Supplemental Information

IncRNA CEBPA-AS1 Overexpression Inhibits Proliferation and Migration and Stimulates Apoptosis of OS Cells via Notch Signaling

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Supplementary Figure 1. LncRNA CEBPA-AS1 influences OS cell migration by regulating NCOR2. A, the results of gRNA activity. Empty vector 1 is the control of the siLncRNA CEBPA-AS1 group. Empty vector 2 is the control of the siNCOR2 group. The indel mutation rate in the siLncRNA CEBPA-AS1 group is 23.2%, and the indel mutation rate in the siNCOR2 group is 26.4%. B-C, RT-qPCR analysis of lncRNA CEBPA-AS1 expression and the mRNA expression of NCOR2 in transfected MG63 and SAOS-2 cells. The above data are measurement data and expressed as standard error of the mean. n = 3. In Panel A, comparisons among multiple groups are analyzed using one-way ANOVA. * p < 0.05 vs. the blank group.
Supplementary Figure 2. LncRNA CEBPA-AS1 elevation or NCOR2 overexpression enhances OS cell apoptosis. A, quantitative analysis of cell cycle distribution in transfected MG63 and SAOS-2 cells. B, RT-qPCR analysis of the mRNA expression of Cyclin D1 in transfected MG63 and SAOS-2 cells. C, western blot analysis of the Cyclin D1 protein expression in transfected MG63 and SAOS-2 cells. The band intensity is assessed. The above data are measurement data and expressed as the mean ± standard deviation. Comparisons among multiple groups are analyzed using one-way ANOVA. All experiments were repeated three times. * \( p < 0.05 \) vs. the blank group. \# \( p < 0.05 \) vs. the siLncRNA CEBPA-AS1 or oe-LncRNA CEBPA-AS1 group.
Supplementary Figure 3. LncRNA CEBPA-AS1 could bind to miR-10b-5p to reverse its inhibition on NCOR2. Activation of NCOR2 inhibits the Notch signaling pathway, thus repressing OS cell proliferation and differentiation and promoting its apoptosis.
## Supplemental Table 1

The primers for RT-qPCR

| Gene       | Forward primer (5’ - 3’)                  | Reverse primer (5’ - 3’)                  |
|------------|-------------------------------------------|-------------------------------------------|
| CEBPA-AS1  | CCCTCGCATTTCTTACCG                       | TTACTCCCTCCCGCTCCAA                      |
| NCOR2      | AAGCAGCGACCGGCTGACCAT                    | TGGCAGGGCGCTGCTCTC                       |
| HES1       | CGGCTAAGGTGTTTGGAGGCT                    | CGCTGTGCTGGTGTTGTAACG                    |
| RBPJ       | CGCATATTGAGTGCAGATG                      | CAGGAAGCGCCATCATTTAT                     |
| Cyclin D1  | TGTTGCAAGGAGGACTT                        | ACGTCAGCCTCCACACTCTT                     |
| MMP-2      | GGTGATGCCTTTGCTTCG                       | TGGAAAGCGGAATGGAAC                      |
| Bel-2      | GGTGCCACCTGTGGTCCACCT                    | CTTCACTTGTGGCCAGATAAG                    |
| Bax        | CGATGTGTTTCTGACGCG                      | TCAGGCTTCTCTTTCCAGA                      |
| GAPDH      | CAGGATCGGTGATGTGAACG                      | ACGACATACTCAGCACCAGCA                    |

Notes: RT-qPCR, reverse transcription quantitative polymerase chain reaction; NCOR2, nuclear receptor corepressors 2; HSE1, hes family bHLH transcription factor 1; RBPJ, recombination signal binding protein for immunoglobulin kappa J region; MMP-2, matrix metalloproteinase-2; Bel-2, B-cell lymphoma-2; Bax, Bel-2-associated X protein.
**Supplementary Table 2**

The RCR primers for gRNA activity

| Gene        | Sense primer (5’ - 3’) | Antisense primer (5’ - 3’) | Length |
|-------------|------------------------|----------------------------|--------|
| CEBPA-AS1   | CCGTAGGACTGGAAGAAA CC  | ATAACACCTCCGCAGACA AA      | 450 bp |
| NCOR2       | CACCCAGTGACCTCTACCT CC | GCACTGGCATTCCAGAGGG TT     | 558 bp |

Notes: PCR, polymerase chain reaction; LncRNA CEBPA-AS1, long non-coding RNA CEBPA-AS1; NCOR2, nuclear receptor corepressors 2.
**Supplementary Materials and Methods**

**Recombinant plasmid construction**

The complete nucleotide sequences of lncRNA CEBPA-AS1 (NR_026887) and NCOR2 (Gene ID: 9612) were identified by the National Center for Biotechnology Information (NCBI), and their gRNA and gDNA were designed and synthesized by Beijing Syngentech Co., Ltd. (Beijing, China) (the gRNA sequence of lncRNA CEBPA-AS1: GCCGTTTGGGACCGGCGGAGNGG; the gRNA sequence of NCOR2: GGCGATGGGGGTGCTGGTGGNGG). The Kpn1 and EcoRI digestion sites were added to both ends of the lncRNA CEBPA-AS1 gDNA and NCOR2 for linkage with the double-digested vector of LentiCRISOR-v2. After the recombinant plasmid had been transferred into the DH5α cells, the positive clone was screened out after which the plasmid was extracted: LentiCRISOR-v2-CEBPA-AS1 and LentiCRISOR-v2-NCOR2.

**Determination of gRNA viability**

After 24 h of transfection, the DNA in the siLncRNA CEBPA-AS1, siNCOR2 and empty vector groups were extracted using a DNA extraction kit (DP304, TIANGEN Biotechnology Co. Ltd, Beijing, China). The primers (Supplementary Table 2) along the target of lncRNA CEBPA-AS1 and NCOR2 on the gRNA designed by Primer 5.0 were synthesized at Beijing Genomics Institute (BGI) (Shenzhen, China). After approximately 500 bp segments had been produced by polymerase chain reaction (PCR) amplification, the T7E1 endonuclease reagent kit (v-solid03, Viewsolid Biotech Co., Ltd., Beijing, China) was employed at 37°C for 30 min. The reaction conditions were as follows: 5 μL PCR product; 1.1 μL 10 × T7E1 Buffer; 4.4 μL ddH2O. The digestion results were subsequently evaluated and analyzed by 2% agarose gel electrophoresis (AGE). The mutation rate was calculated based on the following formula: \[ \text{indel (\%)} = 100 \times (1 - f_{\text{cut}}), \] \[ f_{\text{cut}} = \text{sum of the digested band intensity/sum of the total band intensity}. \]

**Fluorescence in situ hybridization (FISH)**
The locations of lncRNA CEBPA-AS1 and NCOR2 were detected based on the instructions provided by the FISH Tag™ RNA Red Kit (F32952, Invitrogen, Carlsbad, CA, USA) and FISH Tag™ DNA Green Kit (F32954, Invitrogen, Carlsbad, USA). The MG63 cells were then rinsed with PBS, subjected to KCl hypotonic treatment, and then fixed in PBS (pH 7.4, containing 3.6% methanal and 10% acetic acid) for 15 min. The cells were permeabilized using 0.2% - 0.5% Triton X-100 and 5 mm vanadyl ribonucleotide in PBS for 5 min. Next, the cells were rinsed twice with 2 × sodium citrate buffer solution (SCC) and subsequently washed with PBS, followed by dehydration and drying. The cells were hybridized with the corresponding fluorescence-labeled DNA probe overnight at 37℃. The cell nuclei were stained with 4′-6-diamidino-2-phenylindole (DAPI) under conditions void of light for 15 min. All slides were analyzed under a fluorescence microscope (DYF-85, Shanghai dianying optical Instrument Co., Ltd., Shanghai, China).

Propidium iodide (PI) single staining
After removal of the culture medium, the MG63 and G292 cells were resuspended in ethyl alcohol [containing 30 g/L bovine serum albumin (BSA)] at 4℃, fixed for 24 h, and centrifuged with supernatant aspirated. After two PBS washes, the cells were incubated with 1 mL of RNase (50 mg/L) at room temperature under conditions void of light for 30 min. Next, 1 mL of PI (60 mg/L) was added and incubated under conditions void of light for 30 min. Cell cycle distribution was detected using a flow cytometer.

Xenograft tumors in nude mice
A total of 80 Balb/c nude mice (with no significant difference in terms of age or weight) were randomly assigned to the blank (without any treatment), empty vector (transfected with empty vector), siLncRNA CEBPA-AS1 (transfected with LentiCRISOR-v2-CEBPA-AS1 and Cas9 plasmids), siNCOR2 (transfected with LentiCRISOR-v2-NCOR2 and Cas9 plasmids) and siLncRNA CEBPA-AS1 + siNCOR2 groups (8 mice per group, half male and half female). Next, the MG63 cells were detached with trypsin to generate a cell suspension with a density of $3 \times 10^7$
cells/mL. After the local skin of the nude mouse had been disinfected, each mouse was subjected to subcutaneous injection with 0.5 mL cell suspension in the upper thigh region. During this process, a syringe needle was subcutaneously moved a certain distance, and subsequently pushed and pulled out in a slow manner, followed by the application of a slight degree of local pressure for 30 s to prevent leakage of the cell suspension. The generation condition and local inoculation changes were analyzed and recorded. After 4 weeks, the tumor volume was measured and calculated, with a growth curve plotted accordingly. Finally, the nude mice were euthanized under anesthesia, after which the formed tumors were excised and their weights were measured.