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·Original Article·

Possible novel roles of poly(rC)-binding protein 1 in SH-SY5Y neurocytes: an analysis using a dynamic Bayesian network

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Abstract: Objective Poly(rC)-binding protein 1 (PCBP1) belongs to the heterogeneous nuclear ribonucleoprotein family and participates in transcriptional and translational regulation. Previous work has identified transcripts targeted by both knockdown and overexpression of *PCBP1* in SH-SY5Y neuroblastoma cells using a microarray or ProteomeLab™ protein fractionation 2-dimensions (PF-2D) and quadrupole time-of-flight mass spectrometer. The present study aimed to further determine whether these altered transcripts from major pathways (such as Wnt signaling, TGF-β signaling, cell cycling, and apoptosis) and two other genes, *H2AFX* and *H2BFS* (screened by PF-2D), have spatial relationships. **Methods** The genes were studied by qRT-PCR, and dynamic Bayesian network analysis was used to rebuild the coordination network of these transcripts. **Results** PCBP1 controlled the expression or activity of the seven transcripts. Moreover, PCBP1 indirectly regulated *MAP2K2*, *FOS*, *FST*, *TP53* and *WNT7B* through *H2AFX* or regulated these genes through *SAT*. In contrast, *TP53* and *WNT7B* are regulated by other genes. **Conclusion** The seven transcripts and PCBP1 are closely associated in a spatial interaction network.

Keywords: PCBP1; RNA interference; overexpression; Bayesian network

1 Introduction

Poly(rC)-binding protein 1 (PCBP1) or αCP1 is extensively distributed in the nervous system, circulatory system and other tissues. It is a member of the heterogeneous nuclear ribonucleoprotein (hnRNP) family and contains three K-homologous (KH) domains[1]. Different intracellular distribution patterns have been observed for PCBP1 in different cell types. In Calu-6 cells, endogenous PCBP1 occurs throughout the cell but predominantly in the nucleus[2]. Using HeLa nuclear extracts, PCBP1 was determined to be a member of a structure-related gene regulation complex that includes hnRNPs A2/B1, I, K, and L[3,4]. In normal cervical tissues, however, this protein is mainly localized to the cytoplasm[5]. In addition to being an important RNA-binding protein[6], PCBP1 also binds DNA[7]. Although PCBP1 is known to affect the stability of gene expression and axon maturation through post-transcriptional regulation[8-10], its exact functions in neurons are still unclear.

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Our previous studies support the idea that hnRNPs have a very important function in the nervous system[11]. When we knocked down endogenous[12] or overexpressed exogenous *PCBP1*[11] in neuroblastoma SH-SY5Y cells, microarray data showed that many genes were up- or down-regulated. PCBP1 also has a variant function that depends on its involvement in forming different protein-RNA complexes. When axons mature, neurofilament-M (NF-M) expression rises, partly due to increased stability of NF-M mRNA. Such post-transcriptional regulation is partly mediated through the binding of the 47-kDa PCBP1 complex, which may contribute to the increased cytoplasmic levels of NF-M mRNA that accompany axonal maturation[9].

In brief, PCBP1 is a multifunctional molecule due to its *cis*-DNA/RNA-binding activity. An increasing number of studies have demonstrated that the functions of each member of the hnRNP complex are mediated by protein-protein interaction networks[6,13,14]. Previously, a simplified picture and the global cellular level of PCBP1-targeted transcriptional and/or translational molecules were elucidated by knockdown or overexpression studies[11,12]. The objective of the present study was to further elucidate the roles of PCBP1 in neurocytes. Experimentally, *PCBP1* was either knocked down or overexpressed. After real-time reverse-transcription PCR (qRT-PCR) for selectively targeted genes, we constructed a Bayesian network model (a new method for identifying gene regulatory networks[15]) of genes that were oppositely affected by *PCBP1* knockdown or overexpression. Finally, the major interacting genes in the network that were regulated by *PCBP1* included *Homo sapiens H2AFX* (complete name: H2A histone family, member X), *TP53* (tumor protein p53), *FOS* (v-fos FBJ murine osteosarcoma viral oncogene homolog), *FST* (follistatin), *SAT* (spermidine/spermine N1- acetyltransferase 1), *MAP2K2* (mitogen-activated protein kinase kinase 2) and *WNT7B* (wingless-type MMTV integration site family, member 7b).

2 Materials and methods

2.1 Plasmids and cell lines The pcDNA^{TM}4/His C expression vector was from Invitrogen (Carlsbad, CA). The lentivirus carrier, pLentiLox3.7 (pLL3.7), and the packaging plasmids (VSVG, RSV-REV, pMDLg/pRRE) were provided by Peking University Hospital. HEK 293T cells were provided by the Human North Gene Center (Beijing, China). The human neuroblastoma cell line, SH-SY5Y, was from the American Type Culture Collection (CRL-2266), and the cells were cultured in RPMI 1640 supplemented with 15% fetal bovine serum (FBS), 1% double antibiotics, and 10 mmol/L HEPES. The packaging cell line HEK293T was grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 10 mmol/L HEPES, 2 mmol/L *L*-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin.

2.2 *PCBP1* knockdown Plasmids expressing small interfering RNAs were constructed using the pLentiLox3.7 plasmid (XhoI [Code D1094A, TaKaRa] and HpaI [Code D1064A, TaKaRa]), as described in our previous work[12]. Following the manufacturer's instructions (manual part no. 25-0677) for the BLOCK-iT™ Lentiviral RNAi Expression System (Invitrogen), effective recombinant viruses and their corresponding vacant viruses were successfully constructed.

The day before effective or vacant lentivirus infection, 3×10^6 to 5×10^6 SH-SY5Y cells were seeded into each T75 flask. The next day, virus supernatant supplemented with polybrene (8 μg/mL final concentration; Catalog no. S2667, Sigma, St. Louis, MO), and the cells were incubated overnight at 32°C. On the following day, the medium was replaced, and the cells were returned to 37°C. The stably infected cells were then selected using fluorescence-activated cell sorting. After sorting, the infected cells were collected from each experiment and passaged in culture, and their protein and RNA were extracted for further analysis.

2.3 *PCBP1* overexpression The open reading frame region (nucleotides 178–1 248) of human PCBP1 cDNA (GenBank accession no. NM_006196) was cloned into the pcDNA^{TM}4/His C expression vector using PCR amplification oligonucleotides[11]. These primers contained *EcoR*I (D1040A, TaKaRa) restriction digest sites to facilitate their insertion. The primers for *PCBP1* were forward 5'-TTCTAGAATTCATGGATGCCGGTGT GACTGAAAGTG-3', reverse 5'-TTCTAGAATTCAAC

CTACACTGTTCTAGCTGCACC-3'. Positive clones were identified by sequencing.

The day before transfection, SH-SY5Y cells were seeded into T75 flasks at a density of 3.5×10^6 to 4×10^6 cells/flask. For transfection, the cells were grown to 70%–80% confluence, and the medium was then changed to antibiotic-free medium. Transfection was performed with Lipofectamine 2000 (Invitrogen, P/N52758). Lipofectamine 2000 (72 μL) was mixed into 5 mL Opti-MEM®I Reduced Serum Medium (31985-070, Gibco, Carlsbad, CA) without serum. The pcDNA™4/His C-PCBP1 plasmid (24 μg) was mixed into another 5 mL of Opti-MEM®I without serum. Both 5-mL volumes were allowed to incubate for 5 min, and then mixed for 20 min at room temperature. Finally, the mixture was added to the cell cultures. After 8 h, the medium was removed, and the cells were washed with fresh medium containing 15% fetal calf serum. After 48 h, SH-SY5Y cell protein or RNA was extracted for later analysis. As a control, the same procedure was performed with pcDNA™4/His C vectors.

2.4 qRT-PCR qRT-PCR was used to detect the expression of *PCBP1*, selected genes in the target pathways (Wnt signaling, TGF-β signaling, cell cycling, and apoptosis), as well as *H2AFX* and *H2BFS* (screened by protein fractionation 2-dimensions). cDNA was synthesized from an RNA sample (2 μg) using M-MLV reverse transcriptase (M170, Promega Madison, WI). Primers (forward 5'-GGAAGGGCACCACCAGGAGT-3' and reverse 5'-TGCAGCCCCGGACATCTAAG-3') were used to amplify the 18S RNA gene as an internal control[11]. The primers used for amplifying PCBP1-targeted RNAs during qRT-PCR (Opticon® DNA Engine, CFD3200, MJ Research, Watertown, MA) are listed in Table S1.

2.5 Dynamic Bayesian networks (DBNs) A Bayesian network is a probabilistic graphical model that represents a set of variables and their probabilistic independencies[15]. The models of sequences of genes (such as a series of disease treatments that include changes in gene expression levels) are called DBNs. DBN analysis was performed (Genminix Informatics Ltd., Co., Shanghai, China) to construct a possible regulatory network for the selected genes.

Constructing a DBN using time-series data comprises 5 steps[15]: (1) identifying the time-point of the initial expression change (up- or down-regulation) of each gene based on the microarray time-course expression data; (2) limiting potential regulators to those with simultaneous or antecedent expression changes compared with their target genes; (3) estimating the transcriptional time-lag between the potential regulator and its target gene as the time difference between the initial expression changes of the two genes; (4) statistical analysis of the expression relationship between the potential regulator and its target gene in time slices that represent the transcriptional time-lag between the two genes; and (5) predicting the gene regulatory network. A detailed description of the DPN process can be found in reference 15.

Although there is currently no gold standard to determine the threshold for up- or down-regulation, changes of gene expression ≥1.2-fold (up-regulation) and ≤0.7-fold (down-regulation) compared to baseline are typically used as cutoffs. However, this method may inevitably omit genes with small, but potentially important, expression changes. Therefore, in this gene regulatory network modeling, we did not use these cutoffs to assign absolute up- or down-regulation to the expression levels at each time point, but focused on the relative increase or decrease in expression levels, because the main focus of DBNs is to identify correlations between gene expression patterns rather than their absolute levels at any specific time point. The Bayesian network analysis time points of the initial expression change (up- or down-regulation) of a gene could be affected by the cutoffs used to determine significantly up- or down-regulated expression levels.

We selected a number of target genes based on microarray data from the experimental knockdown and overexpression of *PCBP1*. qRT-PCR data from these target genes in the *PCBP1* knockdown and over-expressing cells were used to generate a Bayesian network describing the interactions among these genes (Wolfram Mathematica software, Version 7.0, Champaign, IL).

2.6 Immunofluorescence staining After 24 h of adherence, the cultured SH-SY5Y cells were fixed with 3.2%

paraformaldehyde/PBS at room temperature for 20 min, permeabilized with 0.2% Triton X-100/PBS for 5 min, blocked with 10% FBS, and incubated with goat anti-PCBP1 antibody (1:100; sc16504, Santa Cruz Biotechnologies) overnight at 4°C. Then the coverslips were washed twice with 0.2% Triton X-100/PBS and incubated with Cy3-rabbit anti-goat IgG (1:50; BA1034, Sigma) in the dark for 45 min at 37°C. Hoechst 33258 (5 μg/mL; 861405, Sigma-Aldrich) was used to stain nuclei. The slides were then observed using a Zeiss confocal microscope (LSM 510 SYSTEM, Oberkochen, Germany).

2.7 Western blotting Western blotting was performed according to previous descriptions[11]. Briefly, protein samples (25 μg) were fractionated by 10% SDS/PAGE electrophoresis, transferred onto 0.45 μm nitrocellulose membranes (PA66485, Gelman, Ann Arbor, MI), and incubated with goat anti-PCBP1 (1:100; sc16504, Santa Cruz Biotechnologies, CA), polyclonal rabbit anti-TP53 (1:600; ab31333, Abcam, Cambridge, UK), goat anti-WNT7B (1:100; sc-26363, Santa Cruz Biotechnologies), or mouse anti-β-actin (1:500/1:2000; A5316, Sigma) antibody overnight (4°C). The membranes were then washed for 4 × 3 min in Tris-buffered saline with 0.05% Tween-20 (TBS-T), and incubated with horseradish peroxidase (HRP)-conjugated donkey anti-goat (1:2 000; sc-2020, Santa Cruz Biotechnologies), HRP-conjugated anti-rabbit (1:2 000; Zymed Laboratories, South San Francisco, CA) and HRP-conjugated anti-mouse (1:2 000; sc2318, Santa Cruz Biotechnologies) for 1 h at room temperature. After four washes in TBS-T, the proteins hybridized by the antibody were visualized with a Super Signal® West Pico chemiluminescent substrate (Pierce, Rockford, IL), according to the manufacturer's instructions. For each protein, the experiment was performed independently three times. Protein band densities were quantified by Image Master VDS software (Pharmacia Biotech., Uppsala, Sweden), and normalized to control.

2.8 Statistical analysis for qRT-PCR and Western blotting Data are expressed as means ± SEM. Statistical significance was assessed using one-way analysis of variance followed by the Newman-Keuls *post hoc* test of difference between groups. $P < 0.05$ was considered as statistically significant.

3 Results

3.1 qRT-PCR assays using SH-SY5Y cells qRT-PCR assays were used to measure the levels of the selected mRNAs (Table S1) in both the *PCBP1* knockdown and overexpression groups. The ratios of their expression to that of 18S are listed in Table S2. Eight of the genes (Fig. 1A) participated in the network (Fig. 1E). In the knockdown group, only *H2AFX* was significantly up-regulated, while the effects on *MAP2K2* and *TP53* were insignificant. The other four mRNAs of the knockdown group, *SAT*, *FOS*, *FST* and *WNT7B*, were significantly down-regulated compared with control. In the overexpression group, *H2AFX*, *MAP2K2* and *TP53* were significantly down-regulated, while *SAT*, *FOS* and *FST* showed no significant difference from control. However, *WNT7B* was significantly up-regulated compared to control.

3.2 Spatial interactions of the selected genes using Bayesian network assays Immunofluorescence analyses of PCBP1 distribution in SH-SY5Y cells indicated that it was mostly located in the cytoplasm, with small clusters in the nucleus (Fig. 1B–D). Bayesian assays showed that seven genes and *PCBP1* form an intimate network (Fig. 1E). Their interactions are displayed in Fig. 2A–H. The upstream gene *PCBP1* controls the expression of the other seven genes. After examining each gene, they were divided into upstream (red) and downstream genes (blue), and the regulated chain *PCBP1 – SAT – H2AFX – MAP2K2 – FOS – FST – TP53/WNT7B* was formed.

3.3 Protein levels of TP53 and WNT7B in SH-SY5Y cells The protein levels of TP53 and WNT7B corresponded with the detected mRNA levels ($P = 0.000\ 2$ for WNT7B and 0.000 0 for TP53 in overexpression compared to control; $P = 0.000\ 0$ for WNT7B and 0.483 3 for TP53 in knockdown compared to control) (Fig. 3).

4 Discussion

DBN analysis is an important approach for predicting gene regulatory networks from time course expression

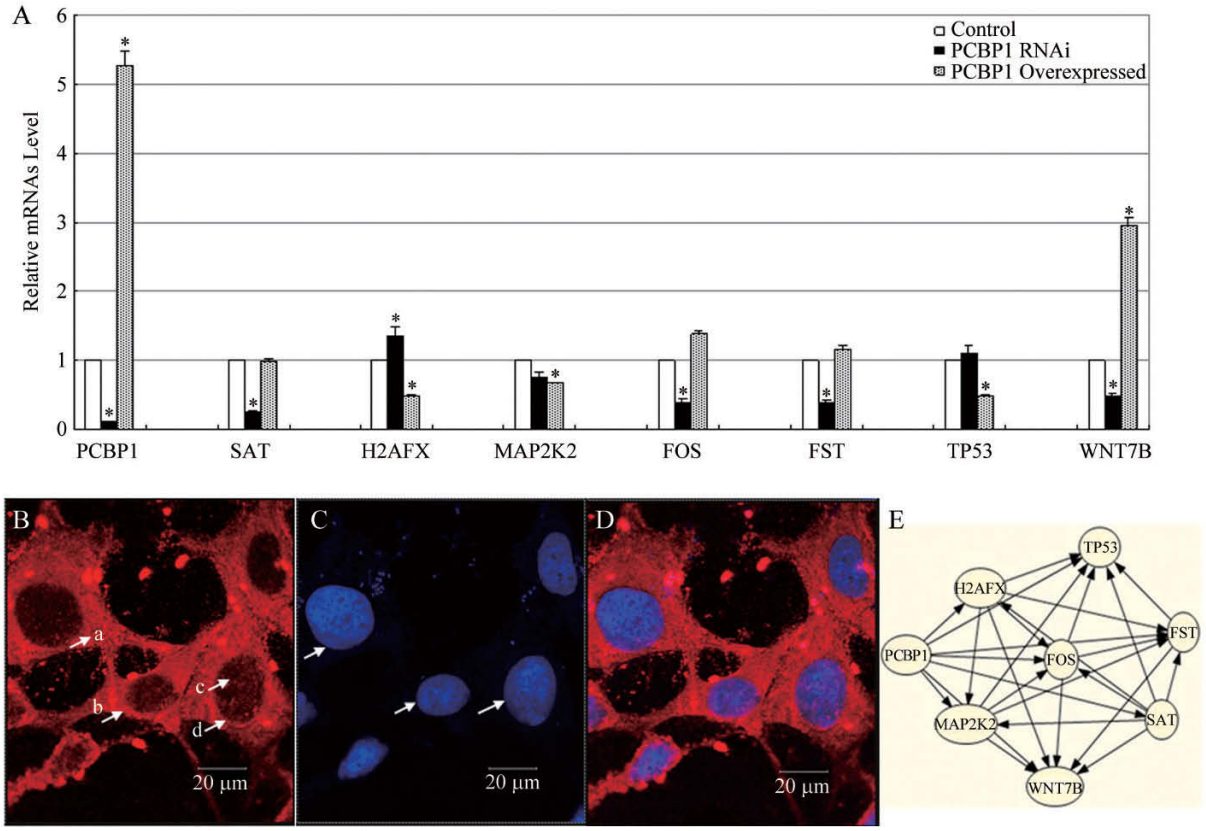

Fig. 1. PCBP1 distribution in SH-SY5Y cells and quantitative analysis of the transcripts of the major targeted genes in the Bayesian network. A: The selected transcripts among microarray results[11,12], in which *PCBP1* was overexpressed (OE) or knocked down (KD), were quantified using qRT-PCR. The relative amount of mRNA for each transcript isolated from the OE or the KD group was compared to the control cells. *Significant difference between the control and OE/KD groups. The *P* values for KO and OE respectively *vs* control were as follows: *PCBP1*, 0.000 4 and 0.000 0; *SAT*, 0.000 2 and 0.487 9; *H2AFX*, 0.013 5 and 0.043 3; *MAP2K2*, 0.134 5 and 0.028 5; *FOS*, 0.004 9 and 0.130 8; *FST*, 0.001 8 and 0.269 4; *TP53*, 0.334 6 and 0.031 6; *WNT7B*, 0.038 1 and 0.010 6. B: Immunofluorescent staining for PCBP1 in SH-SY5Y cells. Arrows indicate the distribution of PCBP1. C: Nuclei stained with Hoechst 33258. Arrows indicate the nuclei of the corresponding cells in B. D: B and C merged. E: Some of the genes in the pathways that had no direct spatial relationship with *PCBP1* were excluded using a Bayesian network assay. The other seven transcripts were located downstream of *PCBP1* and had direct or indirect relationships. Scale bars for B–D, 20 μm.

data[16,17]. However, two fundamental problems, low accuracy of prediction and excessive computational time, significantly reduce the effectiveness of current DBN methods. Zou *et al.* have presented a DBN-based approach with increased accuracy and reduced computational time compared with the existing methods[15]. The present study adopted the latter.

Knocking down endogenous *PCBP1*, in addition to overexpressing exogenous *PCBP1*, provides information about the global gene expression changes and helps to identify the PCBP1-targeted pathways, which may lead to a penetrating investigation of its biological functions[11,12]. Therefore, here we selected genes that participated in the pathways of the knockdown and overexpression groups and those detected by PF-2D, including *H2AFX* and *H2BFS*, and determined whether they had spatial relationships. Our experiments showed that PCBP1 is mainly distributed in the cytoplasm of a neuronal cell line, with a small amount detected in the nucleus. This is consistent with previous studies[7,13]. Besides, our previous work identified a great number of target transcripts of PCBP1 in SH-SY5Y cells, after either knockdown or overexpression of *PCBP1*, by using microarray analysis[11,12]. We knew that PCBP1 is not only an important RNA-binding protein that regulates

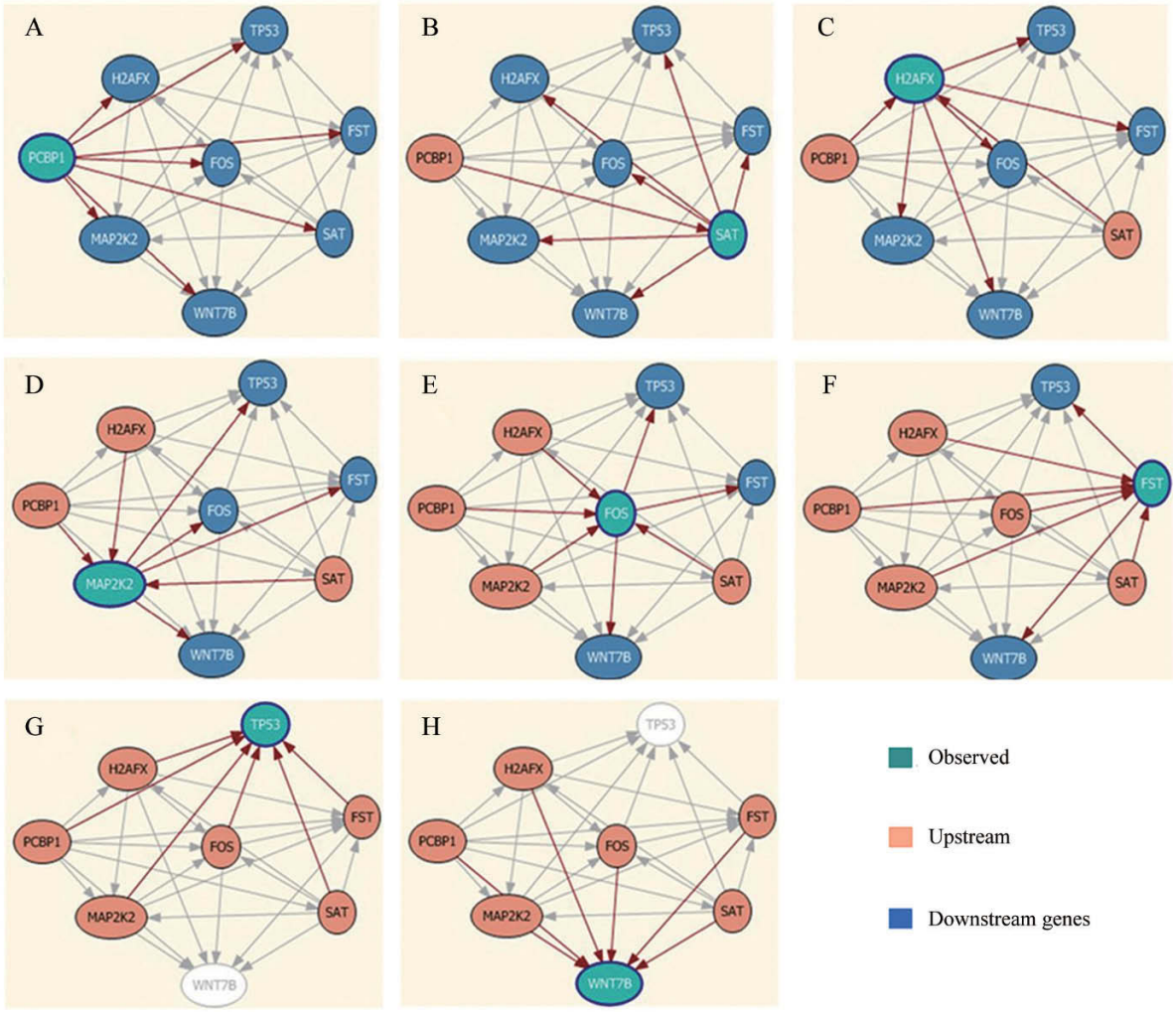

Fig. 2. Dynamic Bayesian networks for PCBP1-associated genes. Based on the RT-PCR data, eight genes including *PCBP1* displayed spatial interactions. A: When *PCBP1* was selected, the other seven genes were indicated to act as its downstream effectors. Then, as each gene was respectively selected, the order of regulation between the genes was determined. B: *SAT* is a downstream gene directly regulated by *PCBP1*. C: The transcripts upstream of *H2AFX* are *PCBP1* and *SAT*. D: In addition to *PCBP1* and *SAT*, *MAP2K2* is also regulated by *H2AFX*. *MAP2K2* and the former three genes (*PCBP1*, *H2AFX* and *SAT*) together regulate FOS (E) and then *FST* (F). *TP53* and *WNT7B* are relatively independent and are regulated respectively by the six genes in the network (red) except for both of them (G and H). Based on this analysis, PCBP1 can control the expression levels or activities of the seven genes among the pathways in which PCBP1 was either knocked down or overexpressed. In contrast, TP53 and WNT7B are presumably regulated by other genes.

the expression of many genes, but also a ssDNA-/dsDNA-binding protein, which is involved in DNA replication and transcription[7]. Meanwhile, it might also participate in protein-protein interactions[6]. The genes we selected, *PCBP1*, *SAT*, *H2AFX*, *FOS*, *FST*, *TP53*, *MAP2K2* and *WNT7B* were intimately associated in a spatial interaction network. They formed the chain *PCBP1 – SAT – H2AFX – MAP2K2 – FOS – FST – TP53/WNT7B* and had complicated relationships[15]. In addition, many participate in known pathways, such as apoptosis and cell cycle pathways (*TP53*), and the TGF-β signaling pathway (*FOS* and *FST*)[11,12,18]. The mRNA levels of *TP53*, *MAP2K2* and *H2AFX* changed inversely with respect to *PCBP1* levels. *H2BFS* and other pathway genes, however, had no direct relationship with *PCBP1* in the Bayesian network analysis. Our results showed that *TP53* was significantly

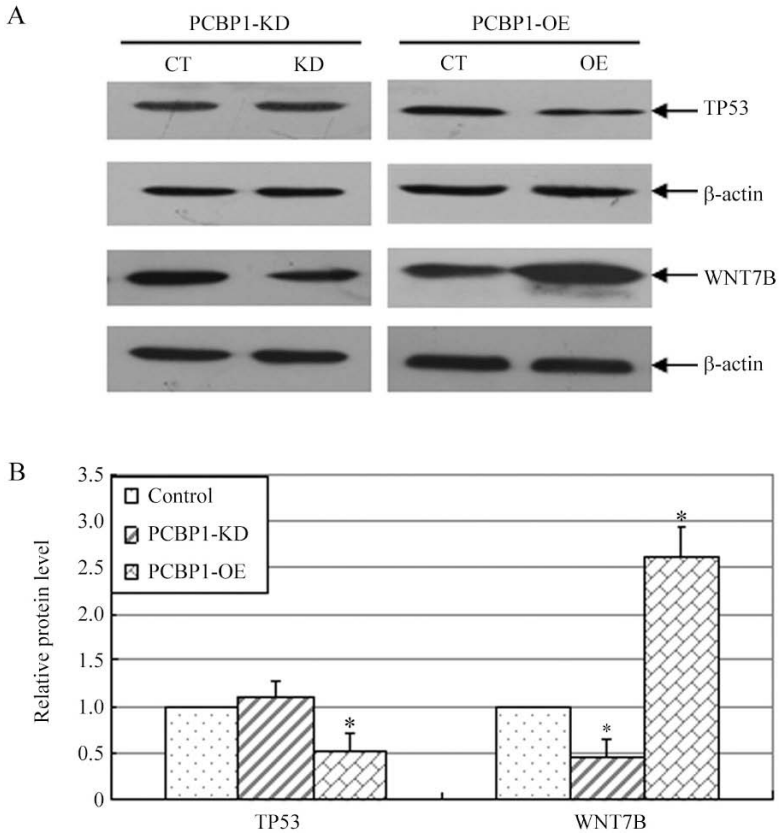

Fig. 3. Western blot analysis of protein levels of TP53 and WNT7B. A: Western blot bands of TP53 and WNT7B in control (CT), *PCBP1* knockdown (KD) and overexpressing (OE) SH-SY5Y cells. β-actin served as an internal control. B: Densitometric analysis of the bands in A. TP53 did not significantly differ between KD and control (P = 0.483 3). *P <0.001 *vs* control.

down-regulated in the *PCBP1* overexpression group. TP53 activation rapidly inhibits protein synthesis[19], and TP53 down-regulation in the *PCBP1* overexpression group suggested that many proteins regulated by TP53 would be increased in SH-SY5Y cells. We previously noted that cell replication is much slower when endogenous *PCBP1* is depleted, but much faster when exogenous *PCBP1* is overexpressed, compared to controls[11,12]. MAP2K2 is involved in the integrin pathway, and members of the integrin-associated pathway are associated with tumor cell infiltration and metastasis[20]. FOS and FST are involved in the TGF-β signaling pathway. In different cells and tissues, the TGF-β signaling pathway plays different roles[21,22]: it stimulates cell division in fibroblasts but inhibits growth in epithelial cells; it also weakens the cell adhesion effect through inducing the tyrosine phosphorylation of β-catenin, which facilitates cell movement and growth[23]. FST is a single-chain gonadal protein that specifically inhibits follicle-stimulating hormone release. *FOS* belongs to the FOS gene family, members of which encode leucine zipper proteins that can dimerize with proteins of the JUN family, forming the transcription factor complex AP-1[24]. Thus, the FOS proteins have been implicated as regulators of cell proliferation, differentiation, and transformation. *WNT7B* was down-regulated in cells with knocked-down *PCBP1* but up-regulated in cells with *PCBP1* overexpression. WNT proteins are a family of locally-acting signaling molecules required in diverse developmental events, including gastrulation, axis formation, cell polarity, stem cell differentiation, and organ development[25]. Some studies suggest that Wnt7B and Wnt8B act in forebrain patterning and morphogenesis[26]. PCBP1 may participate in the

corresponding process by regulating these genes. *SAT* is regulated by *PCBP1* and might also regulate other genes. Previous data have shown that SAT is predominantly localized to mitochondria[27]. SAT overexpression is correlated with tumorigenesis[28]. The basic structural unit of the nucleosome is an octamer of four core histones, H2A, H2B, H3, and H4, around which a 146-bp fragment of DNA is wrapped[29]. In addition to being a structural component of chromatin, histone H2AFX also maintains genomic stability and act as a multifunctional molecule[30]. The core histones form a nuclear multiprotein complex along with acetyltransferases, methyltransferases, hnRNPs, DNA helicase II, β-actin and vimentin, which may be associated with the structural modification of histones[31]. In addition, clinical studies have clearly shown the dose-dependent involvement of H2AFX in tumorigenesis[32]. These results suggest that PCBP1 controls H2AFX expression by regulating its transcription level. PCBP1 overexpression might down-regulate H2AFX, which would lead to DNA replication, but PCBP1 knockdown up-regulated H2AFX, which might retard DNA replication, repair the damaged DNA and induce recovery of damaged cells. The network (Fig. 2A–H) suggests that gene expression is regulated by spatial factors including complicated interactions between proteins and protein complexes. Our results indicate that *PCBP1* is the initial regulator in this kernel network.

In conclusion, our data suggest that PCBP1 plays an important regulatory role not only in planar pathways, but also in spatial networks in the nervous system. Many proteins, including PCBP1, compose an interactive spatial network. In this regional network, *H2AFX* is an important gene that can be reverse-regulated by *PCBP1* and *SAT*. Moreover, *PCBP1* can indirectly regulate its downstream genes through *H2AFX*.

Supplemental Data: Supplemental Data include two tables and can be found online at http://www.neurosci.cn/epData.asp?id=21.

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