migration distance. Scale bars, 500 μ m. (E) Representative images and quantitative analysis of wound-healing assays in H1299 and A549 cells transfected with miR-103a-3p mimics or mimic negative control (mimics NC). Cell migration was quantified by measuring the migration distance. Scale bars, 500 μ m. (F) Representative images and quantitative analysis of basement membrane matrix-coated Transwell invasion assays in H1299 and A549 cells transfected with miR-103a-3p inhibitor or inhibitor NC. Scale bars, 500 μ m. (G) Representative images and quantitative analysis of basement membrane matrix-coated Transwell invasion assays in H1299 and A549 cells transfected with miR-103a-3p mimics or NC mimics. Scale bars, 500 μ m. (H) Western blot analysis of EMT-related protein expression in H1299 and A549 cells transfected with miR-103a-3p inhibitor or negative control. (I) Western blot analysis of EMT markers in H1299 and A549 cells transfected with miR-103a-3p mimics. Statistical significance is indicated as follows: * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001. WT, wild type; MUT, mutant; NC, negative control.

3.6 CircATRNL1 Regulates NSCLC Cells through the miR-103a-3p/IGFBP3 Axis

IGFBP3-WT and IGFBP3-MUT reporter plasmids were constructed, and dual-luciferase reporter assays showed that miR-103a-3p mimics significantly reduced the luciferase activity of IGFBP3-WT but not IGFBP3-MUT, supporting a specific interaction between miR-103a-3p and the predicted binding site within the IGFBP3 3'UTR (Fig. 6A,B). Wound-healing assays showed that CircATRNL1 knockdown suppressed the migration of H1299 and A549 cells, whereas miR-103a-3p inhibition partially reversed this effect. Conversely, CircATRNL1 overexpression promoted cell migration, and this effect was attenuated by miR-

103a-3p mimics (Fig. 6C,D). Consistently, basement membrane matrix-coated Transwell assays showed corresponding changes in cell invasion under the same treatment conditions (Fig. 6E,F). Western blotting in A549 cells further showed that CircATRNL1 knockdown increased E-cadherin expression and decreased N-cadherin, Vimentin, and Snail expression, whereas miR-103a-3p inhibition or IGFBP3 overexpression partially reversed these changes (Fig. 6G,H).

Collectively, these findings indicate that CircATRNL1 promotes NSCLC cell migration, invasion, and EMT-associated molecular changes through the miR-103a-3p/IGFBP3 axis.

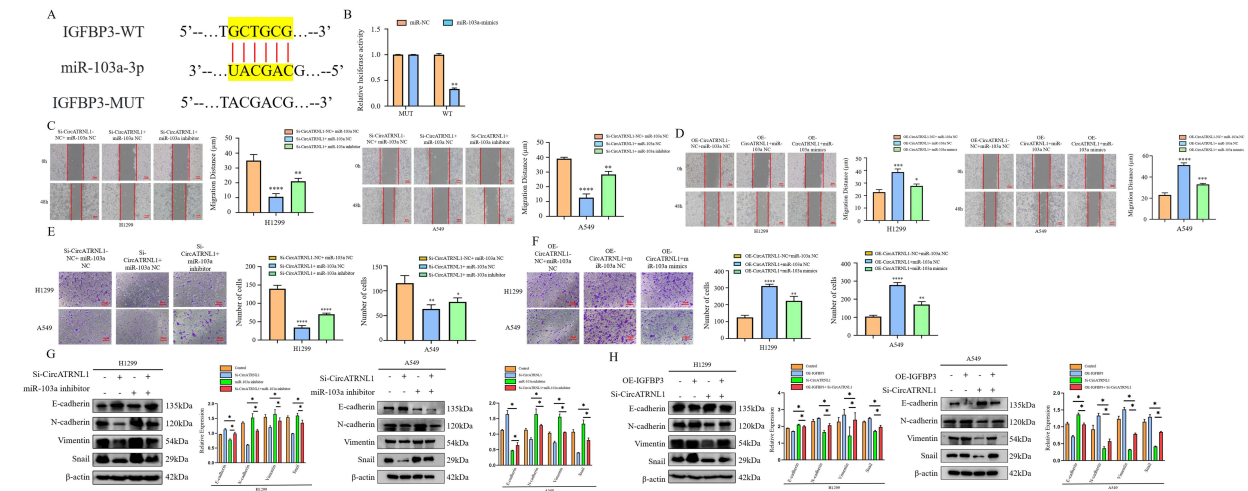


Figure 6: CircATRNL1 regulates NSCLC cells through the miR-103a-3p/IGFBP3 axis. (A) Predicted binding site between miR-103a-3p and the IGFBP3 3'UTR. (B) Dual-luciferase reporter assay in NSCLC cells co-transfected with miR-103a-3p mimics and luciferase reporters containing either wild-type (IGFBP3-WT) or mutant (IGFBP3-MUT) IGFBP3 3'UTR sequences. (C,D) Wound-healing assays in H1299 and A549 cells following transfection with: (1) si-circATRNL1 negative control + miR-103a-3p negative control (Si-circATRNL1-NC + miR-103a-3p NC); (2) si-circATRNL1 + miR-103a-3p negative control (Si-circATRNL1 + miR-103a-3p NC); (3) si-circATRNL1 + miR-103a-3p inhibitor (Si-circATRNL1 + miR-103a-3p inhibitor); or transfection with: (4) circATRNL1 overexpression plasmid negative control + miR-103a-3p negative control (OE-circATRNL1-NC + miR-103a-3p NC); (5) circATRNL1 overexpression plasmid + miR-103a-3p negative control (OE-circATRNL1 + miR-103a-3p NC); (6) circATRNL1 overexpression plasmid + miR-103a-3p mimics (OE-circATRNL1 + miR-103a-3p mimics). Representative wound-healing images were captured 48 h after transfection (Scale bars, 500 μ m). (E,F) Transwell assays performed under the same treatment conditions as in (C,D), with invaded cells stained using 0.1% crystal violet (Scale bars, 500 μ m). (G,H) Western blot analysis of EMT-related proteins in A549 cells following CircATRNL1 knockdown and corresponding rescue experiments. Statistical significance is indicated as follows: * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001. WT, wild type; MUT, mutant; NC, negative control.

3.7 IGFBP3 Promotes EMT-Associated Migration and Invasion through Activation of the PI3K/AKT Signaling Pathway

The PI3K/AKT signaling pathway was subsequently investigated to clarify the downstream mechanism by which IGFBP3 regulates EMT-associated migration and invasion in NSCLC cells. GSEA comparing samples with high and low CircATRNL1 expression revealed significant enrichment of the PI3K/AKT signaling pathway, suggesting that this pathway may be involved in CircATRNL1-associated NSCLC progression (Fig. 7A). Subsequent molecular and functional analyses were therefore performed to determine whether IGFBP3 mediates the effects of CircATRNL1 through regulation of PI3K/AKT signaling. Western blot analysis further demonstrated that IGFBP3 silencing decreased the levels of phosphorylated PI3K and

AKT in H1299 and A549 cells (Fig. 7B), whereas IGFBP3 overexpression produced the opposite effects (Fig. 7C). In contrast, the total protein levels of PI3K and AKT were not markedly altered, indicating that IGFBP3 mainly regulated PI3K/AKT pathway activation rather than total PI3K or AKT expression.

To further determine whether PI3K/AKT signaling was required for the biological effects of IGFBP3, IGFBP3-overexpressing cells were treated with the PI3K inhibitor LY294002. Wound-healing assays showed that IGFBP3 overexpression significantly enhanced the migratory ability of H1299 and A549 cells, whereas LY294002 treatment markedly attenuated this effect (Fig. 7D,E). Similarly, Transwell assays revealed that IGFBP3 overexpression increased the invasive capacity of both cell lines, while LY294002 significantly reduced IGFBP3-induced invasion (Fig. 7D,G). In addition, LY294002 partially reversed IGFBP3-induced EMT, as reflected by increased E-cadherin expression and decreased Vimentin and Snail expression in IGFBP3-overexpressing cells (Fig. 7H).

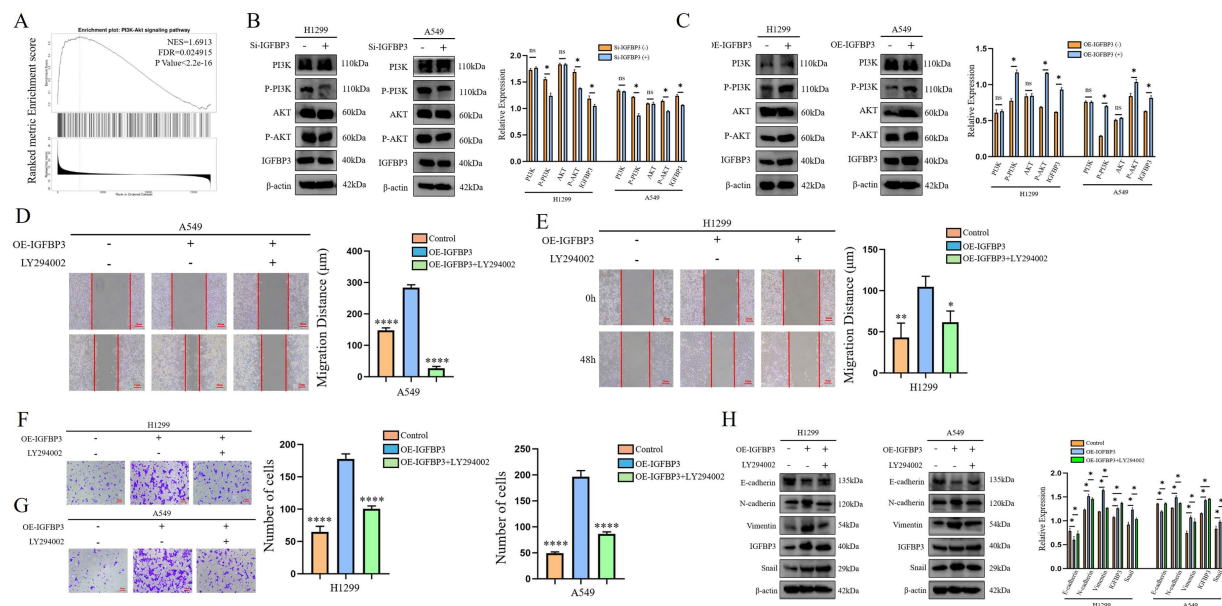


Figure 7: IGFBP3 Promotes EMT-Associated Migration and Invasion Through Activation of the PI3K/AKT Signaling Pathway. (A) Gene set enrichment analysis of the PI3K/AKT signaling pathway. RNA-sequencing samples were divided into IGFBP3-high and IGFBP3-low groups according to the median IGFBP3 expression level, and enrichment analysis was performed using GSEA software (version 4.4.0; Broad Institute, Cambridge, MA, USA). (B) Western blot analysis of PI3K/AKT pathway-related proteins in H1299 and A549 cells following IGFBP3 knockdown. (C) Western blot analysis of PI3K/AKT pathway-related proteins in H1299 and A549 cells following IGFBP3 overexpression. (Scale bars, 500 μ m). (D,E) Wound-healing assays showing the migratory ability of H1299 and A549 cells after IGFBP3 overexpression with or without LY294002 (HY-10108, MCE, 20 μ M, 24 h) treatment. (Scale bars, 500 μ m). (F,G) Transwell invasion assays showing the invasive ability of H1299 and A549 cells after IGFBP3 overexpression with or without LY294002 treatment. (H) Western blot analysis of EMT-related proteins in IGFBP3-overexpressing H1299 and A549 cells treated with or without LY294002. Statistical significance is indicated as follows: * p < 0.05, ** p < 0.01, **** p < 0.0001. NES, normalized enrichment score; FDR, false discovery rate; NC, negative control; GSEA, gene set enrichment analysis; LY294002, PI3K inhibitor.

4 Discussion

Histopathological classification indicates that nearly 85% of lung malignancies belong to the non-small cell lung cancer (NSCLC) category, making it the predominant form of the disease [26]. Growing

evidence that circular RNAs (circRNAs) participate in both tumorigenesis and the subsequent advancement of malignant disease has made these molecules a prominent focus of cancer research [27]. Silencing CircATRNL1 significantly suppressed the motility and migration and invasion in cultured NSCLC cells. Moreover, IGFBP3 was identified as a downstream target regulated by CircATRNL1. Mechanistically, the findings obtained in this study support the possibility that CircATRNL1 modulates the miR-103a-3p/IGFBP3 regulatory axis through a ceRNA-like mechanism, thereby contributing to increased IGFBP3 expression. These findings provide new mechanistic insights into how circATRNL1 contributes to cancer cell invasion and migration in NSCLC.

The migratory and invasive capacities of tumour cells are fundamental biological traits that enable malignant progression and are essential steps in the metastatic cascade [28]. Migration refers to the ability of the dissemination of malignant cells beyond the primary tumour into adjacent tissues or distant anatomical sites, whereas invasion involves breaching the basement membrane, infiltrating adjacent tissues, and gaining access to the vasculature or lymphatic system. Although conceptually distinct, these processes often occur in a coordinated manner and drive tumour evolution and metastatic spread [29]. Enhanced migration and invasion are key malignant phenotypes associated with metastatic progression, therapeutic resistance, and poor prognosis, and increasing evidence indicates that circRNAs play important regulatory roles in these processes. For example, Chen et al. identified circ_0002019 as an oncogenic circRNA in gastric cancer. Its depletion weakened malignant cellular phenotypes and reduced tumor growth, whereas circ_0002019 promoted NF- κ B activation by enhancing PTBP1-dependent stabilization of TNFAIP6 mRNA [30]. Long et al. showed that cytoplasmic circ_0000285 contributes to osteosarcoma progression. Its silencing impaired tumour growth and aggressive cellular phenotypes, whereas its oncogenic activity was mediated, at least in part, through the miR-409-3p/IGFBP3 axis [23]. Zan et al. reported that circCSNK1G1 promotes aggressive phenotypes and glycolytic activity in triple-negative breast cancer through the miR-28-5p/LDHA axis, whereas its silencing enhances apoptosis [31]. Consistent with these findings, our study reveals that circATRNL1 shows elevated expression in NSCLC cells and shows a strong association with the invasive and migratory phenotypes of these cells.

Non-coding RNAs are increasingly recognised as regulators of cancer biology, and Kang and Lee highlighted the broad involvement of microRNAs in lung cancer progression and therapeutic response [32]. Additionally, Oliveto et al. [31] reviewed the roles of microRNAs in translational regulation and cancer, highlighting their involvement in the post-transcriptional control of gene expression and their contribution to tumor development and progression. miRNAs are endogenous non-coding RNAs approximately 18–25 nucleotides in length that play essential regulatory roles in the initiation and progression of various cancers [33]. Accumulating evidence suggests that miRNAs regulate gene expression by interacting with complementary sites in the 3' untranslated regions (3'UTRs) of target transcripts, resulting in translational repression or mRNA destabilisation. Through these mechanisms, miRNAs influence diverse cellular functions, such as cell growth, programmed cell death, motility, invasiveness, and resistance to anticancer treatment [34]. Importantly, miRNAs exhibit functional duality: depending on the tissue context and their downstream targets, they can act as either oncogenes or tumour suppressors. In contrast, Johnson et al. demonstrated that the tumour-suppressive miRNA let-7 is downregulated in NSCLC, accompanied by upregulation of its oncogenic target RAS; enforced expression of let-7 decreases RAS levels and inhibits lung cancer cell growth [35]. Consistently, Yamakuchi et al. found that miR-34a targets SIRT1 and enhances p53 acetylation and activation, thereby inducing apoptosis and reinforcing its role as a key tumour-suppressive miRNA [36]. Given these observations, dysregulation of miRNA expression patterns has emerged as a hallmark of numerous malignancies, significantly contributing to the acquisition of aggressive phenotypes.

In line with this paradigm, our study demonstrates that downregulation of circATRNL1 results in the release of miR-103a-3p, which subsequently suppresses IGFBP3 expression and attenuates the migratory and invasive behaviour of NSCLC cells.

IGFBP3 is an important member of the IGF-binding protein family and participates in the regulation of IGF-dependent and IGF-independent signalling. Although IGFBP3 has traditionally been considered a tumour-suppressive factor in some contexts by limiting IGF/IGF1R signalling, increasing evidence indicates that its biological function is highly context-dependent and may vary according to tumour type, cellular background, and microenvironmental conditions. In NSCLC, the role of IGFBP3 remains complex and may be associated with malignant biological behaviours under specific molecular contexts. Our findings indicate that CircATRNL1 increases IGFBP3 expression, which in turn contributes to the enhanced motility and invasiveness of NSCLC cells. These findings suggest that IGFBP3 may act as a downstream functional mediator of CircATRNL1 in regulating NSCLC cell motility. However, the biological role of IGFBP3 appears to be context-dependent. Unlike the pattern observed in our study, Wang et al. documented that IGFBP3 suppressed lung tumorigenesis and cell growth through inhibition of IGF1 signaling [37]. Kwon et al. found that IGFBP3 reduced telomerase activity in MCF-7 cells, thereby inducing cellular senescence and limiting their proliferative potential [38]. Consistent with our results, Natsuizaka et al. showed that IGFBP3 supports oesophageal tumour growth by limiting oxidative stress under hypoxic conditions [39]. Consistent with the observations described above, our findings support a tumor-promoting function of IGFBP3 in NSCLC. Mechanistically, CircATRNL1 may increase the migration and invasion capacity of NSCLC cells by sequestering miR-103a-3p, which weakens miR-103a-3p-dependent suppression of IGFBP3 and consequently elevates IGFBP3 expression. Taken together, these data implicate the circATRNL1/miR-103a-3p/IGFBP3 regulatory network as a potential oncogenic pathway involved in NSCLC progression.

This study has several limitations. First, most functional and mechanistic findings were obtained from *in vitro* experiments using NSCLC cell lines; therefore, further validation in *in vivo* metastasis models is needed to verify the biological functional contribution of CircATRNL1 during the advancement of NSCLC. Second, although CircATRNL1 promotes cell motility and invasiveness through the miR-103a-3p/IGFBP3/PI3K-AKT axis, the clinical relevance of this regulatory pathway requires further confirmation in larger patient cohorts with complete clinicopathological and prognostic information. Third, the downstream mechanisms by which IGFBP3-mediated PI3K/AKT activation promotes the migratory and invasive phenotypes of NSCLC cells remain to be further explored. Future studies addressing these issues will help clarify the translational significance of the CircATRNL1/miR-103a-3p/IGFBP3 axis in NSCLC.

5 Conclusion

In conclusion, this study suggests that CircATRNL1 may function as an oncogenic circRNA in NSCLC by promoting EMT-associated migration and invasion. Mechanistically, CircATRNL1 may regulate miR-103a-3p through a ceRNA-like mechanism, thereby contributing to the upregulation of IGFBP3. Increased IGFBP3 expression was associated with enhanced PI3K/AKT pathway activation, and pharmacological inhibition of PI3K with LY294002 partially attenuated IGFBP3-induced EMT- and motility-related phenotypes. These findings indicate that the CircATRNL1/miR-103a-3p/IGFBP3 axis may promote NSCLC cell migration and invasion, at least in part, through PI3K/AKT signaling. Further studies, particularly *in vivo* validation and endogenous interaction assays, are warranted to fully clarify the biological and translational significance of this regulatory pathway in NSCLC progression.

Acknowledgement: None.

Funding Statement: This work was supported by the Clinical Medicine Science and Technology Development Fund of Jiangsu University (2021) (Grant No. JLY2021160); the Scientific Research Fund Project of Wannan Medical University (Grant Nos. WK2023ZZD13 and WK2024ZQNZ61); the Anhui Provincial Health Research Project (Grant No. AHWJ2023BAc10056); and the School–Enterprise Cooperation Project of Wannan Medical University (Grant No. XQQL202404).

Author Contributions: The authors confirm contribution to the paper as follows: Conceptualization, Jiawei Liu, Tao Huang and Qi You; methodology, Jiawei Liu, Tao Huang and Shaojin Zhu; formal analysis, Jiawei Liu, Tao Huang and Qi You; investigation, Jiawei Liu, Tao Huang and Qi You; data curation, Jiawei Liu, Tao Huang, Shaojin Zhu and Qi You; writing—original draft preparation, Jiawei Liu, Tao Huang, Qi You, Yuxiang Cao, Shaojin Zhu and Feng Liu; writing—review and editing, Qi You, Yuxiang Cao and Jiawei Liu; visualization, Jiawei Liu, Tao Huang and Qi You; supervision, Feng Liu; project administration, Feng Liu and Shaojin Zhu; funding acquisition, Feng Liu and Shaojin Zhu. All authors reviewed and approved the final version of the manuscript.

Availability of Data and Materials: The data that support the findings of this study are available from the Corresponding Author, [Feng Liu], upon reasonable request.

Ethics Approval: Not applicable.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

ceRNAs	competitive endogenous RNAs
circRNAs	Circular RNAs
EMT	epithelial–mesenchymal transition
IGFBP3	Insulin-like growth factor binding protein 3
NSCLC	Non-small cell lung cancer
RT-qPCR	Reverse transcription quantitative PCR

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