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Cellular nucleic acid binding protein suppresses tumor cell metastasis and induces tumor cell death by downregulating heterogeneous ribonucleoprotein K in fibrosarcoma cells

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ABSTRACT

Background: Cellular nucleic acid binding protein (CNBP) has been implicated in vertebrate craniofacial development and in myotonic dystrophy type 2 (DM2) and sporadic inclusion body myositis (sIBM) human diseases by controlling cell proliferation and survival to mediate neural crest expansion. CNBP has been found to bind single-stranded nucleic acid and promote rearrangements of nucleic acid secondary structure in an ATP-independent manner, acting as a nucleic acid chaperone.
Methods: A variety of methods were used, including cell viability assays, wound-scratch assays, chemotaxis assays, invasion assays, circular dichroic (CD) spectroscopy, NMR spectroscopy, chromatin immunoprecipitation, expression and purification of recombinant human CNBP, electrophoretic mobility shift assay (EMSA), surface plasmon resonance (SPR), fluorescence resonance energy transfer (FRET) analyses, luciferase reporter assay, Western blotting, and isothermal titration calorimetry (ITC).
Results: Up-regulation of CNBP induced human fibrosarcoma cell death and suppressed fibrosarcoma cell motility and invasiveness. It was found that CNBP transcriptionally down-regulated the expression of heterogeneous ribonucleoprotein K (hnRNP K) through its conversion of a G-rich sequence into G-quadruplex in the promoter of hnRNP K. G-quadruplex stabilizing ligand tetra-(*N*-methyl-4-pyridyl) porphyrin (TMPyP4) could interact with and stabilize the G-quadruplex, resulting in downregulation of hnRNP K transcription.
Conclusions: CNBP overexpression caused increase of cell death and suppression of cell metastasis through its induction of G-quadruplex formation in the promoter of hnRNP K resulting in hnRNP K down-regulation.
General significance: The present result provided a new solution for controlling hnRNP K expression, which should shed light on new anticancer drug design and development.

1. Introduction

CNBP is a single-stranded nucleic-acid-binding protein containing strikingly conserved two remarkable structural features: seven tandem CCHC-type zinc knuckle domains (C–Φ–X–C–G–X_3–H–X_4–C, where Φ = aromatic amino acid and X = variable amino acid) and a peculiar arginine/glycine-rich region between the first and the second Zn knuckles, which is highly similar to the arginine–glycine–glycine (RGG) box [1]. CNBP has been initially described as a DNA-binding protein acting as a negative transcriptional regulator in the coordinated control of cholesterol metabolism [2]. As a transcriptional regulator, CNBP has been reported to control the expression of the early promoter–enhancer of the JC virus [3] and the β-myosin heavy chain gene [4]. Conversely, CNBP has also been shown to up-regulate the expression of macrophage colony-stimulating factor (CSF-1) in fibroblasts [5] and c-Myc proto-oncogene [6]. Likely, CNBP may possess nucleic acid chaperone activity to catalyze the rearrangement of nucleic acids via ATP-independent repeated cycles of binding and release, and thus facilitating the folding of its thermodynamically stable three-dimensional structure required for nucleic acid biological function [7–9]. Various reports have implicated the effect of CNBP in vertebrate craniofacial development and in myotonic dystrophy type 2 (DM2) and sporadic inclusion body myositis (sIBM) human diseases [10–13]. Both apparently unrelated biological processes have progressed significantly,

Abbreviations: CD, circular dichroism; ChIP, chromatin immunoprecipitation; CNBP, cellular nucleic-acid-binding protein; EMSA, electrophoretic mobility-shift assay; FAM, 6-carboxyfluorescein; FRET, fluorescence resonance energy transfer; hnRNP K, heterogeneous ribonucleoprotein K; ITC, isothermal titration calorimetry; SPR, surface plasmon resonance; TAMRA, tetramethylrhodamine; TMPyP4, tetra-(*N*-methyl-4-pyridyl) porphyrin

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however, a complete understanding of CNBP function has not been achieved.

hnRNP K is a RNA-binding protein found in the nucleus, cytoplasm, mitochondria, and plasma membrane, and belongs to the family of heterogeneous nuclear ribonucleo-protein (hnRNP) complex [14–16]. hnRNP K has been implicated to function in transcription [17–19], pre-mRNA splicing [20], RNA processing [21], stability [22], and translation [23,24]. Besides, hnRNP K plays key roles in coordinating transcriptional responses to DNA damage [25]. The expression of hnRNP K is increased in several human malignancies: chronic myelogenous leukemia and solid tumors, including cancers of the esophagus, lung, nasopharynx and colorectum [26–30]. Some studies have shown that hnRNP K is indispensable for metastasis and cell death [31,32]. Overall, hnRNP K has been implicated as a potential key player in carcinogenesis, making it an attractive target for anticancer therapies. Although hnRNP K has been the subject of numerous studies, the mechanisms regulating its expression are still largely unknown.

In the present study, we found a new G-rich sequence in the promoter of hnRNP K gene, which can form stable G-quadruplex resulting in downregulation of hnRNP K. We found that elevated CNBP expression down-regulated the transcription and expression of hnRNP K through its conversion of this G-rich sequence into G-quadruplex, resulting in enhanced tumor cell death and suppressed tumor cell metastasis.

2. Materials and methods

2.1. Materials

Human fibrosarcoma cell HT1080 was obtained from the American Type Culture Collection (ATCC) and preserved in our lab. The cell culture was maintained in complete DMEM medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C under humidified atmosphere with 5% CO_2. Cell line was tested negative for mycoplasma contamination prior to and after experimentation. All oligomers/primers (sequences shown in Table S1) used in this study were purchased from Sangon Biotech (China). All DNA oligomers were HPLC-purified and dissolved in double-distilled water before use, except for primers and CD experiments, which were purified by using PAGE (Table S1). All oligonucleotides were first heated at 95 °C for 5 min, and then slowly cooled to room temperature to form G-quadruplex structure. All antibodies were commercially available: β-actin rabbit mAb (#4970S, Cell Signaling), hnRNP K rabbit mAb (#9081S, Cell Signaling), CNBP goat polyclonal antibody (sc-51052X, Santa Cruz), nucleolin mouse mAb (ab13541, Abcam), SP1 rabbit mAb (sc-14027,Santa Cruz), NM23-H2 rabbit mAb (5969-1, Epitomics), Anti-rabbit IgG-HRP (#7074S, Cell Signaling), Anti-mouse IgG-HRP (#7076, cell signaling) and Donkey anti-goat IgG-HRP (sc-2020, Santa Cruz).

2.2. Plasmids

The pET28a-CNBP plasmid was generated by inserting the CNBP cDNA into the pET28a plasmid (Promega) using the Nde I and Xho I restriction sites. The cDNA was obtained by using PCR with the primers: CNBP-pET28a-F and CNBP-pET28a-R (Table S1). The EGFP–CNBP plasmid was generated by inserting the CNBP cDNA into the pEGFP-N3 plasmid (Promega) using the Nde I and BamH I restriction sites. The cDNA was obtained by using PCR with the primers: CNBP–GFP-F and CNBP–GFP-R (Table S1). The wild type psiCheck2-hnRNP K luciferase plasmid was generated by inserting the hnRNP K promoter sequence (from −998 to +50) into the psiCheck-2 reporter vector (Promega) using the Kpn I and Nhe I restriction sites. The promoter oligonucleotide was obtained by using PCR with the primers hnRNP K-WT-F and hnRNP K-WT-R. The mutated-1 (Mut1) psiCheck2-hnRNP K luciferase plasmid was generated by multi-point site-directed mutagenesis from wild type psiCheck2-hnRNP K (WT) luciferase plasmid. All reconstructed plasmids were sequenced and proved to be correct by BLAST.

2.3. Cell viability assays

Cell viability was measured by using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium-bromide) assay. After 24 h or 48 h transfection of pEGFP–CNBP, the medium was removed and the MTT solution (0.5 mg/mL in medium) was added. After 4 h, the MTT solution was removed and tetrazolium salt was dissolved in 100 μL of DMSO/ethanol solution (1:2). Colorimetric measurement was performed at 570 nm by using an ELISA reader.

2.4. Wound-scratch assays

Confluent monolayer HT1080 cells were maintained in serum-containing growth medium for 12 h, and then in serum-free medium for 12 h. A 200 μL plastic pipette tip was used to scratch the monolayers. The wounded cells were then cultured in serum-free medium for an additional 12 h and photographed. Three different points were marked on each plate. The experiment was repeated for three times.

2.5. Chemotaxis assays

Cell migration assays were performed by using 8-μm-pore Transwell inserts (Millipore). HT1080 cells were transfected with pEGFP–CNBP at ~75% confluency in 10-cm dishes. Chemotaxis assays were carried out as that previously described by Inoue et al. [33].

2.6. Invasion assays

Invasion assays were performed with a Cell Invasion Assay Kit (Millipore). HT1080 cells were transfected with pEGFP–CNBP at ~75% confluency in 10-cm dishes. Twenty-four hours after transfection, cells were collected, washed twice with PBS, and suspended in DMEM medium. For invasion assay, 5.0×10^4 cells were seeded in each well and the assay was performed following the manufacturer's instructions. After 24 h of incubation, Matrigel was swabbed off with cotton bud, and cells that invaded across the Matrigel were stained with Cell Stain (Millipore).

2.7. Circular dichroic (CD) spectroscopy and CD-melting experiments

CD measurements were performed on a Chirascan circular dichroism spectrophotometer (Applied Photophysics) at room temperature. CD experiments were carried out with oligonucleotides (5 μM) annealed in 10 mM Tris–HCl, 100 mM KCl, and pH 7.4. CD spectra were collected from 220 to 340 nm with a 0.2 cm path length cylindrical quartz cuvette. A buffer blank correction was made for all spectra. For CD melting temperature experiments, samples were annealed at first, G-quadruplex formation was induced and molar ellipticity at 263 nm was measured over a temperature range of 25–95 °C.

2.8. NMR spectroscopy

Sample for NMR study was dialyzed successively against 60 mM KCl solution and against water. The strand concentration of the NMR samples was 1 mM, and the solutions contained 100 mM KCl and 20 mM potassium phosphate (pH 7.0). NMR experiments were performed on 600 MHz Bruker spectrometers at 25 °C. The imino protons were observed using the 11-echo pulse sequence [34] with the excitation maximum adjusted to the center of the Hoogsteen imino region. Five millimeter NMR tubes were used for extract measurements with a sample volume of 500 μL. Samples analyzed via CD were also used for NMR experiments.

2.9. Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed using Magna ChIPTM kit (Millipore) following the manufacturer's protocol. After 4 days, antibody against CNBP, nucleolin, NM23-H2, and SP1 was used to immunoprecipitate chromatin in HT1080 cells. Rabbit IgG was used for mock immunoprecipitation. Briefly, cells were fixed with 1% formaldehyde for 10 min and then lysed. Chromatin was sheared to an average size of 0.3–0.5 kb using a SCIENTZ-II D sonicator (SCIENTZ), and 1% of lysate was removed as input. ChIP was performed overnight at 4 °C, and immune complexes were collected using protein A magnetic beads provided by the kit. After extensive washing, the DNA was extracted from immunoprecipitated chromatin. Immunoprecipitated DNA samples were amplified by using PCR to show CNBP, nucleolin, NM23-H2, and SP1 occupancy of hnRNP K promoter and negative control in cellulo.

2.10. Expression and purification of recombinant human CNBP

Wild-type human CNBP was expressed as 6× His fusion protein in *Escherichia coli* strain BL21 (DE3) and purified to homogeneity by using affinity chromatography, as described previously [35].

2.11. Electrophoretic mobility shift assay (EMSA)

Briefly, 5 μM 5′-FAM-labeled oligonucleotides were mixed with diverse amount of recombinant CNBP in EMSA binding buffer (10 mM HEPES, 2 mM $MgCl_2$, 1 mM DTT, 5% glycerol, pH 6.9) for 1 h at 37 °C in a final volume of 20 μL. For binding reactions requiring G-quadruplex DNA or single-strand DNA, the oligonucleotides were incubated with CNBP for 1 h at 37 °C in binding buffer with or without 100 mM KCl respectively. After incubation, the reaction mixtures were run in 8% native polyacrylamide gels at 4 °C and 10 $V \cdot cm^{-1}$ in 0.5× TBE buffer (Tris/borate).

2.12. Surface plasmon resonance (SPR)

SPR measurements were performed on a ProteOn XPR36 Protein Interaction Array system (Bio-Rad Laboratories, Hercules, CA) using a GLH chip. In a typical experiment, biotinylated CNBP was immobilized in flow cells 1 and 2, leaving the third flow cell as a blank. Oligonucleotide was prepared with running buffer (12 mM HEPES, 4 mM Tris, 1 mM EDTA, 1.5 mM $MgCl_2$) by serial dilutions from stock solutions. Oligonucleotide was injected at a flow rate of 30 μL/min for 480 s of association phase, and followed by 600 s of disassociation phase at 25 °C. The GLH chip was regenerated with short injection of 50 mM NaOH between consecutive measurements. The final graphs were obtained by subtracting blank sensorgrams. Data were analyzed with ProteOn manager software, using the Langmuir model for fitting kinetic data.

2.13. Fluorescence resonance energy transfer (FRET) analyses

FRET measurements were carried out in 10 mM Tris–HCl buffer, pH 7.4, 100 mM KCl at 25 °C on a Perkin-Elmer LS-55 fluorescence spectrometer. A quartz cuvette with 1 cm path length was used for the spectra recorded at 10 nm excitation and emission slit width. The final DNA concentration was 100 nM with indicated DNA/protein ratio, and the resulting mixture was incubated at 37 °C for 2 h before measurement. Excitation was set at 495 nm, and emission was collected from 500 to 700 nm. A buffer blank was subtracted for all spectra, and the final analysis was carried out by using Origin 8.0.

2.14. Luciferase reporter assay

HT1080 cells were transfected with plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Forty-eight hours after transfection, Renilla and firefly luciferase activities were measured by using a Dual-Glo Luciferase assay system (Promega) according to the manufacturer's protocol with a Synergy 2 microplate reader (BioTek Instruments). The ratio of Renilla to firefly luciferase activity was calculated for each plasmid.

2.15. Western blotting

After appropriate treatment, the cells were washed with PBS, and lysed in 100 mL of lysis buffer (10 mM Tris–HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA, 25 mM NaF, fresh 100 mM Na_3VO_4 and 1 mM dithiothreitol). Equal amounts of protein lysates were loaded and separated by using SDS-PAGE followed by Western blotting.

2.16. Isothermal titration calorimetry (ITC)

ITC measurements were carried out with a VP-ITC titration calorimeter (MicroCal, Northampton, MA), as described previously by Haq et al. [36]. The thermograms (integrated heat/injection data) obtained in ITC experiments were fit with proper model in Origin 8.0.

2.17. Statistical analysis

All data in reporter assays are presented as mean ± SD. Comparisons between the two groups on a single parameter were conducted using Student's *t*-test. Statistical analyses were performed using KaleidaGraph. The criterion for statistical significance was set at $P < 0.05$.

3. Results and discussion

3.1. CNBP enhanced tumor cell death and suppressed tumor cell metastasis

In our studies for the effect of CNBP on tumor cells, we manipulated the expression of its gene in HT1080 human fibrosarcoma cells. At first, we constructed GFP-fusion recombinant CNBP expression plasmid, which was transformed into the tumor cells. The overexpression of CNBP was found to be associated with enhanced tumor cell death (up to ~70%) after 24 h and 48 h (Fig. 1A). Then, we investigated the effect of CNBP on cell motility and invasion by conducting assays for wound healing, non-coated Boyden chamber chemotaxis and Matrigel-coated Boyden chamber invasion. A scratch wound-healing assay indicated that exogenous overexpression of human CNBP in HT1080 cells exhibited significantly decreased tumor cell mobility compared with control vector (Fig. 1B). Exogenous overexpression of human CNBP showed significant abrogation of cell migration as compared with the control vector by using chemotaxis assay (Fig. 1C). Overexpression of human CNBP showed ablation of cell invasion across Matrigel-coated membrane (Fig. 1D). We tried our best to generate CNBP-overexpression stable cell lines, but not successful. It is possible that ectopically expressed CNBP induced cell death (Fig. 1A). Therefore, we used transient transfection method to address cell behavior in the present study. Taken together, our results indicated that elevated expression of CNBP was associated with enhanced tumor cell death and decreased tumor cell metastasis.

3.2. G-quadruplex formed in a region of G-rich strand in hnRNP K promoter

In order to understand the above experimental observation, we carried out mechanistic studies at molecular level. CNBP has been found to bind with single-strand nucleic acids and promote their secondary structure rearrangements in an ATP-independent manner, as a nucleic acid chaperone [7,8]. It has been suggested that CNBP could promote the formation of G-quadruplex in gene promoter region, and thus regulating gene transcription [9]. hnRNP K has been found to be indispensable for tumor cell viability [32] and a target for metastasis [31]. We found that the promoter of the hnRNP K gene contains a G-

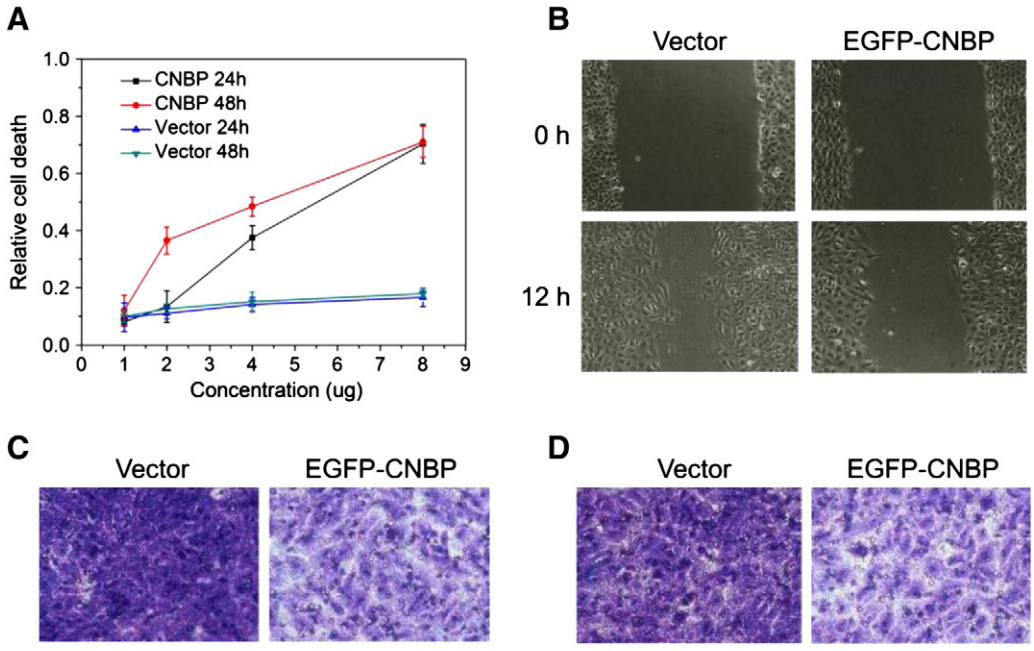

Fig. 1. CNBP regulated the tumor cell death and metastasis. (A) Overexpression of CNBP was associated with enhanced human fibrosarcoma cell death. Colorimetric measurement was performed at 570 nm by using an ELISA reader. (B) Ectopic overexpression of human CNBP in HT1080 human fibrosarcoma cells exhibited significantly decreased tumor cell mobility compared with control vector. Cells were photographed at 0 and 12 h after scratches. (C) The migration properties of the cells were analyzed by using chemotaxis assay with a Boyden chamber. The cells that migrate through the Boyden chamber were stained. The EGFP–CNBP reduced the number of migrating cells compared with vector. (D) The invasive properties of the cells were analyzed by using an invasion assay with a Matrigel-coated Boyden chamber. The cells invaded through the ECM/Matrigel were stained. The overexpression of EGFP–CNBP reduced the number of invasive cells.

rich sequence (G15) on the positive strand (Fig. 2A). In order to study whether this G-rich sequence can fold into a stable G-quadruplex, we carried out biophysical experiments on its corresponding synthetic oligonucleotide. Circular dichroism (CD) spectroscopy has been widely used to characterize the structure of folded nucleic acid quadruplexes [37]. In a solution of 100 mM KCl at pH 7.4, the CD spectra of G15 displayed a positive peak at 263 nm and a negative peak at 240 nm (Fig. 2B), which are characteristic CD signature of a parallel G-quadruplex structure [37,38]. In order to further determine whether G15 sequence forms G-quadruplex structure, we carried out NMR spectroscopy study. NMR spectroscopy can provide rich structural information in which spectral parameters are usually related to quadruplex conformation and dynamics. The quality of the spectrum of the exchangeable proton resonances is a good indication of how amenable the quadruplex spectra will be for structural analysis. The ^{1}H NMR spectrum in 90% H_2O/D_2O can provide a wealth of structural information on the quadruplex. In general, imino proton chemical shifts of over 12.5 ppm are indicative of Watson–Crick base pairs (NH···H hydrogen bonds). Imino proton chemical shifts in the range of 10.5–12 ppm are indicative of guanine NH···O hydrogen bonds that appear in Hoogsteen alignments of the G-quadruplexes [39–42]. In our NMR study, imino proton spectra of d[TGGCGGGAGGCGGGG] (G15) (Fig. 2C) showed sharp peaks in the range of 10.5–12.0 ppm corresponding to guanine imino protons of G-quadruplex structures.

3.3. The G-quadruplex in the promoter of hnRNP K regulated the expression of a reporter gene

Since G-quadruplex formed in the promoter of hnRNP K, we wondered whether this G-quadruplex could affect the expression of hnRNP K. To answer this question, we first carried out reporter assay experiment. As shown in Fig. 3A, a plasmid (psiCheck2-hnRNP K-WT: WT) was constructed with the promoter of hnRNP K inserted into the upstream of a firefly luciferase reporter gene. We also mutated hnRNP K promoter (psi-hnRNP K-Mut1) with psi-hnRNP K-WT as a template through a GGCGGG-to-AACAAC substitution, which disrupted the G-

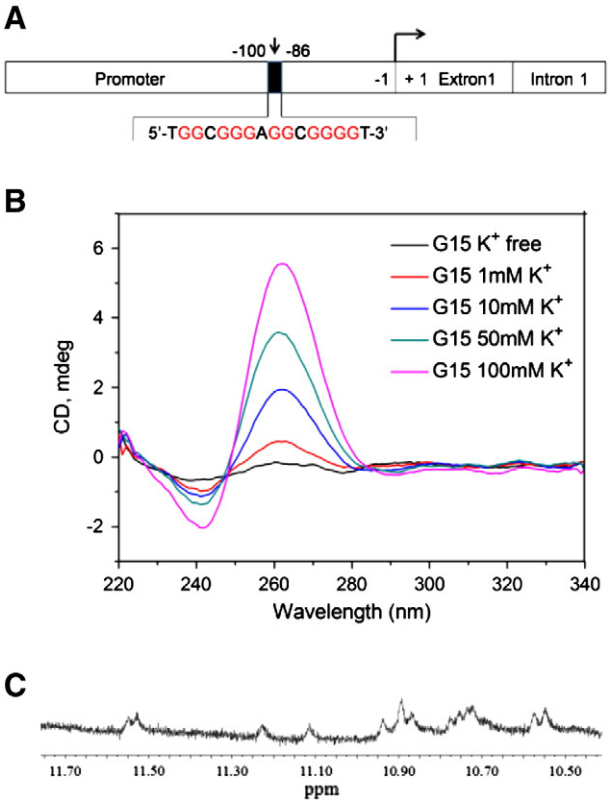

Fig. 2. The hnRNP K promoter and the G-rich region. (A) The hnRNP K promoter spanning from −100 to −86 bp includes a G-rich region, with the putative G-quadruplex forming sequence of the hnRNP K promoter shown in the inset. (B) Circular dichroism spectra of G15. CD analyses were performed on G15 in the absence and presence of various concentrations of KCl. (C) Imino proton spectra of the 15-nt hnRNP K promoter d[TGGC GGGAGGCGGGG] oligonucleotide in KCl at 25 °C.

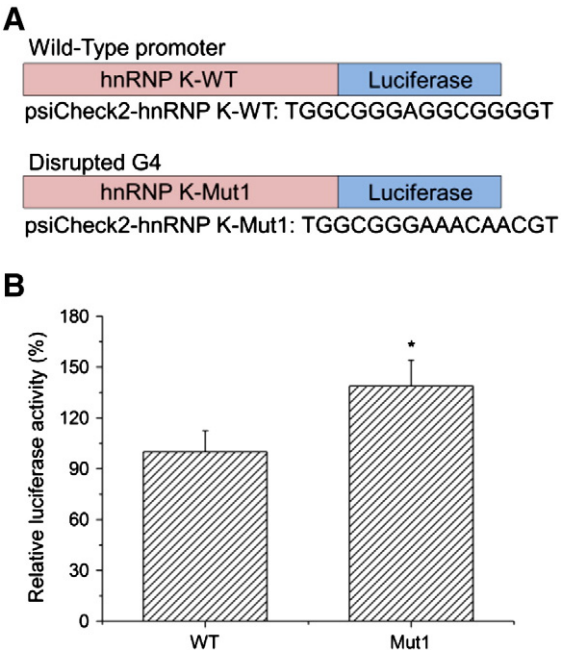

Fig. 3. Effect of the G-quadruplex in the hnRNP K promoter on the transcriptional efficiency. (A) Schematic representation of the plasmids used for reporter gene assays. The hnRNP K promoter contains wild type (WT) sequence or mutated variant (Mut1), which was put into the upstream of a firefly luciferase reporter gene in the psiCheck-2 vector (Promega). (B) Relative transcription efficiency of these two constructs, as judged by quantitation of luciferase activity. Results were normalized relative to the data for the WT group. Error bars represent the s.d. of three independent experiments. *$P < 0.05$.

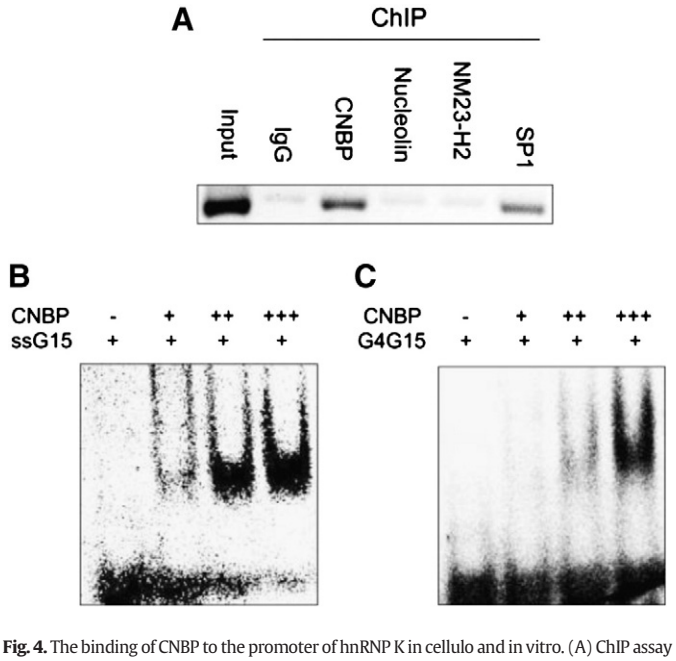

Fig. 4. The binding of CNBP to the promoter of hnRNP K in cellulo and in vitro. (A) ChIP assay of the hnRNP K promoter. Non-specific rabbit IgG (control), CNBP antibody, nucleolin antibody, NM23-H2 antibody and SP1 antibody were used in the immunoprecipitation. Immunoprecipitated DNA samples were amplified by using PCR to show CNBP, nucleolin, NM23-H2, and SP1 occupancy of hnRNP K promoter and negative control in cellulo. (B–C) The binding studies of CNBP with single strand G15 (ssG15) and G-quadruplex G15 (G4G15) in vitro by using EMSA. 5 μM 5′-FAM-labeled oligonucleotides were mixed with diverse amount of recombinant CNBP in EMSA binding buffer (10 mM HEPES, 2 mM $MgCl_2$, 1 mM DTT, 5% glycerol, pH 6.9) for 1 h at 37 °C in a final volume of 20 μL. After incubation, the reaction mixtures were run in 8% native polyacrylamide gels at 4 °C and 10 $V \cdot cm^{-1}$ in 0.5× TBE buffer (Tris/borate).

quadruplex structure and kept the original length of the promoter. We evaluated the efficiency of translation by the standard luminescence assay for luciferase catalytic activity. Fig. 3B shows the relative translation efficiency for each system. Mutation of the G-quadruplex (Mut1) resulted in a 1.4-fold increase in translation efficiency relative to the wild type. This indicates that the G-quadruplex in the promoter of hnRNP K can regulate the expression of luciferase.

3.4. Transcription factors Sp1 and CNBP bind to the promoter of hnRNP K in cellulo

Previous studies have shown that transcription factor Sp1 can bind conserved DNA sequence GGCGG [43,44], while CNBP, NM23-H2, and nucleolin can bind to G-rich sequence and function with G-quadruplex thus regulating gene transcription [45–50]. Further characterizations of these proteins with other G-rich sequences are likely to better understand the role of various DNA structures in gene expression. In order to know whether these proteins bind to the promoter sequence of hnRNP K in cellulo, we performed chromatin immunoprecipitation (ChIP) experiments. As shown in Fig. 4A, IgG antibody (Ab) did not immunoprecipitate DNA–protein complexes containing promoter sequence of hnRNP K (negative control). Our result showed that anti-CNBP Ab and anti-Sp1 Ab did immunoprecipitate a DNA–protein complex containing a promoter sequence of hnRNP K, while anti-nucleolin Ab and anti-NM23-H2 Ab did not immunoprecipitate a DNA–protein complex. Therefore, CNBP and SP1 should bind with the G-rich sequence in hnRNP K gene promoter and possibly regulate hnRNP K gene transcription.

3.5. CNBP bound tightly to single strand G15 and induced its conversion to G-quadruplex

After our ChIP experiment, we would like to further confirm that CNBP could bind to the G15, and in vitro experiment through electrophoretic mobility shift assay (EMSA) was carried out with purified human CNBP. Fig. 4B–C shows that CNBP bound with single strand G15 (ssG15) and G-quadruplex G15 (G15) to form protein–DNA complex. In contrast, CNBP had no binding with T-rich strand and C-rich strand (Fig. S1).

In order to better understand the potential biological function of the G-quadruplex structures associated with the G15 region in the hnRNP K promoter, we studied the effect of CNBP on this G15 region. It is interesting to know whether CNBP is able to induce the G-quadruplex formation in this particular region, since it has been known that CNBP can promote the formation of G-quadruplex in gene promoter region thus regulating gene transcription [9]. The effect of CNBP on the folding of the G-quadruplex was investigated by using CD. Our CD results showed that incubation of CNBP with single-strand G15 resulted in a dose-dependent increase of the positive peak at 263 nm with obvious red shift. In contrast, incubation of single-strand G15 with unspecific protein BSA and CNBP dialysis buffer did not produce obvious change in the CD spectra (Fig. 5A). This result suggests that CNBP induced the formation of parallel G-quadruplex in the promoter of hnRNP K.

To further confirm the CD results, FRET assays were performed. Our FRET assay showed that incubation of CNBP with single-strand G15 (Fig. 5B) caused a CNBP dose-dependent increase of the peak at 580 nm (TAMRA). This corresponds to the proximity of the fluorophore (FAM) and the quencher (TAMRA), which, in this particular case, indicates the formation of G-quadruplex [51]. In the case of incubating single-strand G15 with unspecific protein BSA in CNBP dialysis buffer, no obvious change in the fluorescence spectra was observed (Fig. 5B). Both CD and FRET results indicated that CNBP can bind with this particular G-rich sequence and induce its formation of G-quadruplex.

In order to quantitatively determine the binding affinity of CNBP to the G15–C15 region, SPR experiment was carried out. The binding affinity of the single-strand sequence and its corresponding G-quadruplex structure was determined as shown in Table 1 and Supplementary Fig. 2, and the intensity of the observed response enhanced with the

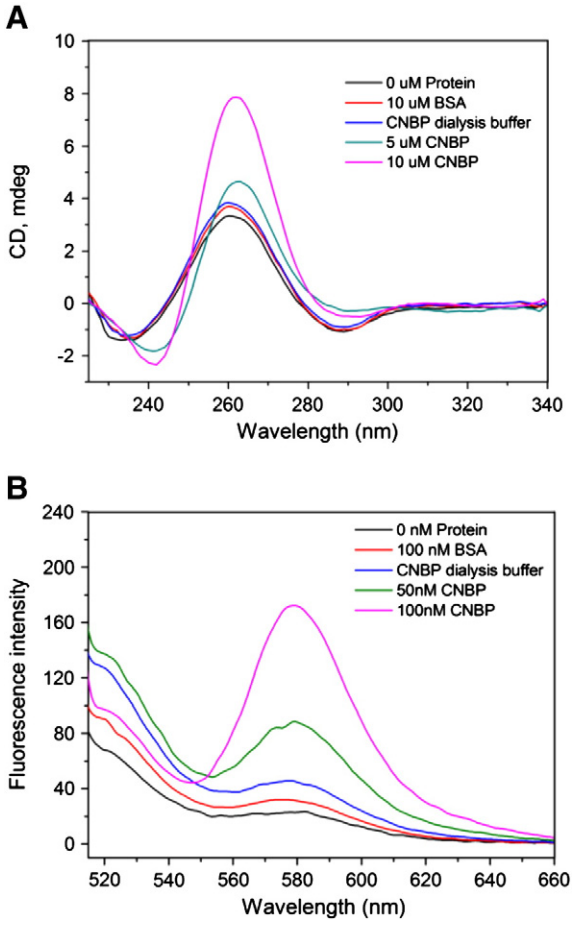

Fig. 5. CNBP bound with a G-rich sequence and induced the formation of G-quadruplex. (A) CD spectra analysis for incubation of CNBP with G15 for the study of G-quadruplex formation in the promoter of hnRNP K. The incubations were carried out with protein and the DNA at 37 °C for 2 h before CD measurements. (B) Fluorescence resonance energy transfer (FRET) analysis for the effect of CNBP on hnRNP K promoter G-quadruplex formation. The incubations were carried out with indicated molar ratio of the protein and the DNA at 37 °C for 2 h before fluorescence measurements. The excitation wavelength was 495 nm, and emission was collected from 500 to 700 nm.

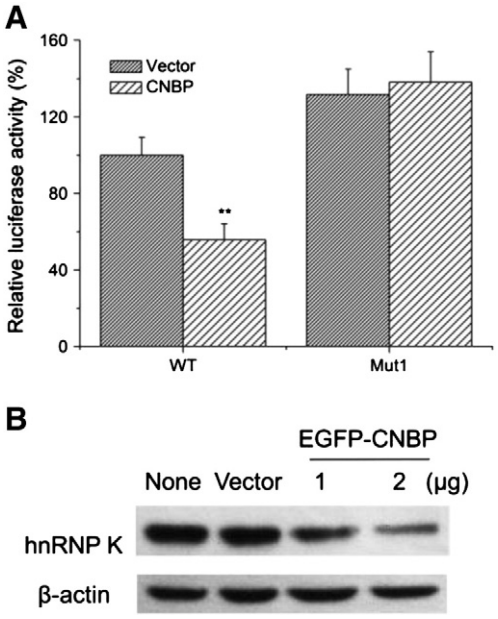

Fig. 6. Effect of ectopic CNBP on endogenous hnRNP K expression mediated through the hnRNP K promoter. (A) Effect of CNBP on the reporter construct containing either wild-type (WT) or mutant (Mut1) hnRNP K promoter for transcriptional activity. CNBP-expressing plasmid or the empty vector (500 ng) was cotransfected with the reporter construct containing either wild-type (WT) or mutant (Mut1) hnRNP K promoter sequence in 96-well plate. The comparative firefly luciferase expressions (firefly/Renilla) of vector or CNBP are shown in the histograms. Error bars represent the s.d. of three independent experiments. *P < 0.05; **P < 0.01. (B) Effect of ectopic CNBP on endogenous hnRNP K expression in HT1080 cells. Western blot analysis of cell lysates from HT1080 cells with various concentrations of pEGFP–CNBP. β-Actin was used as a control.

increasing concentrations of DNA in a dose-dependent manner. By fitting the sensorgrams with a 1:1 Langmuir model, their K_D values were calculated to be 1.54×10^{-9} M (ssG15) and 6.59×10^{-6} M (G4G15) respectively. This result demonstrated that the affinity of CNBP for the single strand G15 (ssG15) is significantly higher than that for G-quadruplex G15 (G4G15). Taken together, our results suggest that CNBP binds to ssG15 with high affinity and induces its conversion to G-quadruplex.

3.6. CNBP regulated the transcription and expression of hnRNP K

Since our above results showed that CNBP bound to the promoter of hnRNP K, we carried out further experiment to examine whether CNBP could affect the transcription and expression of hnRNP K. In our reporter assay experiment, when CNBP was co-transfected with the wild-type reporter plasmid, we detected ~40% decrease of firefly luciferase relative to the Renilla luciferase, compared to the sample transfected with an empty vector as a control (Fig. 6A). In contrast, the hnRNP K expression with Mut-1 promoter was not changed upon CNBP co-transfection. We further investigated the effect of CNBP on the transcription and expression of endogenous hnRNP K. We transiently transformed an increasing amount of CNBP into HT1080 cells, and determined endogenous hnRNP K levels by using RT-PCR and Western blot. As shown in Fig. 6B and Supplementary Fig. 3, hnRNP K transcription and expression were significantly reduced with increasingly expressed CNBP. These data suggest that CNBP can regulate the transcription and expression of hnRNP K, which in turn affects the tumor cell death and metastasis. Therefore, it is possible that CNBP binds to ssG15 and induces its conversion to G-quadruplex, which leads to the reduction of hnRNP K transcription and expression. Interestingly, CNBP and hnRNP K have been shown to co-regulate c-*myc* proto-oncogene transcription by binding specifically to the purine-rich and pyrimidine-rich single strand DNA in c-*myc* NHE III1 promoter region. However, the mechanism of CNBP's regulation of c-*myc* transcription is still elusive. Recently, the study of CNBP's regulation of c-*myc* transcription has shown that CNBP had a two-phase transcriptional regulation of c-*myc* [35]. Our results showed that CNBP also regulated hnRNP K transcription and expression.

Table 1
Calculated dissociation constants (K_D) for the binding of CNBP with ssG15 and G4G15.

Oligonucleotide	ka, M^{-1} s^{-1}	K_D, s^{-1}	K_D, M^{-1}
ssG15	$6.41E^5$	$9.87E^{-4}$	$1.54E^{-9}$
G4G15	$4.20E^2$	$2.77E^{-3}$	$6.59E^{-6}$

3.7. G-quadruplex-stabilizing ligand TMPyP4 repressed hnRNP K transcription

In order to further understand the role of this G-quadruplex in regulating the transcription and expression of hnRNP K, we next examined the interaction of a well-known G-quadruplex-interactive ligand tetra-(*N*-methyl-4-pyridyl) porphyrin (TMPyP4) with the G-quadruplex. TMPyP4 (Fig. 7A) is a known G-quadruplex stabilizing ligand, which can stack externally to the G-quadruplex and interact with the loop nucleotides [52], while its positional isomer 5,10,15,20-tetra-(*N*-methyl-2-pyridyl)porphine (TMPyP2) possesses a much weaker biological activity [53]. CD-melting assay showed that TMPyP4 stabilized G15 G-quadruplex, and the ≥*Tm* is 5 °C (Fig. 7B). ITC is a

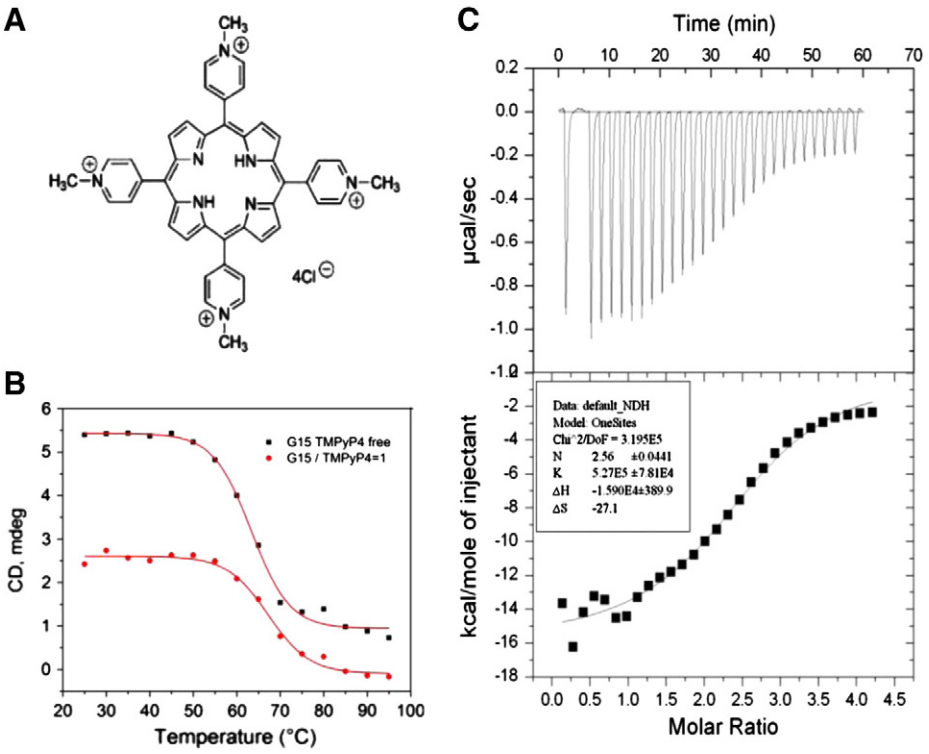

Fig. 7. G-quadruplex-interactive ligand TMPyP4 bound and stabilized G-quadruplex G15. (A) The structure of TMPyP4. (B) Circular dichroic melting curves of G15, and G15 with TMPyP4 monitored at 263 nm in the presence of 10 mM KCl. (C) Isothermal titration calorimetry (ITC) raw calorimetric data for titration of a DNA solution in Na-BPES buffer (5 μM G4G15) with serial 10 μL aliquot injections of 0.3 mM TMPyP4 solution at 25 °C, showing the exothermic properties. Fitted isotherms as a function of [ligand]/[DNA] molar ratio, showing stoichiometry of 3:1 for the binding. All interaction heats were corrected for dilution effects.

sensitive technique for probing bimolecular processes, which can provide direct information about the binding affinity and stoichiometry as well as the key thermodynamic parameters [36,54]. The interaction of TMPyP4 with the G-quadruplex G15 was examined at 25 °C. Fig. 5C shows the results of calorimetric titrations for the interaction of TMPyP4 with the G-quadruplex G15, and the binding stoichiometry of 3:1 for TMPyP4 molecules per quadruplex was obtained. The binding affinity of TMPyP4 to G-quadruplex G15 was determined to be 5.3×10^5 M^{-1} (Fig. 7C).

Since our above results showed that CNBP bound with the G15 in hnRNP K promoter, we would like to know whether TMPyP4 could block CNBP's access to the G15. EMSA was carried out as shown in Fig. 8A, which indicated that TMPyP4 effectively interrupted the binding of CNBP with the G15. It is possible that this blocking effect was individually or jointly caused by CNBP's inability to bind with the G-quadruplex folded by TMPyP4, or TMPyP4 competing with CNBP for the binding motif of G-quadruplex G15, or TMPyP4 directly interacting with CNBP and inhibiting its binding to G-quadruplex G15. This blocking effect was further studied under in cellulo condition through ChIP experiment. Our results indicated that TMPyP4 effectively interrupted protein–DNA binding interaction compared to the control with DMSO, as shown in Fig. 8B.

Since TMPyP4 stabilized and interacted with the biologically relevant G-quadruplex structure formed in the promoter of hnRNP K, we further studied its biological effect on hnRNP K transcription and expression. We investigated the effect of TMPyP4 on the transcription of hnRNP K by using luciferase assay with a hnRNP K luciferase reporter plasmid. HT1080 cells were transfected with plasmid WT and Mut1, and treated with varying concentrations of TMPyP4 (Fig. 8C). We measured the expression of firefly luciferase relative to the Renilla luciferase with dual luciferase assay, 48 h after transfection. At all concentrations used, TMPyP4 reduced the expression of firefly luciferase activity possibly by binding with the G-quadruplex in the wild-type (WT) promoter of hnRNP K (Fig. 8C). In contrast, the hnRNP K expression with the mut-1 promoter had no obvious response to TMPyP4 treatment. We further investigated the effect of TMPyP4 on the expression of hnRNP K by using Western blot. Fig. 8D showed that the treatment of HT1080 cells with TMPyP4 caused the down-regulation of hnRNP K expression, without significant effect on CNBP expression. These results strongly suggested that the stabilization of the G15 G-quadruplex in the promoter of hnRNP K repressed hnRNP K transcription and expression. Therefore, the G15 plays regulatory role in hnRNP K expression.

In summary, we found that CNBP overexpression caused the increase of cell death and suppression of cell metastasis through its down-regulation of hnRNP K expression. Our study showed that CNBP bound with a G-rich sequence in hnRNP K promoter and induced its formation of G-quadruplex thus suppressing hnRNP K transcription. The present result provided a new solution for controlling hnRNP K expression with the G15 as a drug target, which should shed light on new anticancer drug design and development.

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Appendix A. Supplementary data

Supporting information available: oligonucleotide sequences used in this study (Supplementary Table 1), binding studies of CNBP with DNA by using EMSA (Supplementary Fig. 1) and SPR (Supplementary Fig. 2), and regulation of hnRNP K mRNA expression by CNBP. Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2014.02.025.

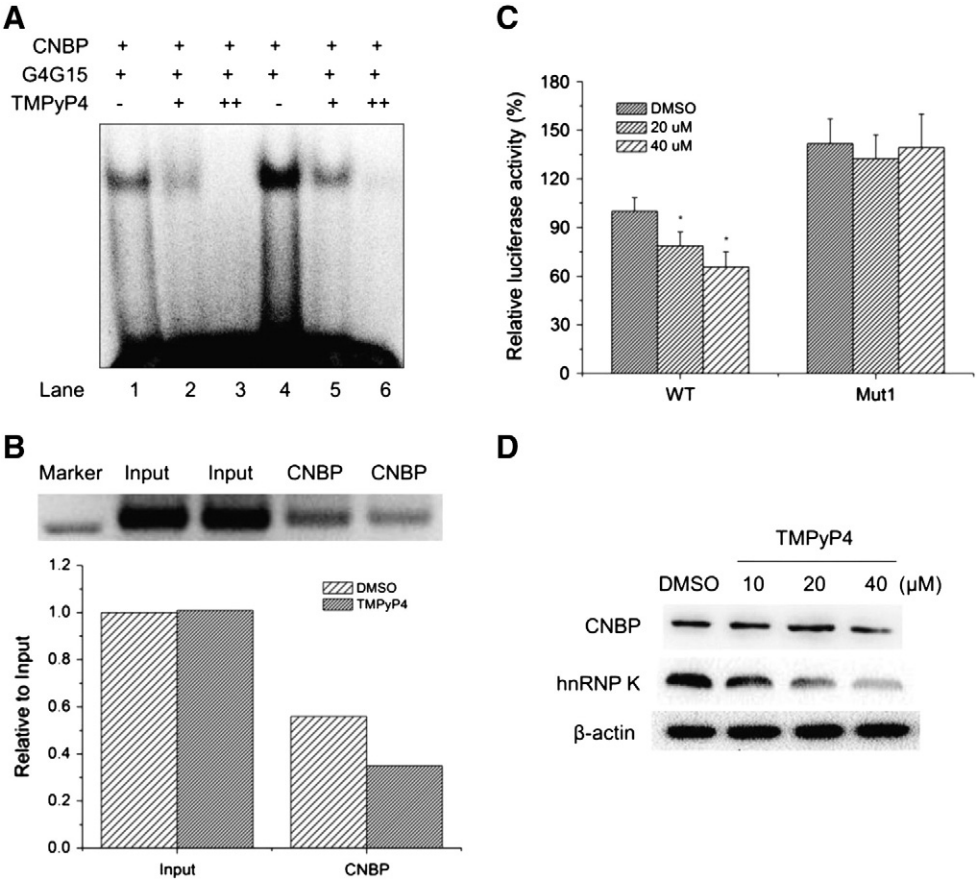

Fig. 8. Effect of quadruplex-stabilizing ligand TMPyP4 on hnRNP K expression. (A) EMSA for TMPyP4 to block the binding of CNBP to G-quadruplex G15. 5 μM of labeled G-quadruplex G15 and CNBP was used in all samples, and the concentration of TMPyP4 was 5 and 10 μM (marked as + and ++ respectively). G-quadruplex G15 was incubated with TMPyP4 before the mixture was incubated with CNBP for lanes 2 and 3. TMPyP4 and CNBP were co-incubated prior to the addition of G-quadruplex G15 for lanes 5 and 6. (B) The in cellulo effect of TMPyP4 studied with ChIP assays. ChIP assays were carried out using antibody against CNBP in HT1080 cells treated with 20 μM TMPyP4 or 0.1% DMSO as a control. Immunoprecipitated DNA samples were amplified by using PCR to show CNBP occupancy of hnRNP K promoter. The quantification of the ChIP result was shown below. (C) Dual luciferase assay to determine the effect of TMPyP4 on the reporter construct containing either wild-type (WT) or mutant (Mut1) hnRNP K promoter for transcriptional activity. The comparative firefly luciferase expressions (firefly/Renilla) with DMSO or TMPyP4 are shown in the histograms. Error bars represent the s.d. of three independent experiments. *$P < 0.05$. (D) Effect of 20 and 40 μM TMPyP4 on hnRNP K protein expression levels measured by using Western blot with 0.1% DMSO as a control.

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