Article

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# *E2F1* Silencing Enhances Cisplatin-induced Apoptosis and DNA Damage in Retinoblastoma: *CENPE* Overexpression as a Compensatory Mechanism

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**Background: The oncogenic gene E2F transcription factor 1 (*E2F1*) plays a key role in tumors. This study was designed to explore the effect of *E2F1* gene expression manipulation and cisplatin on retinoblastoma cells and delineate the underlying mechanism.**
**Methods: *E2F1* expression in retinoblastoma cells was determined using quantitative real-time polymerase chain reaction (qRT-PCR). After *E2F1* knockdown, cisplatin-treated cells presenting a malignant phenotype were identified through several methods, such as MTT assay, determination of half-maximal inhibitory concentration ($IC_{50}$) values, flow cytometry, and comet assay. The effects of *E2F1* silencing with/without cisplatin on downstream target genes were explored using qRT-PCR and Western blotting. To elucidate the mechanism involving *E2F1* and centromere protein E (*CENPE*) in retinoblastoma, rescue experiments were conducted using cells overexpressing *CENPE*.**
**Results: *E2F1* was highly expressed in retinoblastoma cells ($p < 0.05$). *E2F1* silencing improved the effectiveness of cisplatin in suppressing viability, as well as promoting apoptosis, increasing G1 phase and DNA damage of retinoblastoma cells ($p < 0.05$). Upregulation of *CENPE* expression partially reversed the effects of *E2F1* silencing on cisplatin-treated cells ($p < 0.05$).**
**Conclusion: *E2F1* silencing enhances the effect of cisplatin in retinoblastoma by inhibiting *CENPE*.**

**Keywords:** E2F transcription factor 1; cisplatin; sensitivity; retinoblastoma

## Introduction

Originating from the embryonic retinal nerve tissue, retinoblastoma is a congenital intraocular malignant tumor leading to retinal detachment, necrosis and invasion of the optic nerve system, characterized by a low incidence but predominant occurrence during the infancy stage [1,2]. It has been reported that 60% of retinoblastoma patients with an average age of 24 months have bilateral multifocal disease and 40% with an average age of 15 months have single focal disease [3]. Owing to the complexity of retinoblastoma treatment, a multitude of factors, such as tumor size, patient age, tumor metastasis risk, chemotherapy history, drug toxicity, and tumor type, should be taken into consideration during clinical decision-making [4].

Since the 1970s, platinum drugs cisplatin, carboplatin and oxaliplatin have been widely used as chemotherapeutic agents in cancer treatment [5]. Combination chemotherapy based on cisplatin has shown significant anti-tumor activity in a variety of cancers, including ovarian, cervical and lung cancers, but secondary resistance of tumor cells to cisplatin can have a serious impact on clinical outcomes and prognosis [6]. Surgery coupled with chemotherapy serves as the primary treatment strategy for retinoblastoma, but the consequent drug resistance developed after this therapeutic approach often leads to poor prognosis, resulting in tumor relapse in around 45% of children [7–9]. Besides, the mechanism behind the development of cisplatin resistance in retinoblastoma remains incompletely deciphered due to its relatively low incidence compared to other tumors. Therefore, new or adjacent retinoblastoma therapy strategies are needed.

Currently, there is an emerging picture of the etiologies and mechanisms underlying the development of retinoblastoma, which are plausibly linked with mutations in genes and oncogenes [10]. Research into the pathogenesis and genetics of the disease are imperative to improve patients' survival. E2F transcription factor 1 (*E2F1*) belongs to the E2F family that plays key and extensive roles in many processes of the cell cycle, including DNA repair, DNA replication, cell differentiation, proliferation and apoptosis [11,12]. *E2F1* is highly expressed in many tumors and plays instrumental roles in the development and progression of tumors [13–15]. Previous findings have laid the groundwork for *E2F1* as a novel anti-tumor therapeutic target. Moreover, previous study showed that *E2F1* induced neuroblastoma cell migration and invasion via activation of centromere protein E (*CENPE*) [16]. *CENPE* is involved in regulating the development of various cancers (medulloblastoma, cervical cancer, lung adenocarcinoma) [17–

19]. This finding points to an interaction relationship between *E2F1* and *CENPE*. Nevertheless, how *E2F1*/*CENPE* influences retinoblastoma cells has rarely been reported.

Over the past few decades, molecular biology studies have revealed the anti-cancer role of cisplatin is closely associated with the inhibition of DNA replication and RNA transcription, which arrests cancer cells at G2 phase and promotes apoptosis [20–22]. Therefore, in the current study, we investigated the effect of *E2F1* combined with cisplatin on retinoblastoma cells, and explored the underlying mechanisms, in order to find potential therapies.

## Methods

### *Cell Culture and Cisplatin Treatment*

The normal retinal pigmented epithelium cell line ARPE-19 (CRL-2302) and two retinoblastoma cell lines WERI-RB1 (HTB-169) and Y79 (HTB-18) were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). Retinoblastoma cell lines HXO-RB44 and SO-RB50 were provided by the Xiangya Medical College of Central South University (Changsha, China) and the Department of Pathology of the Zhongshan Ophthalmic Center, Sun Yat-sen University (Guangzhou, China), respectively. All cell lines were maintained in RPMI-1640 medium (R7388, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; 30-2020, ATCC, Rockville, MD, USA) and incubated in a 5% $CO_2$-infused incubator set at 37 °C (E0467, Beyotime, Shanghai, China). The cells were harvested upon reaching over 80% confluence. All cell lines were authenticated by short tandem repeat (STR) analysis and were negative for mycoplasma growth.

In drug treatment experiment, cisplatin (D8810, Solarbio, Beijing, China) was dissolved in the solution of N, N-dimethylformamide (DMF-DMA; D4670, Solarbio, Beijing, China). Based on a previous report, WERI-RB1 or Y79 cells were treated with cisplatin at concentrations of 0, 1, 2, 3 and 5 µg/mL for 48 h [23,24].

### *Cell Transfection*

The *E2F1* siRNA (5′-AGAUGGUUAUGGUGAUCAATT-3′) and its negative control siRNA (5′-UUCUCCGAACGUGUCACGUTT-3′) were synthesized by Sangon Biotech company (Shanghai, China), and the pCMV6-Entry (PS100001) vector for *CENPE* overexpression (RC224375, **Supplementary File 1**) was constructed by OriGene company (Rockville, MD, USA). Upon reaching 70–90% confluence, WERI-RB1 or Y79 cells seeded on a 24-well plate were transfected with vectors at 37 °C for a duration of 48 h in the presence of Lipo2000 Transfection Reagent (GK20005, GlpBio, Montclair, CA, USA). Next, transfection efficiency was estimated by means of quantitative real-time polymerase chain reaction (qRT-PCR).

### *qRT-PCR*

Total RNA was isolated from the cells using TRI Reagent (93289, Sigma-Aldrich, St. Louis, MO, USA), and then reverse-transcribed to cDNA with RevertAid First Strand (K1622, Thermo Fermentas, Waltham, MA, USA). Subsequently, the cDNA samples were utilized in a qRT-PCR experiment conducted using a real-time PCR system (7500, Thermo Fisher, Waltham, MA, USA) with SYBR Green qPCR Master Mix (K1070, Apexbio, Houston, TX, USA). Quantitative results were analyzed using the $2^{-\Delta\Delta Ct}$ method [25]. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as the reference gene. Table 1 shows the sequences of primers used in the gene expression study.

**Table 1. Primers used in qRT-PCR.**

| Gene (Human) | Sequence (5′ - 3′) |
|---|---|
| *E2F1* F | ACCAGGGTTTCCAGAGATGC |
| *E2F1* R | CACCACACAGACTCCTTCCC |
| *CENPE* F | GATTCTGCCATACAAGGCTACAA |
| *CENPE* R | TGCCCTGGGTATAACTCCCAA |
| *SMC2* F | TCTCAGGTTCGGGCTTCTAAT |
| *SMC2* R | CCTGTACTCTGGTGTTGTTGG |
| *SMC4* F | CGCCTCCAGCAATGACCAAT |
| *SMC4* R | CCCCAGCATAGGATTTGAAGTT |
| *MCM4* F | GACGTAGAGGCGAGGATTCC |
| *MCM4* R | GCTGGGAGTGCCGTATGTC |
| *GAPDH* F | AGAAGGCTGGGGCTCATTTG |
| *GAPDH* R | AGGGGCCATCCACAGTCTTC |

Abbreviations: qRT-PCR, quantitative real-time polymerase chain reaction; F, forward; R, reverse; *E2F1*, E2F transcription factor 1; *CENPE*, centromere protein E; *SMC2*, structural maintenance of chromosomes 2; *SMC4*, structural maintenance of chromosomes 4; *MCM4*, minichromosome maintenance complex component 4; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

### *Western Blotting*

After cisplatin treatment or transfection, total proteins of the WERI-RB1 and Y79 cells were isolated with RIPA buffer (R0010, Solarbio, Beijing, China), and their concentrations were then detected using bicinchoninic acid (BCA) Protein Assay Kit (PC0020, Solarbio, Beijing, China). After running 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the separated protein bands were transferred from the gels to the polyvinylidene fluoride (PVDF) membranes (IPVH00010, Millipore, Billerica, MA, USA). The membranes were then blocked by immersing them in blocking buffer (SW3010, Solarbio, Beijing, China) for 1 h at room temperature. After blocking, the membranes were incubated with diluted primary antibody solutions (Table 2) at 4 °C overnight. On the next day, the membranes were washed with phosphate-buffered

**Table 2. Antibodies for Western blotting.**

| Antibody | Catalog no. | Molecular weight (kDa) | Dilution factor | Manufacturer |
|---|---|---|---|---|
| E2F1 | ab4070 | 47 | 1/500 | abcam (UK) |
| Anti-γH2AX | ab26350 | 16 | 1/1000 | abcam (UK) |
| Anti-CENPE | ab5093 | 312 | 1/1000 | abcam (UK) |
| Anti-GAPDH | ab8245 | 36 | 1/1000 | abcam (UK) |
| Goat anti-mouse | ab205719 | — | 1/2000 | abcam (UK) |

γH2AX, histone H2AX on serine 139.

saline (PBS; C0221A, Beyotime, Shanghai, China), prior to incubation with secondary antibody solution (Table 2) for 1 h at room temperature. Following blot visualization by an enhanced chemiluminescence (ECL) system (EI600, Beyotime, Shanghai, China) with ECL kit (P0018M, Beyotime, Shanghai, China), measurement of relative protein levels was conducted utilizing the ImageJ software (version 1.8.0, National Institutes of Health, Bethesda, MD, USA).

### *MTT Assay and Determination of Half-maximal Inhibitory Concentration*

We applied MTT (3-[4, 5-Dimethylthylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide) assay to evaluate the viability of transfected and cisplatin-treated cells. Briefly, the transfected cells ($1 \times 10^4$/well) in a 96-well plate were separately incubated with 0, 1, 2, 3 and 5 µg/mL cisplatin for 48 h under the incubation conditions of 37 °C and 5% $CO_2$. Afterwards, 10 µL MTT reagent (M1020, Solarbio, Beijing, China) was added to the cells for a 4-h incubation. Formazan solution was added before determining cell viability by measuring absorbance at the wavelength of 490 nm using a microplate reader (MR-96A, Mindray, Shenzhen, China).

Based upon the cell viability data obtained from the MTT assays, we determined the half-maximal inhibitory concentration ($IC_{50}$) of cisplatin in tumor cells by performing the Probit regression analysis in the Statistical Product and Service Solutions (SPSS) software (version 18.0, International Business Machines Corporation, Armonk, NY, USA).

### *Comet Assay*

Subsequently, comet assay kit (C2041S, Beyotime, Shanghai, China) was employed to detect DNA damage of transfected and cisplatin-treated cells. The principle of comet assay in assessing the severity of DNA damage lies in the detection of amount and size of DNA fragments following cell damage and the subsequent DNA fragmentation; as a general rule of thumb, the more severe a cell's DNA damage is, the more and smaller the DNA fragments are. Through visualization of electrophoresis results in this assay, the DNA fragments form a tail known as 'comet'.

At the beginning of the experiment, the cell density was adjusted to $2 \times 10^5$ cells/mL. Normal melting-point agarose gel (100 µL) was spread on glass slides and cured at 4 °C for 15 min. A cell suspension in low-melting-point agarose was dropped onto slides precoated with 1% normal-melting-point agarose. The coverslip was removed, and the cells were lysed in pre-cooled cell lysate at 4 °C for 1.5 h. After pouring off the supernatant, the electrophoresis buffer was poured in and left for half an hour. Next, electrophoresis was run for 20 min at 25 V and 300 mA. Then, after electrophoresis, place the glass slides in a Petri dish, add neutral buffer solution to submerge the slides, and incubate at 4 °C for neutralization 1–3 times, each time for 5–10 min. Discard the neutral buffer solution afterward. Finally, upon addition of 20 µL propidium iodide solution, the slide was covered, and observed and imaged under a microscope (CX23, Olympus, Tokyo, Japan).

### *Flow Cytometry Assay*

Flow cytometry assay was performed to evaluate the apoptosis of WERI-RB1 or Y79 cells. After transfection, the cells were suspended, seeded in 24-well plate ($1 \times 10^6$/well), and exposed to 3 µg/mL cisplatin for 48 h (37 °C, 5% $CO_2$). Following a 5-min centrifugation, the cells were rinsed with PBS and dyed using Annexin V-FITC Apoptosis Detection Kit (CA1020, Solarbio, Beijing, China) at room temperature in the dark. After the staining process, the cells were washed with PBS again, prior to observation and analysis using a flow cytometer (CytoFLEX, Beckman, Beverly, MA, USA) within 1 h.

### *Cell Cycle Analysis*

Cell cycle analysis was performed using a cell cycle kit (C1052, Beyotime, Shanghai, China). The suspended cells were fixed in ice-cold 70% ethanol at 4 °C for 2 h. After ethanol removal by centrifugation, cells were stained with propidium iodide stain solution in the dark for 30 min at room temperature. Flow cytometry (CytoFLEX, Beckman, Beverly, MA, USA) was performed to evaluate the fluorescence at 488 nm excitation wavelength.

### *ChIP Assay*

ABclonal's ChIP Kit (RK20258, ABclonal, Wuhan, China) was employed to perform ChIP assay. The cells ($2 \times 10^7$) were mixed with 1% formaldehyde in an ice bath for 15 min. Cells were then resolubilized in the cell swelling buffer (1×)+PIC prior to centrifugation (5000 ×*g*, 5 min) to precipitate nucleus. Cells were then sonicated and centrifuged (12,000 ×*g*, 10 min) to obtain crosslinked chro-

matin samples, which were then reacted with anti-E2F1 (1:100, #3742, CST) and anti-rabbit IgG (1:1000, #7074, CST). The reaction solution was added into Protein A/G Magnetic Beads (HY-K0202, MedChemExpress, Shanghai, China) and incubated for 2 h. Chromatin samples were obtained by elution and purified by DNA purification kit (RK30100, ABclonal, Wuhan, China). The results of ChIP experiment were quantitatively analyzed by means of qPCR. Primers for the promoter of *CENPE* are as follows: (forward) 5′-GGAAAATGGCCAGGACCCTC-3′; (reverse) 5′-CCCCTCTCCTGTTTAGCAGT-3′.

### *Statistical Analysis*

Biological triplicates were performed for all experiments. The data were analyzed using GraphPad Prism software (version 8.0, GraphPad, San Diego, CA, USA) and are expressed as mean ± standard deviation in this paper. Data were compared between the two groups using independent sample *t*-test. For comparisons among multiple groups, one-way analysis of variance (ANOVA) followed by a Tukey's test was used. Results with $p < 0.05$ were regarded as statistically significant.

## Results

### *E2F1 Silencing Enhanced the Inhibitory Effect of Cisplatin on Retinoblastoma Cells Viability*

The qRT-PCR assay results showed that *E2F1* was highly expressed in retinoblastoma cells relative to APRE-19 cells ($p < 0.05$, Fig. 1A). A decline of *E2F1* expression, as shown in Fig. 1B–D, indicates that the transfection was successful ($p < 0.05$). Further, the viability of retinoblastoma cells (WERI-RB1 and Y79) was inhibited by cisplatin treatment at different concentration. Moreover, *E2F1* silencing enhanced the inhibitory effect of cisplatin ($p < 0.05$, Fig. 1E) and decreased the $IC_{50}$ value ($p < 0.05$, Fig. 1F).

Compared with its negative control, *E2F1* depletion could significantly exacerbate cell apoptosis (Fig. 2A–D, $p < 0.05$), an effect comparable to that of cisplatin (Fig. 2A–D, $p < 0.05$). Moreover, silencing *E2F1* amplified the apoptotic effect of cisplatin (Fig. 2A–D, $p < 0.05$). Then, the flow cytometry assay also demonstrated that the silencing of *E2F1* promoted the effect of cisplatin, marked by an increased number of cells in the G0/G1 phase and an increased peak value of the G0/G1 phase, which indicates the cells were being arrested in the G0/G1 phase ($p < 0.05$, Fig. 3A–D).

### *E2F1 Silencing Exacerbated Cisplatin-induced DNA Damage in Retinoblastoma Cells*

To further explore the underlying mechanisms, we surveyed the effects of *E2F1* downregulation on DNA damage. Comet assay showed that the cell tailing was obvious after *E2F1* downregulation or cisplatin treatment. The cell tailing became more obvious under the combined effects of *E2F1* downregulation and cisplatin treatment (Fig. 4A,B). Western blot results showed that the histone H2AX on serine 139 ($\gamma$H2AX) expression was increased after cisplatin treatment or *E2F1* siRNA transfection ($p < 0.05$, Fig. 5A–D). Collectively, the silencing of *E2F1* exacerbated cisplatin-induced DNA damage in retinoblastoma cells.

### *E2F1 Silencing Decreased the Expressions of its Downstream Target Genes, and Enhanced the Inhibitory Effect of Cisplatin on E2F1 and CENPE Expression*

Through qRT-PCR, we found that the expression of *E2F1*, *CENPE*, structural maintenance of chromosomes 2 (*SMC2*), structural maintenance of chromosomes 4 (*SMC4*) and minichromosome maintenance complex component 4 (*MCM4*) was reduced substantially after transfection of the cells with *E2F1* siRNA. Of these four genes, the decrease of *CENPE* expression stood as the most obvious (Fig. 6A,B, $p < 0.05$). The ChIP experiment verified the interaction between *E2F1* and *CENPE* (Fig. 6C). In addition, this in-depth research revealed that the suppressive effect of cisplatin on *CENPE* expression was substantially augmented by *E2F1* silencing (Fig. 6D–G, $p < 0.05$).

### *Overexpression of CENPE Partially Reversed the Effects of E2F1 siRNA on Cisplatin-treated Cells*

For elucidating the mechanism behind cisplatin sensitivity underpinned by *CENPE* and *E2F1*, we performed rescue experiments after transfecting the cells with *CENPE* overexpression vector. Considerable upregulation of *E2F1* was detected in the *CENPE* overexpression group (Fig. 7A, $p < 0.05$). An increase of *CENPE* expression was detected, as shown in Fig. 7B–D, indicating successful transfection ($p < 0.05$). The viability of cells treated with various concentrations of cisplatin improved by *CENPE* overexpression; meanwhile, *CENPE* overexpression also attenuated the suppressive impact of *E2F1* silencing on viability (Fig. 7E, $p < 0.05$). Besides, overexpressed *CENPE* led to an increase in the $IC_{50}$ values and reversed the effect of *E2F1* silencing on the $IC_{50}$ values (Fig. 7F, $p < 0.05$).

Relative to the cells transfected solely with *E2F1* siRNA, cells transfected with both *E2F1* siRNA and *CENPE* overexpression vectors presented a significant reduction in apoptosis rate and cell proportion in G1 phase (Figs. 8,9, $p < 0.05$). Meanwhile, *E2F1* silencing combined with cisplatin treatment caused remarkably increased cell apoptosis as well as cell proportion in G1 phase, which was partially reversed by *CENPE* overexpression (Figs. 8,9, $p < 0.05$).

Moreover, the comet assay showed that there was no obvious cell tailing after *CENPE* overexpression; however, *E2F1* silencing combined with cisplatin resulted in obvious cell tailing, which was partially reversed by *CENPE* overexpression (Fig. 10). Further, Western blot results showed

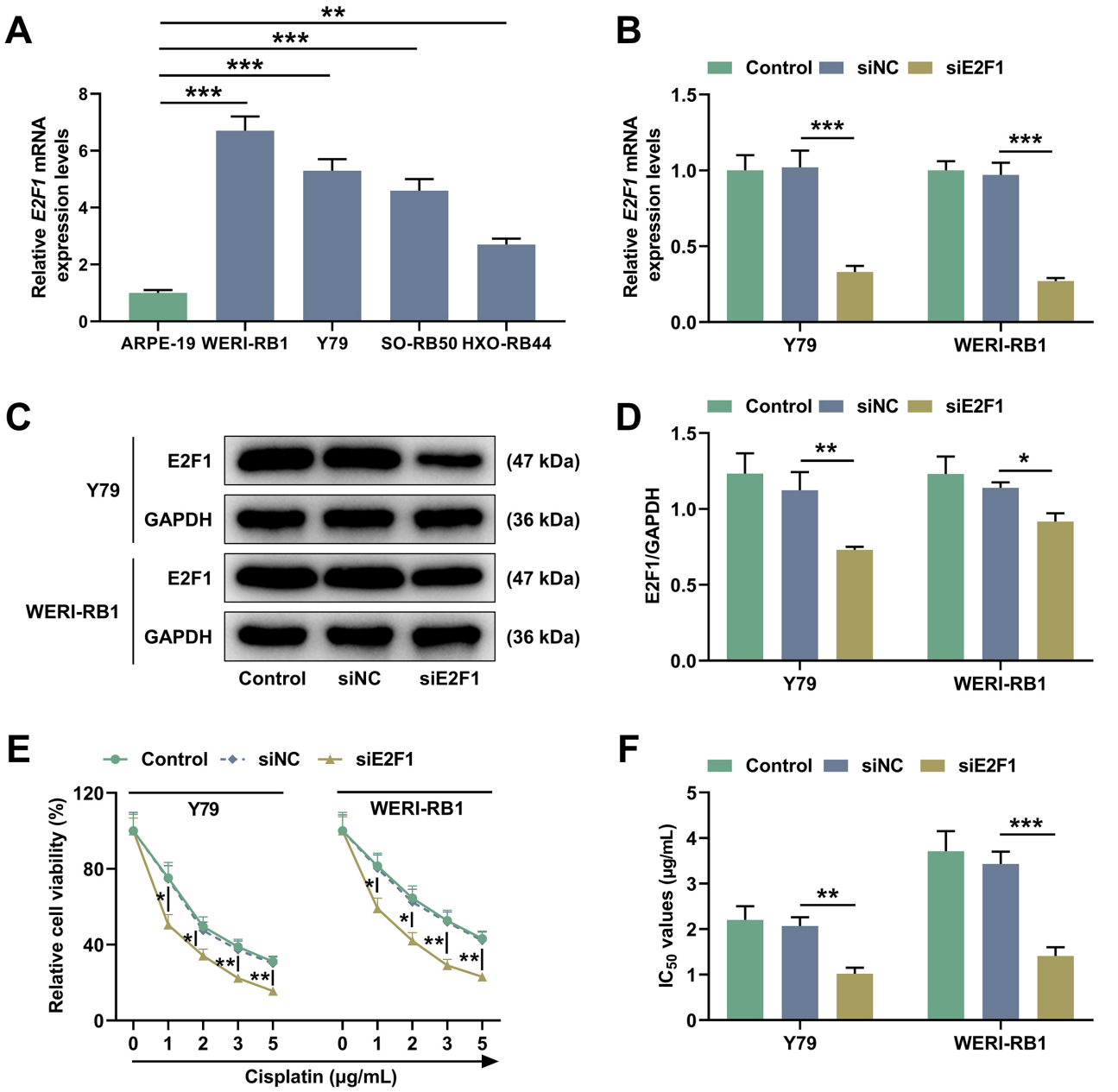


**Fig. 1. *E2F1* silencing enhanced the inhibitory effect of cisplatin on retinoblastoma cells viability.** (A) The mRNA expression of *E2F1* in APRE-19 cells and retinoblastoma cells was measured using qRT-PCR with *GAPDH* serving as the reference gene. (B–D) *E2F1* expression was detected by means of qRT-PCR and Western blotting after transfecting the WERI-RB1 and Y79 cells with *E2F1* siRNA (with GAPDH as the loading control). (E) Viability of cells exposed to different concentrations of cisplatin (measured using MTT assays). (F) $IC_{50}$ values of cisplatin to the tested cells (determined using Probit regression analysis). Biological triplicates were performed for these experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Abbreviations: *E2F1*, E2F transcription factor 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; $IC_{50}$, Half-maximal inhibitory concentration; qRT-PCR, quantitative real-time polymerase chain reaction; NC, negative control.

that *CENPE* overexpression downregulated the γH2AX expression, reversing the combined effect of *E2F1* silencing and cisplatin treatment (Fig. 11, $p < 0.05$).

## Discussion

In this study, we confirmed that *E2F1* silencing could augment the inhibitory effect of cisplatin on retinoblastoma cell by inhibiting *CENPE*. Further investigations unveiled that *E2F1* silencing potentiated the repressive impact of cis-

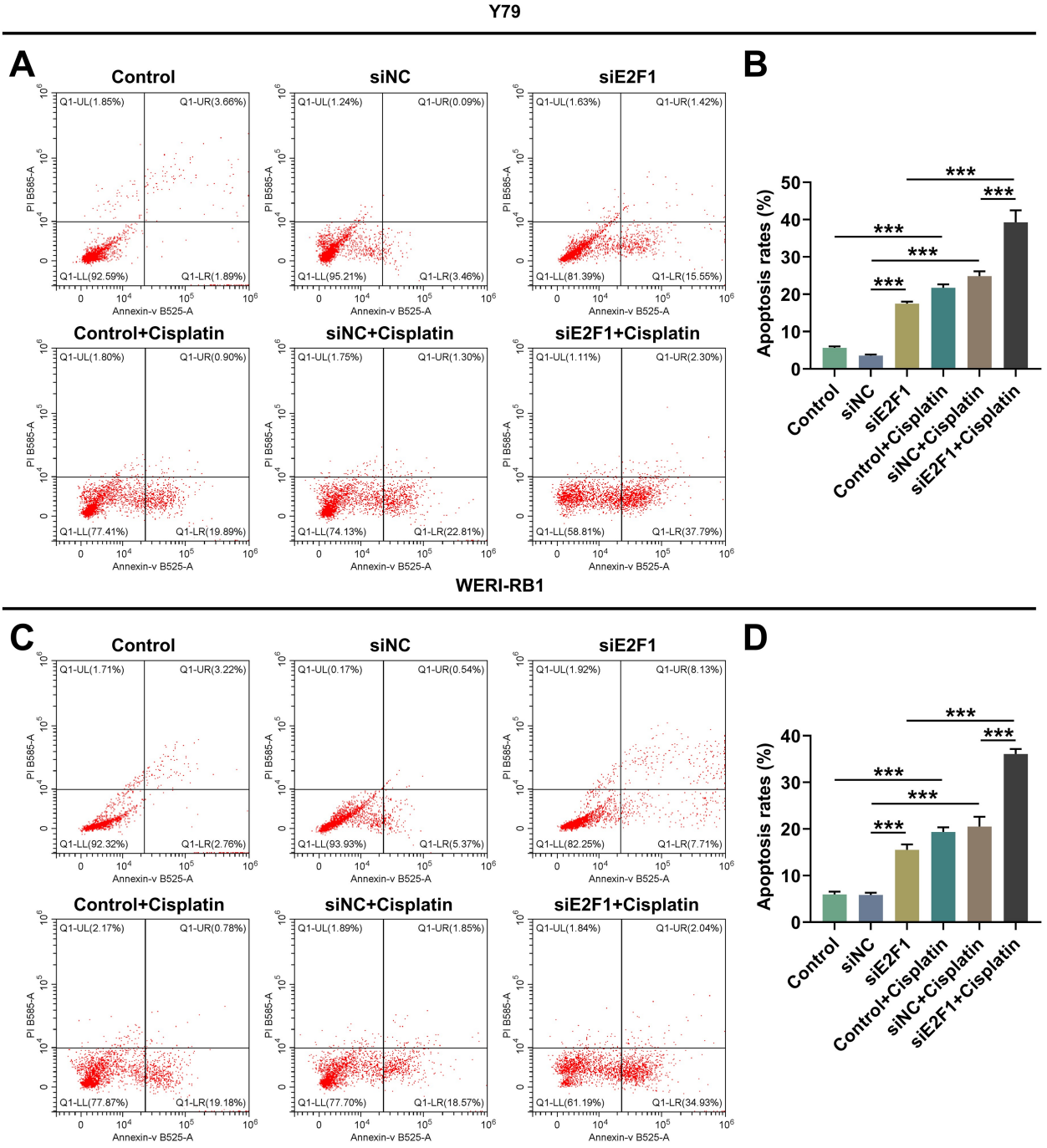


**Fig. 2. *E2F1* silencing strengthened the apoptotic effect of cisplatin.** Transfection of *E2F1* siRNA into the WERI-RB1 and Y79 cells was conducted. (A–D) Representative images of flow cytometry assay of Y79 (A) and WERI-RB1 cells (C) with quantification results (B,D). Biological triplicates were performed for these experiments. $^{***}p < 0.001$. Abbreviations: *E2F1*, E2F transcription factor 1; NC, negative control.

platin on viability, migration and invasion, as well as promoted apoptosis of retinoblastoma cells. More importantly, the influence of *E2F1* on retinoblastoma was associated with inhibition of *CENPE*-mediated DNA damage.

As a broad-spectrum, highly effective anti-cancer drug, cisplatin is applied in the first-line treatment of tumors including retinoblastoma, lung cancer and ovarian cancer [26]. Nonetheless, most patients are initially sensitive to cisplatin, but eventually develop secondary resistance to the drug, which constitutes a major obstacle for cancer treatment [27]. Therefore, the increasing resistance of tumors to cisplatin warrants enhancing the drug's efficacy and improving the disease prognosis.

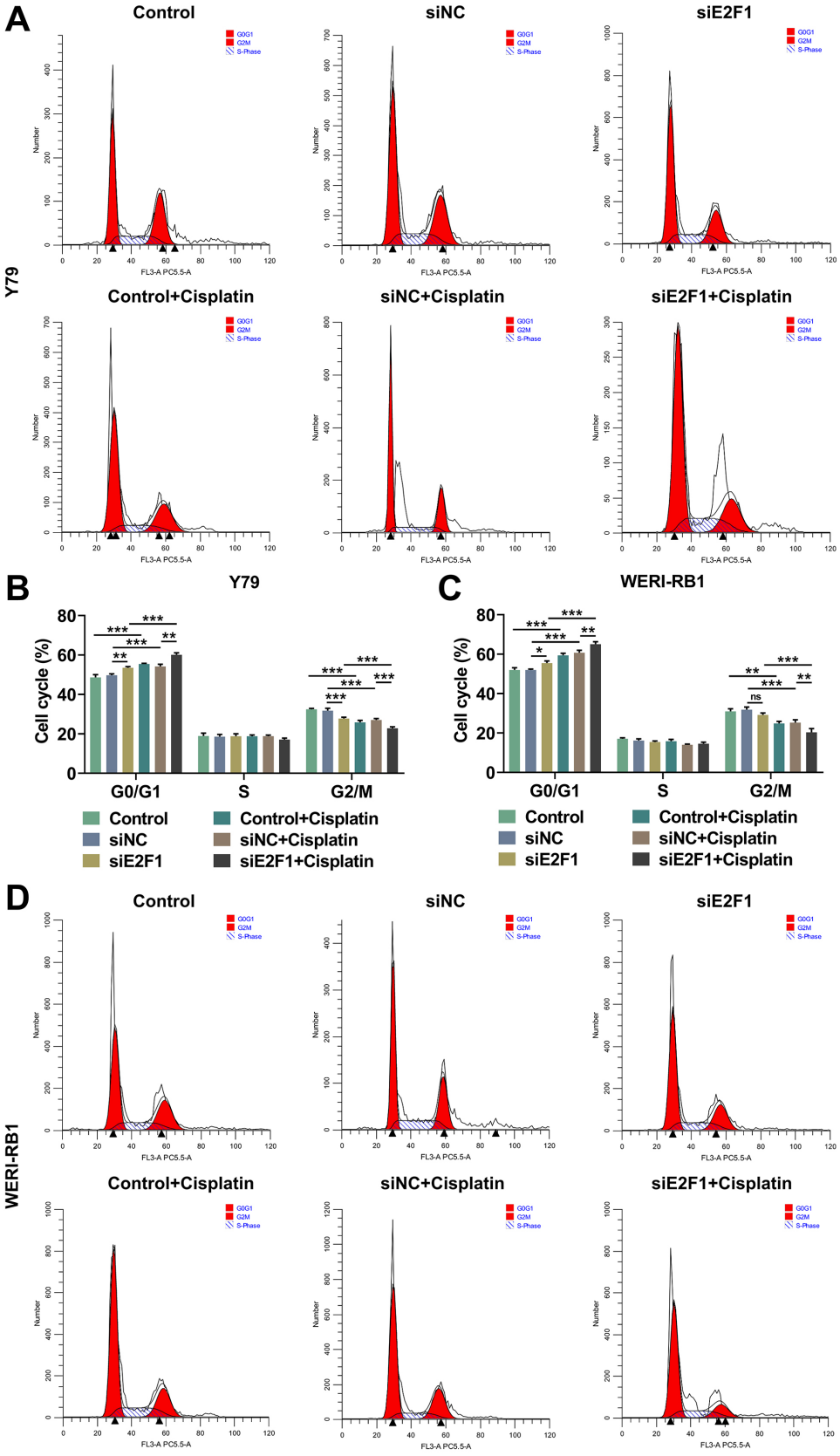


**Fig. 3. *E2F1* silencing augmented the effect of cisplatin on cell cycle.** Cell cycle was analyzed using flow cytometry after *E2F1* silencing and/or cisplatin treatment. (A–D) Representative images of flow cytometry assay of Y79 (A) and WERI-RB1 cells (D) with quantification results (B,C). Biological triplicates were performed for these experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; ns, not significant. Abbreviations: *E2F1*, E2F transcription factor 1; NC, negative control.

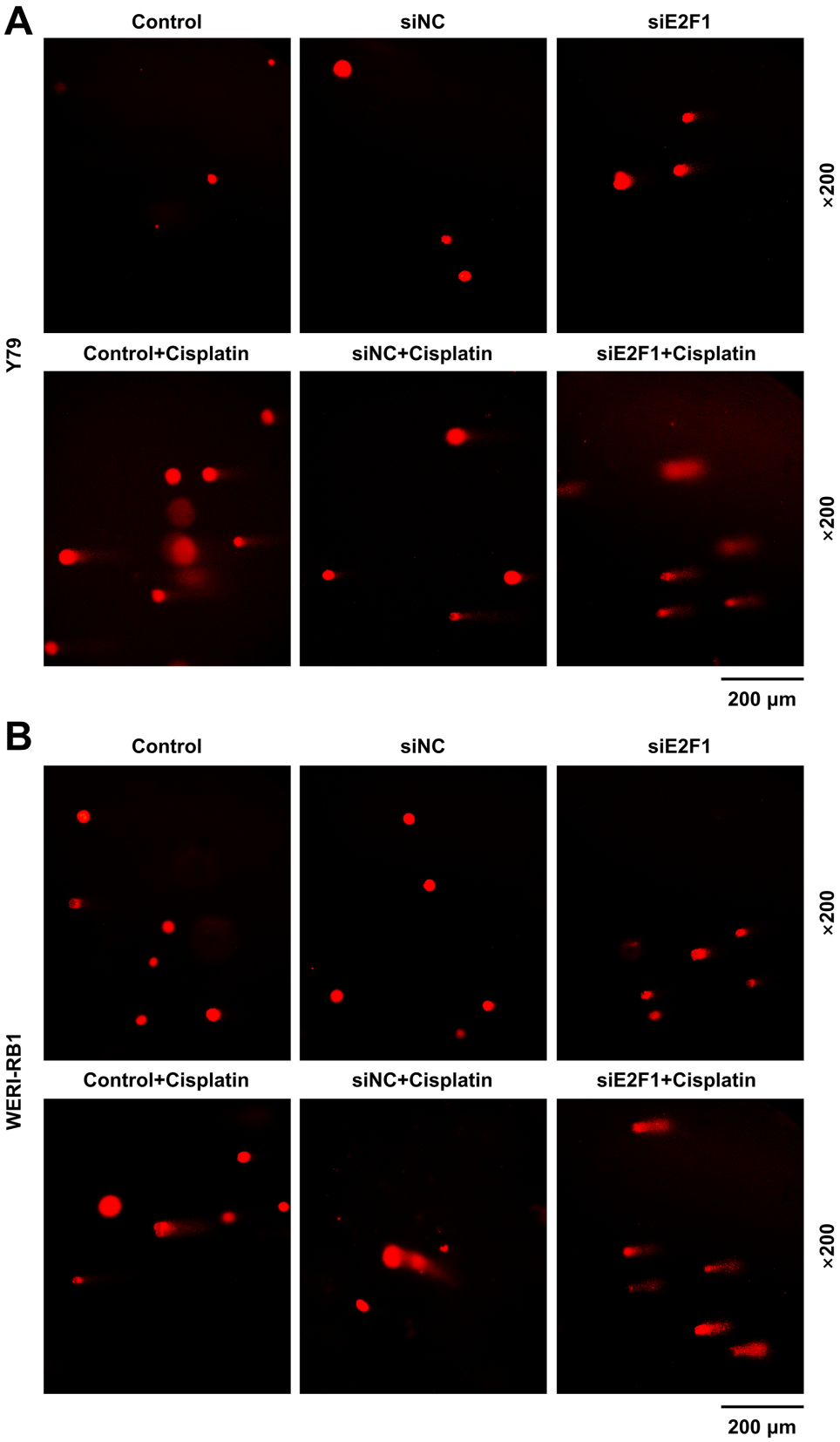


**Fig. 4. *E2F1* silencing exacerbated cisplatin-induced DNA damage in retinoblastoma cells.** DNA damage in Y79 (A) and WERI-RB1 cells (B) was detected by means of comet assay after *E2F1* silencing and/or cisplatin treatment. Abbreviations: *E2F1*, E2F transcription factor 1; NC, negative control.

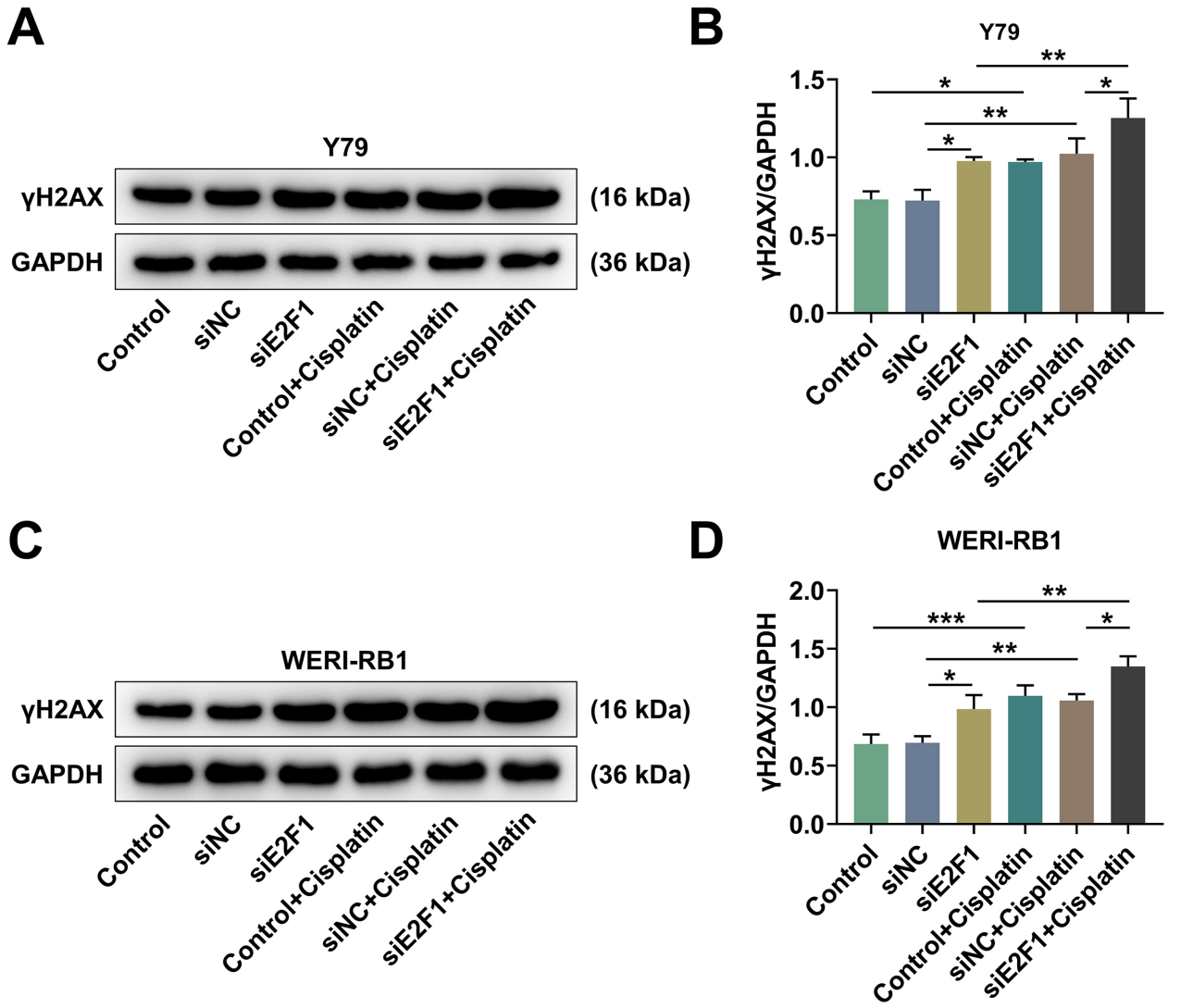


**Fig. 5. *E2F1* silencing enhanced cisplatin-induced γH2AX upregulation.** The protein expression of γH2AX in Y79 (A,B) and WERI-RB1 cells (C,D) was detected by means of Western blotting after *E2F1* silencing and/or cisplatin treatment. Biological triplicates were performed for these experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Abbreviations: *E2F1*, E2F transcription factor 1; γH2AX, histone H2AX on serine 139; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NC, negative control.

Reportedly, *E2F1* is highly expressed in many types of tumor tissues and cells and acts as a cancer-promoting gene; however, in a small number of tumors, these attributes or properties are reversed [28]. It has been found that *E2F1* expression was abnormally upregulated in retinoblastoma cells and involved in disease progression [29], which coinciding with our results. Further, most retinoblastoma patients carry mutations in the retinoblastoma protein 1 gene (*RB1*), and the inactivation of RB1 increases E2F expression, leading to uncontrolled cell proliferation [30]. Moreover, *E2F1* activates transcription of cell cycle-related kinases [31]. A previous study has shown that ERINA promotes cell cycle progression by interacting with E2F1, thereby preventing E2F1 from binding to the tumor suppressor RB1 [32]. Therefore, to investigate its underlying mechanism, we silenced the *E2F1* expression in tumor cells in the present study. By studying malignant phenotypes, we demonstrated that *E2F1* silencing combined with cisplatin delivered higher effectiveness in suppressing cell viability and G1 phase, as well as promoting apoptosis, relative to treatment employing solely cisplatin.

Furthermore, Farra *et al.* [33] suggested that *E2F1*, which regulates the Yes-associated protein (YAP)-*E2F1* DNA damage response pathway, may be a critical target in ovarian cancer. Given that tumor cells are characterized by rapid proliferation, most chemotherapeutic drugs work by directly or indirectly inducing irreversible damage to the DNA strand containing genes that are frequently expressed during proliferation, leading to cytotoxicity and cell death [34]. Previous research suggested that long-term use

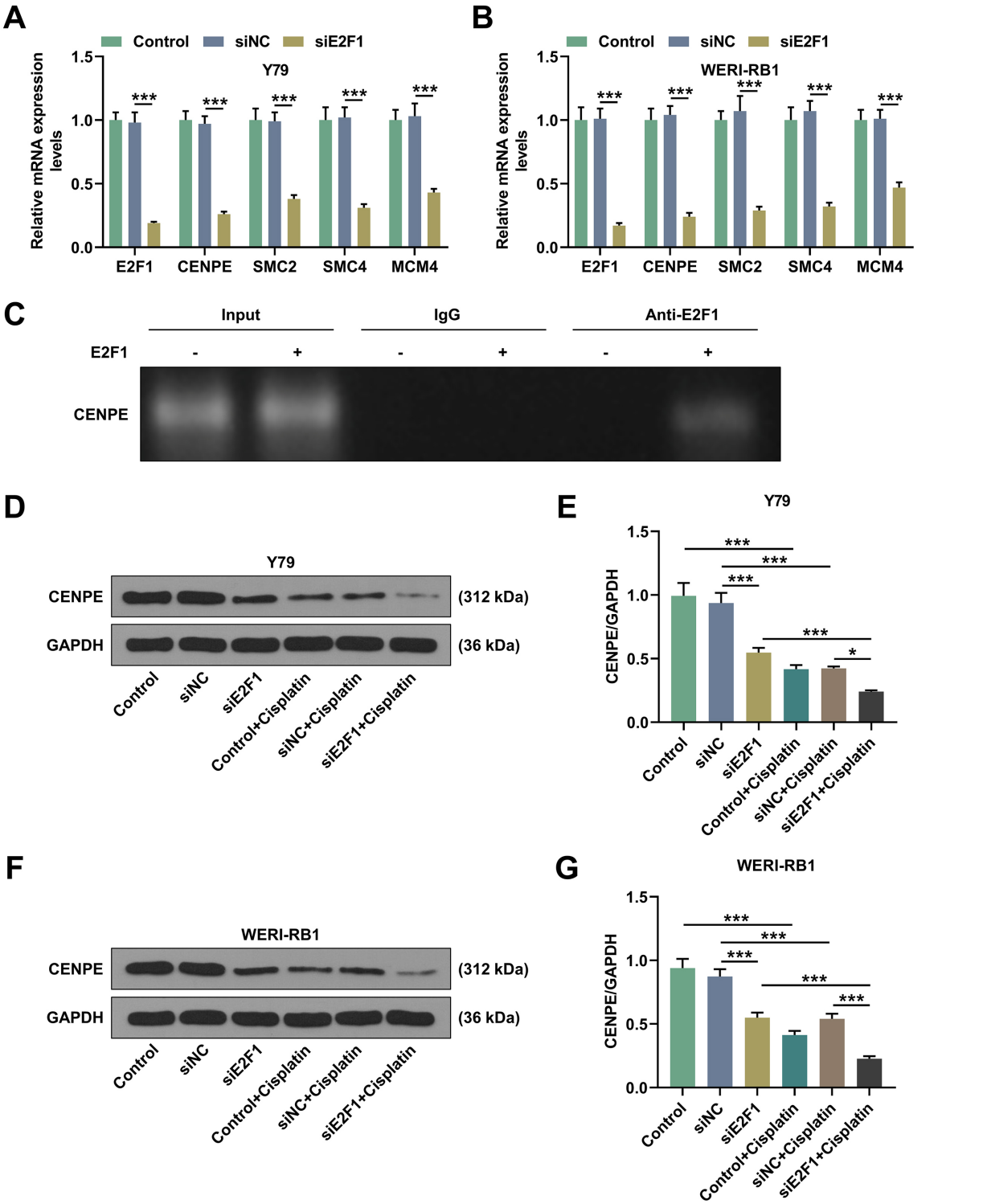


**Fig. 6. The reduced expression of *CENPE*, *SMC2*, *SMC4* and *MCM4* genes concomitant with enhanced inhibitory effect of cisplatin following the *E2F1* silencing.** Transfection of *E2F1* siRNA into WERI-RB1 and Y79 cells was completed. (A,B) The expression of *CENPE*, *SMC2*, *SMC4* and *MCM4* (determined using qRT-PCR, with *GAPDH* as reference gene). (C) The ChIP experiment verified the interaction between E2F1 and CENPE. (D–G) CENPE protein level following cisplatin treatment (measured using Western blotting, with GAPDH as reference control). Biological triplicates were performed for these experiments. $^{*}p < 0.05$, $^{***}p < 0.001$. Abbreviations: *CENPE*, centromere protein E; *E2F1*, E2F transcription factor 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; *MCM4*, minichromosome maintenance complex component 4; NC, negative control; qRT-PCR, quantitative real-time polymerase chain reaction; *SMC2*, structural maintenance of chromosomes 2; *SMC4*, structural maintenance of chromosomes 4.

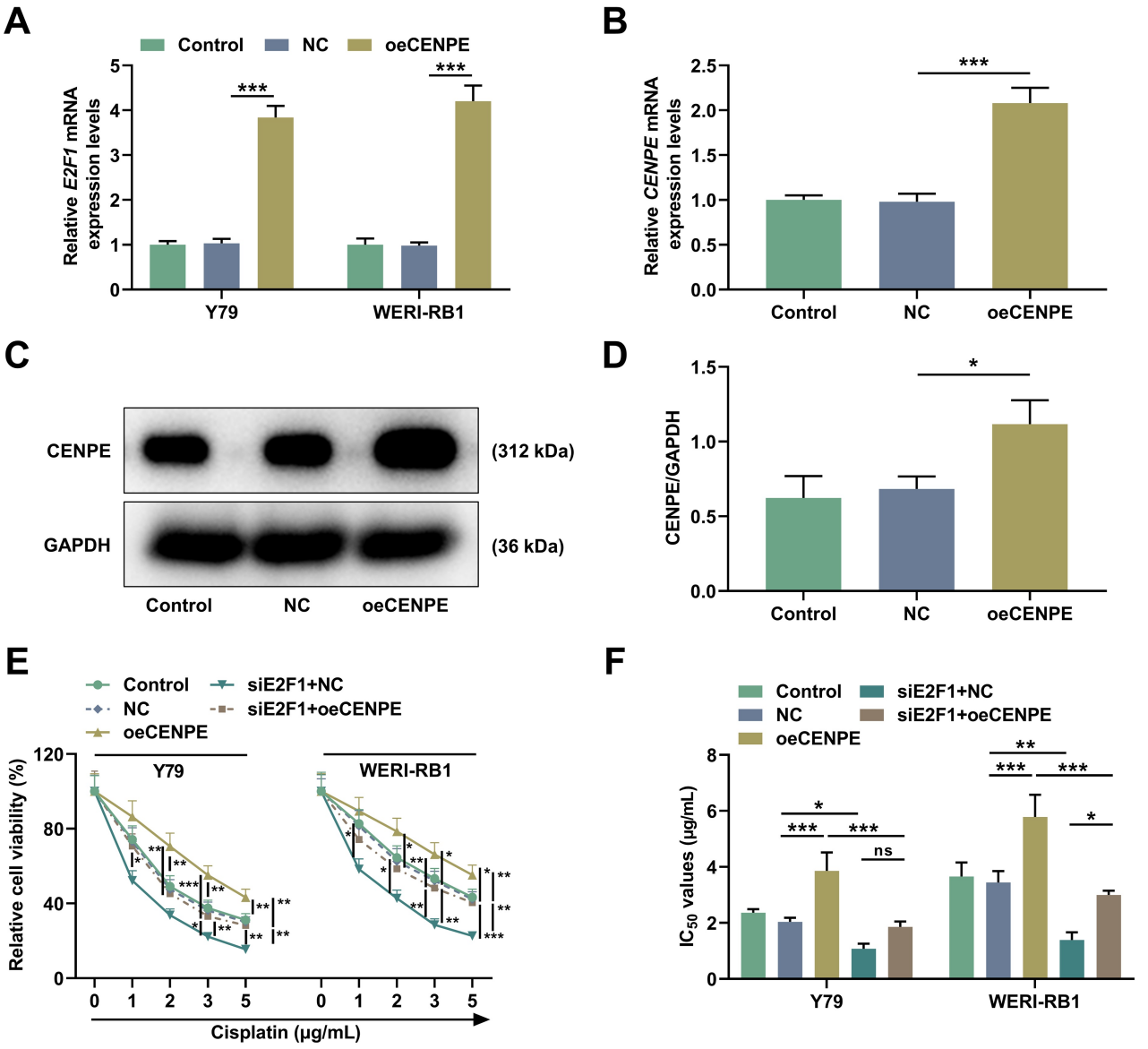


**Fig. 7. *CENPE* overexpression elevated *E2F1* expression and partially reversed the suppressive effect of *E2F1* siRNA on viability of cells treated with cisplatin.** Both the WERI-RB1 and Y79 cells were transfected *CENPE* overexpression vector. (A) *E2F1* expression (determined using qRT-PCR, with *GAPDH* as reference gene). (B–D) *CENPE* expression (assessed using qRT-PCR and western blot, with GAPDH as reference gene). (E) Cell viability at different concentrations of cisplatin (measured using MTT assays). (F) Half-maximal inhibitory concentration ($IC_{50}$) values of cisplatin to cells (measured using Probit regression analysis). Biological triplicates were performed for these experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; ns, not significant. Abbreviations: *CENPE*, centromere protein E; *E2F1*, E2F transcription factor 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NC, negative control; qRT-PCR, quantitative real-time polymerase chain reaction.

of cisplatin can lead to a decrease in the ability of tumor cells to repair DNA damage [35,36]. Moreover, with the occurrence of DNA double-strand breaks, H2AX histones located near the DNA breaks are rapidly phosphorylated to produce γH2AX, and its content is positively correlated with the degree of DNA damage. This study showed that silencing *E2F1*, in combination with cisplatin, can increase γH2AX expression and promote DNA damage.

Moreover, previous study showed that SMC2, SMC4, MCM4, *E2F1* and *CENPE* are all E2F target genes in retinoblastoma tumors [5]. The interactions of the SMC complex (such as SMC2-SMC4), or MCM4 in functions both related to the DNA damage [37,38]. The downregulation of SMC2 induces DNA damage, leading to apoptosis in human neuroblastoma cells [39]. It has been reported that *CENPE* depletion induces endogenous DNA damage accumulation [40]. Therefore, we further focused on the

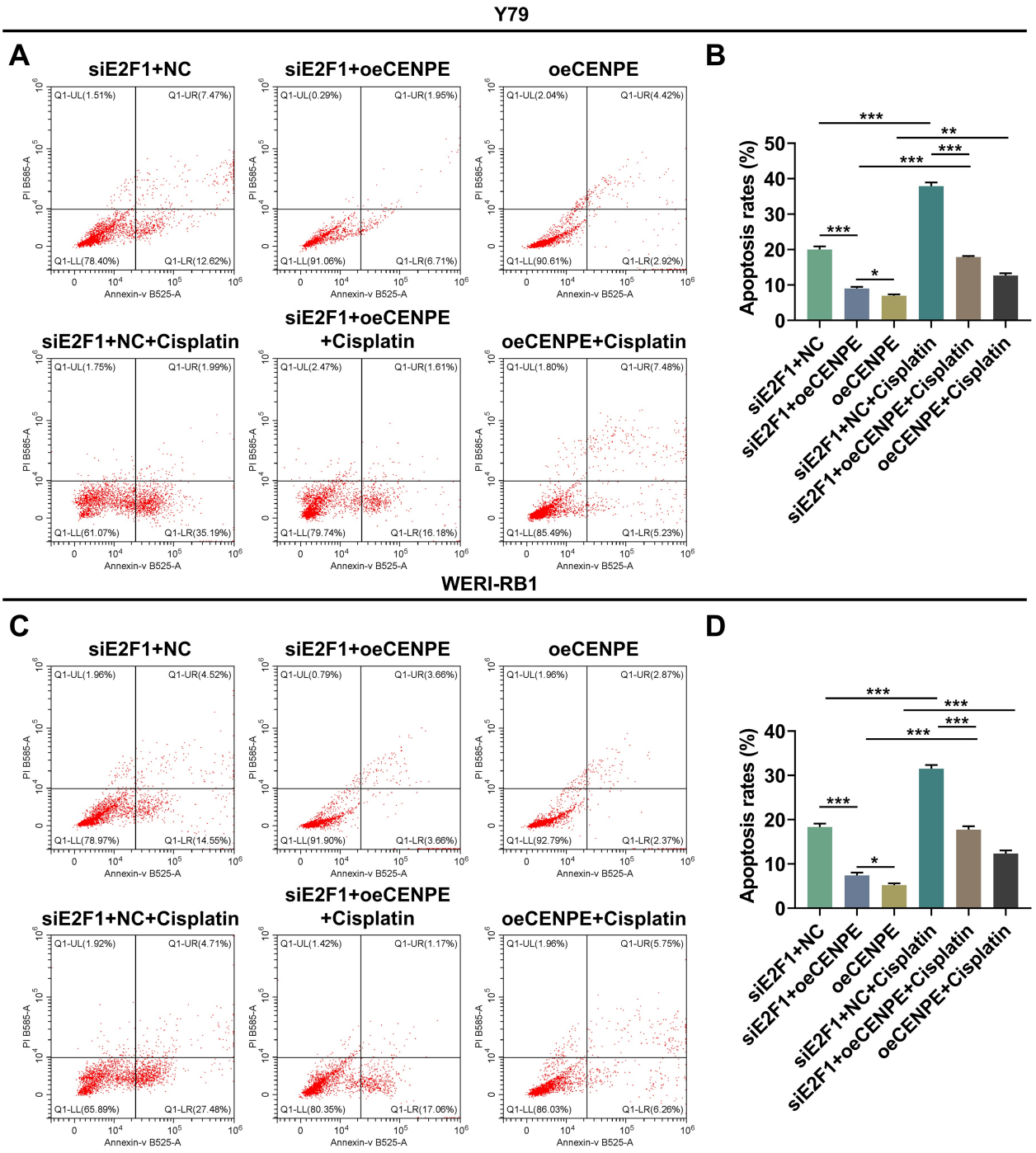


**Fig. 8. *CENPE* overexpression partially reversed the effect of *E2F1* siRNA on apoptosis of cisplatin-treated cells.** The transfection of *CENPE* overexpression vector was carried out in WERI-RB1 and Y79 cells. (A–D) Representative images of flow cytometry assay for Y79 (A) and WERI-RB1 cells (C) with quantification results (B,D). Biological triplicates were performed for these experiments. $^{*}p < 0.05$, $^{***}p < 0.001$. Abbreviations: *CENPE*, centromere protein E; *E2F1*, E2F transcription factor 1; NC, negative control.

expression of the SMC2, SMC4, MCM4, and CENPE after *E2F1* silencing. The qRT-PCR results indicated that *CENPE*, *SMC2*, *SMC4* and *MCM4* levels were reduced substantially by *E2F1* silencing, with *CENPE* presenting the most pronounced downregulation, which augmented the effect of cisplatin on cells. *CENPE* belongs to centromere regulatory gene and impacts mitosis [41]. Shi *et al*. [42] reported that *CENPE* level is elevated in retinoblastoma and can serve as a potential therapeutic target for retinoblastoma. In acute myeloid leukemia, knocking down *CENPE* can significantly enhance cell apoptosis [43]. Kinesin family member 18A (KIF18A) promotes the tumor growth of

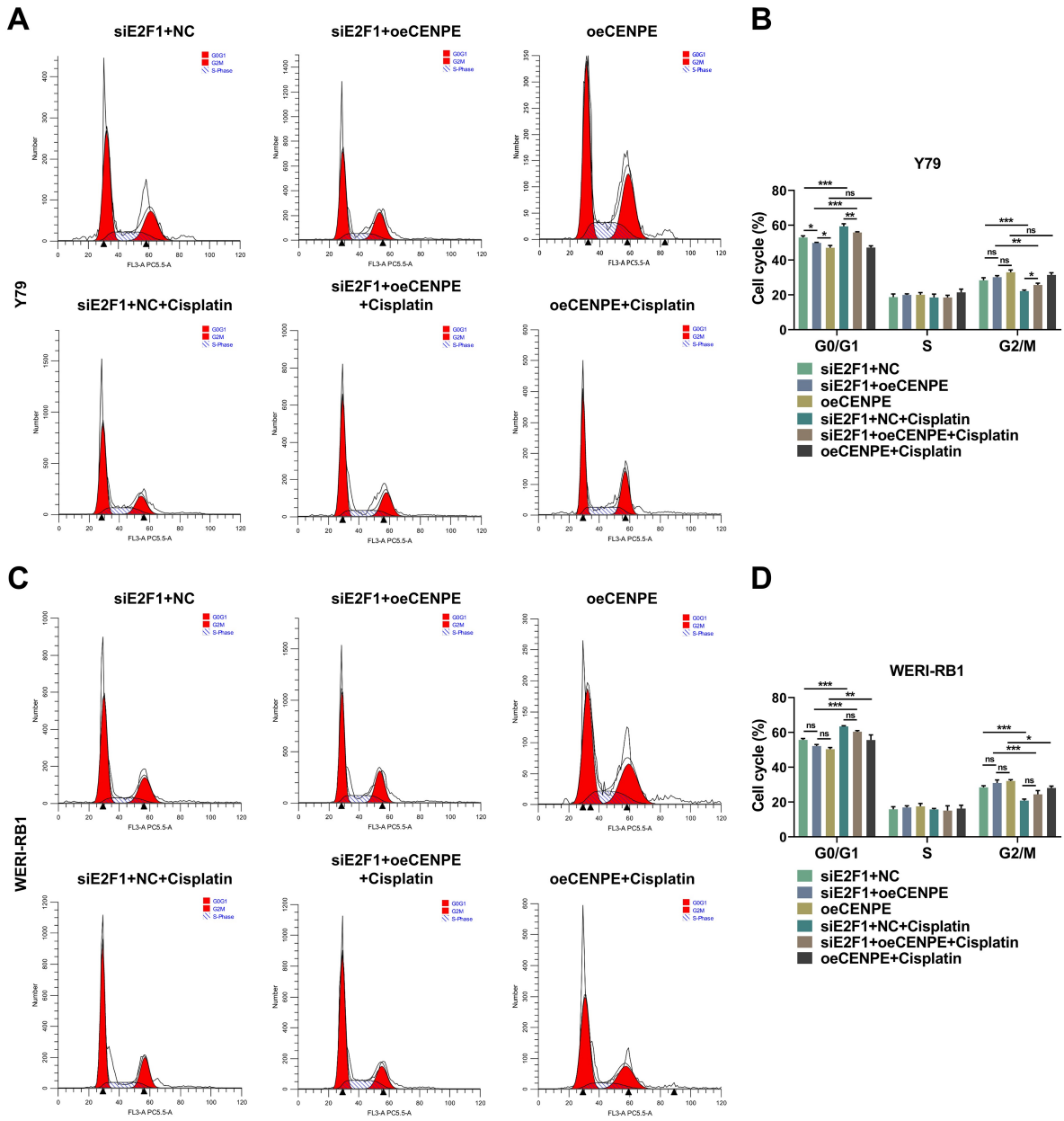


**Fig. 9. *CENPE* overexpression partially reversed the effect of *E2F1* siRNA on cell cycle of cisplatin-treated cells.** The cell cycle was detected by means of flow cytometry after transfection of the cells with *E2F1* silencing and/or *CENPE* overexpression plasmid combined with/without cisplatin treatment. (A–D) Representative images of flow cytometry assay for Y79 (A) and WERI-RB1 cells (C) with quantification results (B,D). Biological triplicates were performed for these experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; ns, not significant. Abbreviations: *CENPE*, centromere protein E; *E2F1*, E2F transcription factor 1; NC, negative control.

cervical squamous cell carcinoma by regulating *CENPE* to modulate the phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) signaling pathway [44]. In addition, *CENPE* promotes the proliferation and chemoresistance of acute myeloid leukemia [45]. Our results showed that up-regulation of *CENPE* partially offset the suppressive effects of *E2F1* silencing on cisplatin-treated cells.

Interestingly, previous study also showed that the increased proliferation capability observed in drug-sensitive ovarian cancer cells when co-cultured with drug-resistant cells may be attributed to the overexpression and activity of *E2F1* [46]. Moreover, CENPE can enhance the proliferation and chemoresistance of acute myeloid leukemia [42]. This indicates that *E2F1* might affect sensitivity of retinoblastoma to cisplatin through regulation of *CENPE*—a theme that underscores the need for further investigations. Therefore, the establishment of a drug-resistant cell line model *in vitro* will aid in the future investigation into the mechanism underlying drug resistance involving *E2F1* and *CENPE*.

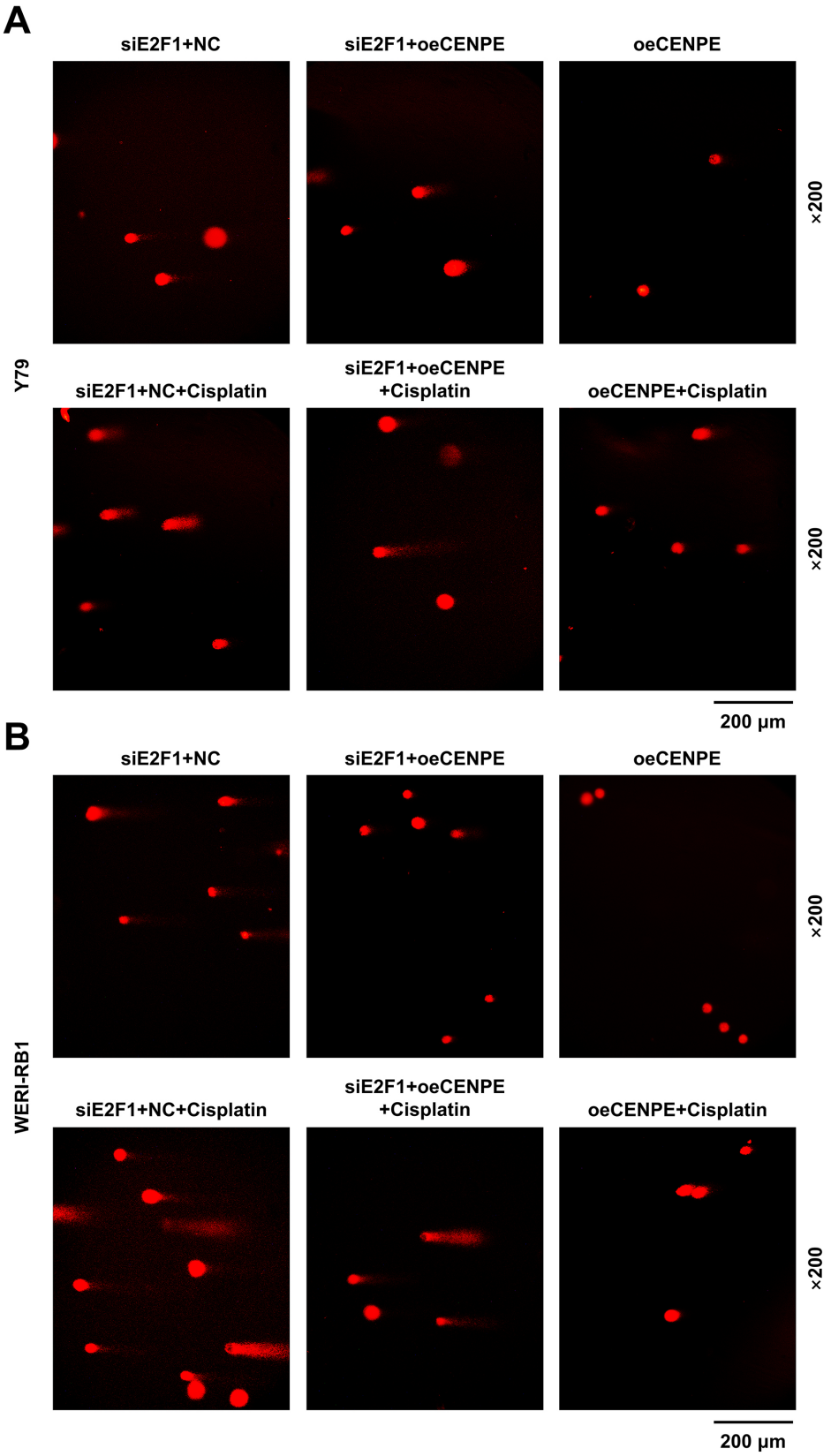


**Fig. 10. *CENPE* overexpression partially reversed the effect of *E2F1* siRNA on DNA damage of cisplatin-treated cells.** DNA damage was detected by means of comet assay after transfection of the Y79 (A) and WERI-RB1 cells (B) with *E2F1* silencing and/or *CENPE* overexpression plasmid combined with/without cisplatin treatment. Abbreviations: *CENPE*, centromere protein E; *E2F1*, E2F transcription factor 1; NC, negative control.

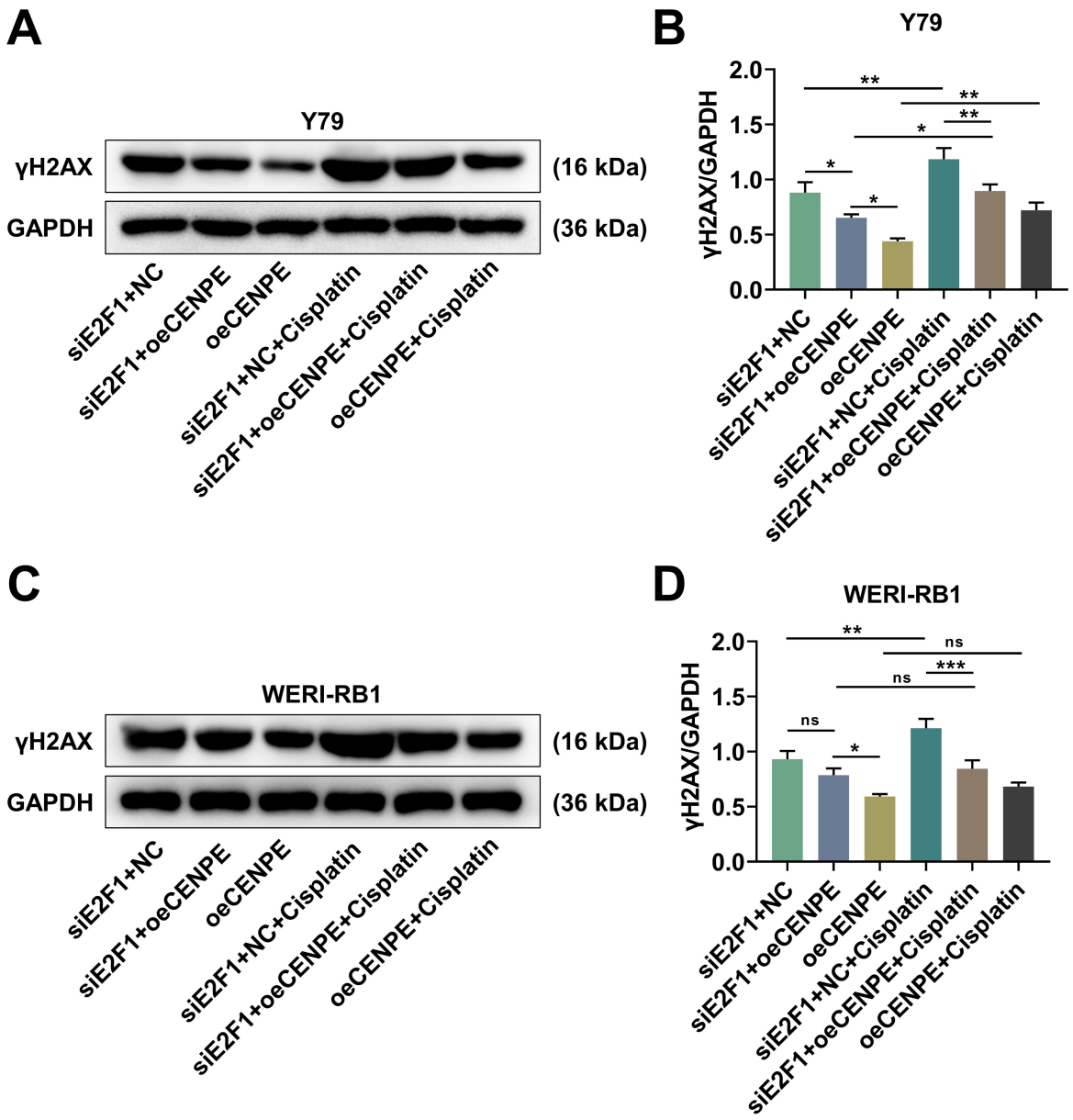


**Fig. 11. *CENPE* overexpression partially reversed the effect of *E2F1* siRNA on γH2AX expression of cisplatin-treated cells.** The expression of γH2AX was detected by means of Western blotting after transfection of the Y79 (A,B) and WERI-RB1 cells (C,D) with *E2F1* silencing and/or *CENPE* overexpression plasmid combined with/without cisplatin treatment. Biological triplicates were performed for these experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; ns, not significant. Abbreviations: *CENPE*, centromere protein E; *E2F1*, E2F transcription factor 1; γH2AX, histone H2AX on serine 139; NC, negative control.

One of the limitations of this study is the absence of *in vivo* experiments, which will be conducted in the future to verify the findings of this study. Further, the protein expression of SMC2, SMC4, and MCM4 requires further investigation. Despite the evaluation of $IC_{50}$ value and DNA damage response in this study, how E2F1 silencing specifically alters cisplatin uptake, efflux, or DNA repair pathways was not explored in details; therefore, more verification experiments are necessary.

## Conclusion

In conclusion, compared to *E2F1* silencing or cisplatin treatment alone, combined approach provides a more effective avenue for inhibiting *CENPE* expression and promoting DNA damage and apoptosis in retinoblastoma cells. This study provides a new theoretical and experimental basis for the therapeutic effect of cisplatin in retinoblastoma.

## Availability of Data and Materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

## Author Contributions

Substantial contributions to conception and design: YTL. Data acquisition, data analysis and interpretation: QSY, SZL, ZXZ, XYH, MF. Drafting the article or critically revising it for important intellectual content: All authors. Final approval of the version to be published: All authors. Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of the work are appropriately investigated and resolved: All authors.

## Ethics Approval and Consent to Participate

Not applicable.

## Acknowledgment

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## Conflict of Interest

The authors declare no conflict of interest.

## Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.24976/Discov.Med.202537198.113.

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