E2F1/CKS2/PTEN signaling axis regulates malignant phenotypes in pediatric retinoblastoma

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Retinoblastoma (RB) is the most common pediatric intraocular malignancy and is a serious vision- and life-threatening disease. The biallelic mutation of the retinoblastoma gene RB1 is the initial event in the malignant transformation of RB, but the exact molecular mechanism is still unclear. E2F transcription factors can be activated by RB1 loss of function and lead to uncontrolled cell division. Among E2F family numbers, E2F1 has higher expression abundance than E2F2 and E2F3 in RB clinical samples. By integrating E2F1 ChIP-seq data, RNA-seq profiling from RB samples and RNA-seq profiling upon E2F1 knockdown, together with pathway analysis, literature searching and experimental validation, we identified Cyclin-dependent kinases regulatory subunit 2 (CKS2) as a novel regulator in regulating tumor-associated phenotypes in RB. CKS2 exhibited aberrantly higher expression in RB. Depletion of CKS2 in Y79 retinoblastoma cell line led to reduced cell proliferation, delayed DNA replication and decreased clonogenic growth. Downregulation of CKS2 also slowed tumor xenograft growth in nude mice. Importantly, reversed expression of CKS2 rescued cancer-associated phenotypes. Mechanistically, transcription factor E2F1 enhanced CKS2 expression through binding to its promoter and CKS2 regulated the cancer-associated PI3K–AKT pathway. This study discovered E2F1/CKS2/PTEN signaling axis regulates malignant phenotypes in pediatric retinoblastoma, and CKS2 may serve as a potential therapeutic target for this disease.

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signaling axis regulates malignant phenotypes in RB and CKS2 may serve as a potential therapeutic target for retinoblastoma.

RESULTS
Global transcriptional dysregulation in retinoblastoma
To determine the differences in mRNA expression level between RB and retina, the transcriptomes of five RB and five normal retina samples were analyzed using RNA-seq. To confirm the reliability of pathogenic and normal tissues, principal component analysis (PCA) was applied to these ten samples. The result showed a clear distinction between the tumor and non-tumor samples (Fig. 1A), suggesting that the transcriptome contained important information to distinguish these two conditions. The separation of tumor and non-tumor samples remained true when we combined our expression profiles with Rajasekaran’s dataset [25], the first in-depth RNA-seq resource of retinoblastoma (Supplemental Fig. S1A, B). Next, we explored differentially expressed genes (DEGs) between RB and normal controls. 3779 upregulated and 3865 downregulated DEGs were identified in tumor samples (Fig. 1B, C). Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses revealed that upregulated genes in tumor samples were enriched in pathways including cell cycle, DNA replication, and other cancer-associated pathways (Fig. 1D), and downregulated genes were enriched in pathways related to light stimulus and eye...
development, which are retina specific (Fig. 1E). Some cancer-related pathways and corresponding upregulated genes were listed in Fig. 1F. Ten cancer-associated genes were randomly chosen and their differential expression levels were validated by reverse transcription followed by quantitative polymerase chain reaction (qRT-PCR; Fig. 1G) using clinical samples, in line with the results in RNA-seq data (Supplemental Fig. S2). These results indicate that RB undergoes dramatic transcriptomic changes, some of which are in cancer-associated genes.

**Screening of cancer-related genes directly regulated by transcription factor E2F1**

It is widely known that E2F transcription factors (E2F1-3) are activated by RB1 loss of function and augment expression of cell-proliferation-associated genes leading to uncontrolled cell division [9–15]. Gene set enrichment analysis (GSEA) using our RNA-seq data showed that the upregulated genes were related to DNA repair and cell cycle progression (Supplemental Fig. S3A–D). The upregulated genes in RB were enriched in E2F targets and the G2M checkpoint gene set (Fig. 2A), consistent with previous knowledge [9–15]. We validated expression of E2F family members (E2F1-3) by qRT-PCR using clinical samples. Result showed that E2F1 had higher expression level than E2F2 and E2F3 (Fig. 2B), implying that E2F1 might play a more important role than the other two family members in malignant transformation. Following this clue, screening cancer-related genes directly regulated by transcription factor E2F1 was the crucial step to explore the underlying mechanism of malignant phenotypes in RB. In order to achieve this purpose, we performed E2F1 chromatin immunoprecipitation sequencing (ChIP-seq) in Y79 retinoblastoma cell line (E2F1 ChIP-seq schematic diagram shown at Fig. 2C). RNA-seq libraries before and after knocking down (KD) E2F1 in the same cell line were also constructed and sequenced. Knockdown efficiency of E2F1 was evaluated by Western blotting (Fig. 2D). By overlapping three groups of genes, including those having E2F1 ChIP-seq peaks in Y79 cells (directly bound by E2F1 at the promoter area), those showing downregulation upon E2F1 knockdown in Y79 cells (potentially regulated by E2F1 at the level of transcription) and those exhibiting upregulation in RB samples compared with normal controls (upregulated in RB clinical samples), we found that 120 genes were potentially bound and directly regulated by E2F1 in RB cells (Fig. 2E). Pathway enrichment analysis revealed that those genes were enriched in cancer-relevant pathways, including p53 signaling pathway, small cell lung cancer, cell cycle and base excision repair (Fig. 2F). Some corresponding genes were listed in Fig. 2G. Four genes that were relatively highly expressed from each term were selected for initial functional screening and ChIP-seq peaks of four representative genes showed strong binding of E2F1 near the promoter regions (Fig. 2H). Lentivirus knockdown vectors for these four genes were constructed and transfected to Y79 cells. Knockdown of corresponding genes was confirmed by qRT-PCR (Fig. 2I; Supplemental Table S1) and Western blotting (Fig. 2J). To determine which gene had the greatest effect on slowing the proliferation rate than the other genes when knocking down (Fig. 2K), we focused on CKS2 to explore its function and mechanism in RB.

**Transcription factor E2F1 enhances CKS2 expression through binding to its promoter**

To explore which transcription factor (TF) potentially bound to the promoter region of CKS2, different databases were used including PROMO [26], USCS Genome Browser [27], ISMARA [28] and JASPAR [29]. We speculated that the potentially functional TFs should be differentially expressed between RB and normal controls in our RB clinical RNA-seq data (Supplemental Fig. S4). The potential binding sites of CKS2 promoter region with different TFs were predicted by ChIPBase v2.0 (http://ma.sysu.edu.cn/chipbase) [30]. Four transcription factors, including E2F1, FOS, PAX5 and EGR1, were selected as candidates. To evaluate whether these candidate TFs could bind to the promoter region of CKS2, a dual-luciferase vector psiCHECK2 with CKS2’s wild-type (WT) or mutated promoters (Mut), was constructed, and then was transfected into Y79 cells for comparison. TFs predicted binding sites in CKS2’s promoter were replaced by a palindromic sequence (Shown at Supplemental Fig. S5). Vector with mutation at E2F1 binding sites (E2F1_Mut in CKS2_promotor) showed significantly reduced luciferase activity than WT and other mutant groups (FOS_Mut in CKS2_promotor, PAX5_Mut in CKS2_promotor and EGR1_Mut in CKS2_promotor) (Fig. 3A), indicating that TF E2F1 might directly bind to CKS2’s promoter region to regulate its target gene’s expression. We also observed the similar result when vectors were transfected into 293T cells (Supplemental Fig. S6). To further explore the relationship between E2F1 and CKS2, based on online tool GENPIA [31], which integrates RNA-seq data from the Cancer Genome Atlas (TCGA), we observed that both E2F1 and CKS2 were highly expressed compared to the normal tissues in 24 and 23 cancer types, respectively (Supplemental Figs. S7 and S8). E2F1 and CKS2 were both highly expressed in 21 cancer types (Supplemental Figs. S7 and S8) and such positive correlation was also observed in our RB clinical data (Fig. 1G). To confirm that E2F1 did play a causal role in promoting transcription of CKS2, we knocked down E2F1 with two short hairpin RNAs (shRNAs) in human retinoblastoma cells (Y79 and WERI-Rb-1) and found that expression of CKS2 was significantly decreased in both RNA and protein levels (Fig. 3B–D). We also knocked down E2F1 in other cancer cell lines (human hepatocellular carcinoma, QGY-7701; human glioma, U343; human non-small-cell lung carcinoma, A549; human pancreatic carcinoma, AsPC-1; human cervical carcinoma, HeLa) in which E2F1 and CKS2 were both highly expressed based on TCGA database (Supplemental Figs. S7 and S8) and exhibited positive co-expression between E2F1 and CKS2 (Fig. 3B, C). The lines of evidence showed that expression of CKS2 reduced correspondingly upon knockdown of E2F1 (Fig. 3A–D). Further, we used ChIP Base v2.0 to uncover that the potential binding site of E2F1 located within one kilobase (kb) upstream of CKS2 transcription start site and ChIP-seq peaks were also displayed in this region containing a canonical E2F binding site (SSGC with S = C or G) [32] using our ChIP-seq data (Y79 cells) and ChIP Base v2.0 data (MCF7 cells) [30] (Fig. 2H; Fig. 3E; Supplemental Fig. S9), supporting the potential binding of E2F1 to the promoter of CKS2. To confirm the direct binding, we performed ChIP coupled with PCR (ChIP-PCR) and quantitative PCR (ChIP-qPCR) assay in Y79 cell line. The ChIP-PCR and ChIP-qPCR primer pair was designed spanning the “SSGC” motif (S = G or C) within the ChIP-seq peak region (Fig. 3E; Supplemental Table S1). The result manifested an enrichment of E2F1 binding signals to this region when compared with a non-specific IgG control (Fig. 3E). Together, these lines of evidence indicate that E2F1 directly binds to the promoter of CKS2 to regulate its expression.

**CKS2 exhibits aberrant higher expression in retinoblastoma and promotes cell proliferation and tumor formation**

After demonstrating that CKS2 had aberrantly higher expression in RB samples compared to normal controls (Fig. 1G), we asked whether CKS2 contributed to cancer-associated phenotypes. To test this, we knocked down CKS2 with two shRNAs in Y79 cells (Supplemental Table S1). Knockdown efficiency of CKS2 in Y79 cells was evaluated by qRT-PCR and Western blotting (Fig. 4A, B). CCK-8 assay suggested that downregulation of CKS2 led to delayed cell proliferation (Fig. 4C). Cell colony formation assay demonstrated that CKS2 depletion repressed clonogenic growth of Y79 cells (Fig. 4D). EdU staining indicated that KD of CKS2 resulted in decreased DNA replication rate (Fig. 4E). Depletion of CKS2 delayed tumor xenograft growth of Y79 cells in nude mice.
The mean tumor weight of the CKS2_KD group was significantly reduced (Fig. 4G). Moreover, recovery of CKS2 by lentivirus-mediated overexpression of CKS2 in CKS2-KD cells (CKS2_Rescue) restored expression of CKS2 in both RNA and protein levels (Fig. 4H, I) and rescued cancer-associated phenotypes including cell proliferation, colony formation, EdU staining and tumor xenograft growth (Fig. 4J–N). We also knocked down CKS2 with two shRNAs in human retinoblastoma WERI-Rb-1 cells (Supplemental Fig. S10A) and found that downregulation of CKS2 in this cell type led to similar phenotypes as well including...
Fig. 2 Screening cancer-related genes directly regulated by transcription factor E2F1. A Representative GSEA plots showing that upregulated DEGs were enriched in E2F targets (top) and the G2M checkpoint (bottom) in the RB versus retinal samples. B E2F1-3 were validated by qRT-PCR using clinical samples, in line with blue dot (normal tissues) and red dot (RB samples). C Schematic diagram of E2F1 ChIP-seq in Y79 cells. D Knockdown efficiency of E2F1 was evaluated by western blotting. E The diagram of overlapping gene numbers among E2F1 ChIP-seq profiling, RNA-seq profiling of E2F1-KD (down-regulated genes) and RNA-seq profiling of RB (upregulated genes), created with BioRender website. F, G The pathway analysis of cancer-related genes directly regulated by E2F1 (F) and their corresponding upregulated genes (G). H ChIP-seq tracks of four representative genes. I J Four representative candidate genes showingdownregulation by Lentivirus knockdown in Y79 cells were evaluated by qRT-PCR (I) and western blotting (J). GAPDH or beta Actin served as an internal control. K Proliferation rate of Y79 cells without (Scramble and Ctrl) and with gene downregulation (PCNA, APAF1, APEX1, CKS2) were analyzed by a Cell Counting Kit-8 (CCK-8) assay. ***P < 0.001; **P < 0.01; *P < 0.05, by two-tailed t-test.

Fig. 3 E2F1 binds to CKS2 promotor and regulates its expression. A Luciferase activity of CKS2's wild-type (WT) and mutated (Mut) promotor in Y79 cells. The mutation was achieved by replacing a palindromic sequence to a specific TF potential binding site. B Knockdown of E2F1 in Y79, WERI-Rb-1, QGY, U343, A549, AsPC-1 and HeLa human cells evaluated by qRT-PCR. GAPDH served as the internal control. C Validation of CKS2 expression after E2F1 knockdown in Y79, WERI-Rb-1, QGY, U343, A549, AsPC-1 and HeLa human cells evaluated by qRT-PCR. GAPDH served as the internal control. D Western blotting showed that the protein expression of CKS2 was significantly reduced in E2F1-KD Y79 (up panel) and WERI-Rb-1 (down panel) cells. GAPDH served as the internal control. E E2F1 binding to the promotor region of CKS2 was evaluated by ChiP-PCR and ChiP-qPCR in Y79 cells. The black arrow represents the transcription direction. The ChiP-PCR and ChiP-qPCR primer pairs (F and R, forward and reverse, respectively) were designed spanning the "SSCGC" motif (S = G or C) within the ChiP-seq peak region. The same amount of DNA was applied for ChiP-PCR (left panel) and ChiP-qPCR (right panel). ***P < 0.001; **P < 0.01; *P < 0.05, by two-tailed t-test.

cell proliferation, colony formation and EdU staining (Supplemental Fig. S10B–D). These results indicate that CKS2 is a novel contributor to cancer-related phenotypes in RB.

CKS2 regulates cancer-associated PI3K–AKT pathway

To explore the downstream signaling pathway of CKS2 in explaining the malignant phenotypes of RB, RNA-seq analyses were performed in Y79 cells under scramble, control, CKS2-KD1 and CKS2-KD2 conditions (for the vectors construction, see details in the "Method" section). Gene expression analysis found 566 upregulated and 257 down-regulated genes in CKS2-KD cells (Fig. 5A, B; fold change > 2, false discovery rate (FDR) < 0.05). GO analysis identified enrichment of some proliferation-related biological processes among CKS2-dependent genes including upregulated and downregulated genes (Fig. 5C), in line with the reduced proliferation rate (Fig. 4C–G). PTEN, a well-known tumor suppressor gene and the most crucial negative regulator of the PI3K–AKT signaling pathway [33–36], showed an increased protein level in CKS2-KD cells (Fig. 5D) in line with the result of CKS2-KD RNA-seq data (Supplemental Fig. S11). Rescue CKS2 by lentivirus-mediated overexpression of CKS2 in CKS2-KD cells reversed PTEN protein level (Fig. 5D), indicating that CKS2 acts upstream of PTEN. We also quantified two key proteins (AKT and S6) and their phosphorylation states in the PI3K–AKT–mTOR signaling pathway and found that phosphorylation of AKT (p-AKT) and S6 (p-S6) both decreased upon CKS2 knockdown (Fig. 5D). Recovery of CKS2 by lentivirus-mediated overexpression of CKS2 in CKS2-KD cells significantly reversed the levels of p-AKT and p-S6 (Fig. 5D). Knockdown of PTEN in CKS2-KD cells did not impair CKS2 protein level, but partially rescued cancer-associated phenotypes including cell proliferation, colony formation and EdU staining (Fig. 5E–H), suggesting that PTEN act downstream of CKS2. To
validate that PTEN was indeed downstream of RB-E2F1, we observed that E2F1 knockdown had the same effect of CKS2 depletion on PTEN protein levels in Y79 cells (Fig. 5D, I). Moreover, overexpression of PTEN in Y79 cells suppressed the cell proliferation independently including colony formation and EdU staining (Fig. 5J–L). These data support that CKS2 can affect the activity of the PI3K–AKT pathway and may explain why CKS2 regulates the retinoblastoma-associated phenotypes.

**DISCUSSION**

Retinoblastoma is a rare type of intraocular malignancy that usually develops in early childhood, and is a serious vision- and life-threatening disease. Previous few microarray [23, 24] and RNA-seq [25] studies have uncovered global gene expression changes between RB and control samples. However, lack of RB samples without chemotherapy and normal retinal samples from young donors as age-matched controls are two possible restrictions to
global gene-expression research for RB. Additionally, these transcriptomic analyses have only discovered an association but not causality between gene expression and RB. The previous RNA-seq study used six RB samples and two control samples (age at 12 and 22) [25]. In our study, we sequenced five RB samples and five control samples. As age-matched normal samples are difficult to obtain since most RB patients are age under five years, the normal retinal samples used in this study were from people aged 17–40 years. If age is the major contributor to gene expression, we would expect the first principal component (PC) regarding expression profiling among all these samples (both RB and normal retina) would show an age-dependent manner. However, when carrying out principal components analysis (PCA), we found that the normal retinal and RB samples could be separated very well (Fig. 1A), suggesting that tumorigenesis was the major contributor of the transcriptome difference. Combing previously published RNA-
Fig. 5 CKS2 regulates cancer-associated PI3K-AKT signaling pathway. A Volcano plot of gene-level expression changes after CKS2 knockdown. The orange and green dots represent genes with an at least two-fold increase or decrease in expression level in CKS2-KD cells compared with control cells. Orange and blue denote increased and decreased expression, respectively. B Heatmap of log2-transformed expression profiles for DEGs compared CKS2-KD cells with control cells. Orange and blue denote increased and decreased expression expression, respectively. C GO enrichment analysis for all DEGs showed in (C). D Western blotting validations of PI3K-AKT-mTOR signaling pathway-associated proteins in CKS2-KD and CKS2-Rescue cells compared with corresponding control Y79 cells. E Western blotting was used to validate the knockdown of PTEN in CKS2 down-regulated Y79 cells. F–H Knockdown of PTEN in CKS2 down-regulated Y79 cells reversed cell proliferation (F), colony formation (G) and EdU staining assay (H). Representative images at left panel and quantification of percent EdU+ cells at right panel, shown as mean ± SEM, ***p < 0.001, ***p < 0.001, n ≥ 3. I Western blotting was used to validate expression of PTEN in E2F1 knockdown Y79 cells. J Lentivirus overexpression of PTEN (PTEN-OE) in Y79 cells was validated by Western blotting. GAPDH served as the internal control. K, L Colony formation assay (K) and EdU staining assay (L). Representative images at left panel and quantification of percent EdU+ cells at right panel, shown as mean ± SEM, ***p < 0.001, ***p < 0.001, n ≥ 3 of Y79 cells between Ctrl group and PTEN-OE group. ***p < 0.001; **p < 0.01; *p < 0.05, by two-tailed t-test.

Fig. 6 Working model for E2F1/CKS2/PTEN signaling axis in regulating malignant phenotypes of RB. CKS2 exhibits aberrant higher expression in retinoblastoma compared with normal controls. Upregulated transcription factor E2F1 bound to upstream of CKS2 transcription start site and promoted CKS2 protein production, leading to cancer-associated phenotypes, possibly by regulating PI3K-AKT signaling pathway (Supplemental Figs. S6 and S7), which might be a possible reason why CKS2 always frequently elevated in many cancers. Working model for CKS2 as a novel regulator in regulating malignant phenotypes of RB was shown at Fig. 6.

In conclusion, our study uncovered the crucial role of E2F1/CKS2/PTEN signaling axis in regulating malignant phenotypes of RB and CKS2 might become a novel therapeutic target for RB in the future.

MATERIALS AND METHODS
Clinical sample collection
A total of ten clinical samples were obtained from Eye & ENT Hospital of Fudan University, Shanghai, China. Five normal retinal samples were acquired from donors aged 17–40 years. Five RB samples were collected from patients aged 1–4 years who had not received radiotherapy, chemotherapy or other related therapies before surgery. The diagnosis was validated according to clinical manifestation and pathological examination. The whole process was approved by Eye & ENT Hospital of Fudan University.

RNA-seq data analysis
The raw paired-end reads obtained from RNA-seq experiments were filtering out low-quality reads, and then aligned to human reference genome sequence (UCSC hg19 assembly) using STAR [42] with default settings. To choose genes with accurate expression value, we consider genes whose FPKM (fragments per kilobase of exon model per million reads mapped) > 1 in at least one sample for subsequent analysis. Differentially expressed gene analysis was performed using edgeR [43] and a statistical cutoff of FDR < 0.05 and fold change > 2 was applied to obtain regulated genes. GO analysis, KEGG analysis and gene set enrichment analysis (GSEA) were
performed by clusterProfiler [44]. Hallmark gene signature sets maintained by the Molecular Signatures Database (MSigDB) [45] were used.

Cell culture, vector construction and transfection

Human 293T, HeLa, QGY, U343, A549, AsPC-1, Y79 and WERI-Rb-1 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) or RPMI 1640 medium with 10% fetal bovine serum (FBS) at 37 °C in a 5% CO2 incubator. For PCNA, APAF1, APEX1, CKS2 and E2F1-KD vector construction, two pair of annealed shRNA oligonucleotides were cloned into pLVKO.1 plasmid for each gene with EcoRI and AgeI restriction enzymes. A pair of shRNAs (Supplemental Table S1) Non-targeting a known gene was used as scramble and pLVKO.1 empty vector that transfected into Y79 cells served as control. To obtain CKS2 and PTEN overexpression vector, the full coding sequences without stop codon sequence was inserted into PCDH_EF1_MC-5_T2A_Puro vector (PCDH). PCDH empty vector transfected into target cells served as overexpression experiment control. For lentivirus transduction, 293T cells were seeded in six-well plates with approximately 70% confluence and transfected with vectors using Lipofectamine 3000 (Thermo Fisher Scientific). After 24 h (h) culture, superantigen of the virus was collected to infect Y79 and other target cells (WERI-Rb-1, QGY, U343, A549, AsPC-1 and HeLa). To achieve stably transformed cells, 1640 or DMEM medium with 3 μg/ml puromycin was used for cell culture.

qRT-PCR and western blotting

Total RNA and protein of each sample were extracted using TRIzol reagent (Ambion), and reversely transcribed into cDNA using random primers. Expression of the tested genes was quantified by qRT-PCR using 2 ngaChamQ Universal SYBR qPCR Master Mix and normalized to GAPDH (Roche Light Cycler). All primer sequence information is listed in Supplemental Table S1. The protein concentration was evaluated by BCA protein assay kit (Vayme Biotechnology). Primary antibodies involved in CKS2 (Abcam, cat.No: ab155078, 1:1000), APEX1 (Proteintech, cat.No: 10203-1-AP, 1:1000), APAF1 (Cell Signaling Technology, cat.No: #8969, 1:1000), PCNA (Arigo, cat.No: ARG62605, 1:5000), GAPDH (IProteintech, cat.No: HRP-60004, 1:5000), β-Actin (Proteintech, cat.No: HRP-60008, 1:5000), E2F1 (Abcam, cat.No: ab179445, 1:1000), AKT (Cell Signaling Technology, cat.No: 4691, 1:1000), p-AKT (Cell Signaling Technology, cat.No: 4060, 1:1000), S6 (Cell Signaling Technology, cat.No: 2217, 1:1000), p-S6 (Cell Signaling Technology, cat.No: 2211, 1:1000), and PTEN (Proteintech, cat.No: 22034-1-AP, 1:1000) were used for Western blotting. Full length western blot scans for the cropped images presented in supplemental material file.

Colony formation assay

To assess the colony formation ability of the RB cell line (Y79 and WERI-Rb-1) with or without gene perturbation, six-well plates were treated with 0.1 μg/ml poly-L-lysine for 2 h and target cells were seeded at 1500 cells per well with RPMI 1640 medium with 10% FBS. After ten days of cell culture, cells were fixed in 4% paraformaldehyde for 30 min (min) and stained with crystal violet staining solution for 1 h. Different wells with or without CKS2 perturbed cell clones were taken pictures after staining.

Cell proliferating rate assay/CKK-8 assay

CKK-8 is a simple, rapid, sensitive colorimetric assay for the evaluation of the number of viable cells. Y79 cells with or without CKS2 gene perturbation were cultured and assayed for cell proliferation using the CCK-8 kit according to the protocol provided by vendor Dojindo.

EdU staining assay

Validation of DNA replication was performed according to the protocol of KeyFluo488 Click-IT EdU Kit (KeyGEN Biotechnology). Briefly, 24-well plates were treated with 0.1 μg/ml poly-L-lysine for 2 h, and Y79 cells with or without CKS2 gene perturbation were seeded to approximate 60% confluence. After cells adhered, 4% formaldehyde was used to treat the cells for 30 min. After three washes with 3% bovine serum albumin in phosphate-buffered saline, cells were incubated with staining solution for 2 h in the dark. After EdU staining, cell nuclei were stained with 5 μg/ml 4′,6-diamidino-2-phenylindole solution at room temperature for 30 min, and then analyzed by inverted fluorescence microscopy (Olympus IX73).

Tumor xenograft model

Animal experiments were approved by the Institutional Research Ethics Committee of Eye & ENT Hospital of Fudan University. Y79 cells (1 × 105) with or without CKS2 gene perturbation were subcutaneously injected into 5-week-old nude mice (each group n = 4). Tumor volume was monitored by calipers every two days and calculated according to the formula: tumor volume = (length × width²)/2. After 5 weeks, all nude mice were sacrificed, and tumor xenografts were dissected and weighed.

Luciferase assays

To evaluate whether target of TFs could bind to the promoter region of CKS2, a dual luciferase vector psiCHECK2 (Promega, cat. no. C8021) with CKS2’s wild-type (WT) or mutated promoters (Mut) was constructed. The promoter (WT or Mut) of CKS2 was cloned into the downstream of the Renilla luciferase translation stop codon using the Xhol and Pmel restriction enzyme sites, and the firefly reporter cassette served as an intra-plasmid transfection normalization reporter. After 24-well plates treated with 0.1 mg/ml poly-L-lysine for 2 h, Y79 cells were seeded into a 24-well plate at approximate 60% confluence. After overnight, the cells were transfected with psiCHECK2 vectors (CKS2’s WT or mutated promoters) using Lipofectamine 3000 (Thermo Fisher Scientific). Thirty hours post transfection, Renilla and firefly luciferase activities were measured by the Dual-Luciferase Reporter 1000 Assay System (Promega).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed using the Simple ChiP® Plus Sonication ChiP Kit using Y79 cells according to the manufacturer’s protocol (Cell Signaling Technology). After ChiP-seq constructed, the library was sequenced using an Illumina HiSeq2000 platform. Primer sequence information for ChIP-qPCR is also listed in Supplemental Table S1.

DATA AVAILABILITY

The raw RNA-seq data from this study have been submitted to the NCBI BioProject database (https://www.ncbi.nlm.nih.gov/bioproject/) under accession number PRJNA752257.

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AUTHOR CONTRIBUTIONS

CZ and TN designed the study. MC, JH, LW, and PY performed the experiments. MC and ZZ performed the bioinformatics and statistical analyses. JC, JQ and TN supervised the bioinformatics and statistical analyses. The manuscript was initially drafted by MC and ZZ and then was revised by CZ and NT All authors read and approved the final manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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