ABBS

Human ZCCHC12 activates AP-1 and CREB signaling as a transcriptional co-activator

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Mouse zinc finger CCHC domain containing 12 gene (*ZCCHC12*) has been identified as a transcriptional co-activator of bone morphogenetic protein (BMP) signaling, and human *ZCCHC12* was reported to be related to non-syndromic X-linked mental retardation (NS-XLMR). However, the details of how human *ZCCHC12* involve in the NS-XLMR still remain unclear. In this study, we identified a novel nuclear localization signal (NLS) in the middle of human ZCCHC12 protein which is responsible for the nuclear localization. Multiple-tissue northern blot analysis indicated that *ZCCHC12* is highly expressed in human brain. Furthermore, *in situ* hybridization showed that *ZCCHC12* is specifically expressed in neuroepithelium of forebrain, midbrain, and diencephalon regions of mouse E10.5 embryos. Luciferase reporter assays demonstrated that ZCCHC12 enhanced the transcriptional activities of activator protein 1 (AP-1) and cAMP response element binding protein (CREB) as a co-activator. In conclusion, we identified a new NLS in ZCCHC12 and figured out that ZCCHC12 functions as a transcriptional co-activator of AP-1 and CREB.

Introduction

Zinc finger proteins constitute the most abundant protein superfamily in eukaryotes. Their functions are extraordinarily diverse, including DNA recognition, RNA packaging, transcriptional activation, regulation of apoptosis, protein folding and assembly, and lipid binding [1]. Up to now, at least 20 different classes of zinc finger motifs have been identified. They differ in the number of bound zinc ions, and in the identity and space of the ligating amino acids [2]. The most clearly identified zinc finger motif is C2H2 type zinc finger, and a lot of C2H2 zinc finger containing proteins such as Zfp191 can bind to specific DNA sequences [3]. Another zinc finger motif, CX2CX4HX4C (CCHC zinc finger, X can be any amino acid), has single-strand nucleic acid binding activity, which is mostly present in retroviral gag proteins (nucleocapsid) and essential for RNA binding, genomic packaging, and infectivity of retrovirus [4–7]. Many other eukaryotic proteins are also found to function through CCHC zinc finger mediated DNA binding and protein–protein interactions. For example, CNBP (CCHC-type zinc finger, nucleic acid binding protein) specifically binds to sterol regulatory element (SRE) to regulate the transcription of downstream target genes [8]; ZCCHC11 interacts with TIFA (TRAF-interacting protein with a Forkhead-associated domain) and suppresses TRAF6-dependent NF-κB activation during LPS treatment [9]; ZCCHC9 binds to NF-κB promoter and suppresses MAPK signal pathway [10]; and ZCCHC8 interacts with SKIV2L2 and RBM7 to involve in RNA processing/degradation [11]. In brief, eukaryotic CCHC zinc finger proteins have multiple functions such as transcription regulation and RNA processing.

Mouse ZCCHC12, also named as Smad-interacting zinc finger protein 1 (Sizn1), was recently identified as a positive modulator of dorsal–ventral patterning in forebrain during embryogenesis. It was a transcriptional co-activator of bone morphogenetic protein (BMP) signaling, encoding a 402-amino acid protein with an

N-terminal paraneoplastic antigen (MA)-homologous region, a single CCHC zinc finger motif, and a putative nuclear localization sequence [12]. Sequences of *ZCCHC12* from *Mus musculus*, *Homo sapiens*, *Rattus norvegicus*, *Bos taurus*, and *Equus caballus* have been reported in the NCBI database. Human ZCCHC12 shares 74% identity with mouse ZCCHC12, and similarly locates on chromosome X. Four different sequence variants in human *ZCCHC12* were characterized in 11 individuals with non-syndromic X-linked mental retardation (NS-XLMR), implicating *ZCCHC12* as a novel candidate gene for XLMR [13].

The majority of NS-XLMR-related proteins can be categorized into three distinct pathways: (i) Rho GTPases pathway modulating neuronal differentiation and synaptic plasticity; (ii) Rab GTPases pathway regulating synaptic vesicle cycling; (iii) gene expression regulation through chromatin remodeling, gene transcription, and RNA splicing/export/degradation [14]. However, the details of how human *ZCCHC12* involves in the NS-XLMR still remain unclear. And it is still unknown whether *ZCCHC12* involves in the regulation of other major transcription factors. Activator protein 1 (AP-1) and cAMP response element binding protein (CREB) are two crucial and well-characterized transcription factors. AP-1 is important for gene regulation during central nervous system dysfunction and development [15], and CREB-dependent gene expression is critical for a variety of functions in the developing and mature nervous system [16]. As both CREB and AP-1 cooperate with BMP signaling [17,18], we speculate that ZCCHC12 may affect their transcriptional activities.

In this study, we independently cloned the human *ZCCHC12* gene and analyzed its tissue expression pattern and subcellular localization. Human ZCCHC12 contains a CCHC zinc finger motif at its C-terminal and two putative nuclear localization signals (NLSs); it showed punctate distribution in the nucleus and acted as a positive regulator of transcription factors CREB and AP-1. Furthermore, ZCCHC12 is demonstrated to interact with c-Jun, which implicates that it may play the role of co-activator in transcription regulation through associating with CBP.

Materials and Methods

Plasmid constructions
The full-length human *ZCCHC12* (GenBank accession no. NM_173798) was cloned from a human brain cDNA library (Invitrogen, Carlsbad, CA, USA) by RT−PCR

using *Eco*RI site containing forward primer and *Kpn*I site containing reverse primer: forward, 5′-CCGAATTCGGATGGCTAGCATCATTGCAC-3′; reverse, 5′-GCGGTACCTTACAGTGGCTCAGAGAAGT-3′. The PCR products were ligated into pMD18-T vector (Takara, Dalian, China), and then subcloned into *Eco*RI and *Kpn*I sites of pCMV-Myc vector (Clontech, Palo Alto, CA, USA). To generate probes for whole-mount *in situ* hybridization, the coding region of mouse *ZCCHC12* (NM_028325) was amplified and inserted into *Eco*RI and *Xho*I sites of pBluescript II KS(−) (Stratagene, La Jolla, CA, USA).

Truncated human ZCCHC12 (1-N, 1-C, 2-N, and 2-C) were amplified from full-length human *ZCCHC12* by PCR, and subcloned into *Eco*RI and *Kpn*I sites of pCMV-Myc using previously described forward or reverse primers and the following primers: reverse primer of 1-N, 5′-GCGGTACCTCATTCCTCCCTTACCATTCTG-3′; forward primer of 1-C, 5′-CCGAATTCGGGAGGATTGGGATGATGCTA-3′; reverse primer of 2-N, 5′-GCGGTACCTCAACCACCACTGGTGGAAGG-3′; forward primer of 2-C, 5′-CCGAATTCGGTCTGGCTATAAGAATAACGA-3′.

Coding regions of human *c-Jun* (NM_002228), *c-Fos* (NM_005252), and *CREB* (NM_004379) were amplified from a human brain cDNA library, and cloned in frame with the Gal4-DNA binding domain into *Eco*RI and *Sal*I sites of pCMV-BD (Stratagene). Primers are: c-Jun forward, 5′-CGGAATTCATGACTGCAAAGATGGA-3′; c-Jun reverse, 5′-GCGTCGACCTCAAAATGTTTGCAAC-3′; c-Fos forward, 5′-CGGAATTCATGATGTTCTCGGGCTTCAA-3′; c-Fos reverse, 5′-GCGTCGACGTCACAGGGCCAGCAGCG-3′; CREB forward, 5′-CGGAATTCATGACCATGGAATCTGG-3′; CREB reverse, 5′-GCGTCGACGTTAATCTGATTTGTGG-3′. All the above constructs were confirmed by DNA sequencing.

Northern blot
Human multiple-tissue northern blot was performed as described previously [19] using α-^{32}P-ATP labeled full-length human ZCCHC12 as probe (Clontech). Briefly, the blot was prehybridized at 65°C for 2 h in 10× Denhardt's solution containing 2× saline sodium citrate (SSC), 1% SDS, and 100 μg/ml salmon sperm DNA, and then hybridized with the labeled ZCCHC12 probe at 65°C overnight in the solution containing 50% formamide, 2× SSC, 1% SDS, and 100 μg/ml salmon sperm DNA. After hybridization, the membrane was washed twice in 2× SSC and 1% SDS at room temperature for

30 min, twice in 1× SSC and 0.5% SDS at 65°C for 40 min, and once in 0.1× SSC and 0.1% SDS at 65°C for 40 min. The stripped membrane was reprobed with α-^{32}P-ATP labeled β-actin for loading control. Film (Kodak, Vancouver, WA, USA) was exposed at −80°C overnight.

Quantitative RT–PCR

Total RNA was isolated from various cell lines using TRIzol reagent (Invitrogen), and treated with RNase-free DNase to remove chromosomal DNA contamination. Subsequently, the cDNAs were synthesized according to the manufacturer's instruction (Qiagen, Valencia, CA, USA). PCR amplification was performed using the following primers: ZCCHC12-sense, 5′-ATCATTGCACGTGTCGGTAACA-3′; ZCCHC12-antisense, 5′-TGGCCATTATAGGACCGAGACTT-3′; β-actin-sense, 5′-AGAGCTACGAGCTGCCTGAC-3′; β-actin-antisense, 5′-AGCACTGTGTTGGCGTACAG-3′. PCR conditions for 30 cycles were: 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The PCR products of *ZCCHC12* and *β-actin* were 105 and 183 bp, respectively. The expression of *ZCCHC12* was normalized by the expression of *β-actin*.

Whole-mount *in situ* hybridization

Anti-sense and sense RNA probes, labeled with digoxigenin-UTP (Roche, Mannheim, Germany), were generated according to the manufacturer's instruction by *in vitro* transcription of linearized pBluescript II KS-ZCCHC12 with T7 or T3 RNA polymerase (Roche). For *in situ* hybridization, mouse embryos of E10.5 were dissected and fixed in 4% paraformaldehyde (PFA)/PBS at 4°C overnight. After washing twice with diethypyrocarbonate (DEPC) treated PBS containing 0.1% Tween-20 (PBST) for 10 min each, the mouse embryos were serially dehydrated in 25%, 50%, 75% methanol/DEPC-PBST, and twice in 100% methanol, then bleached with methanol containing 6% hydrogen peroxide for 1 h, and washed twice with PBST for 15 min each. Afterwards, the samples were treated with 100 μg/ml proteinase K (Sigma, St Louis, MO, USA) in PBST for 15 min at room temperature, refixed with 0.2% glutaraldehyde/4% PFA in PBST for 20 min, and washed twice with DEPC/PBST for 15 min each. Next, the embryos were subjected to hybridization with digoxigenin riboprobes, antibody incubation, and subsequent washes. Hybridized RNA probes were detected by alkaline phosphatase-conjugated anti-digoxigenin antibody (1:5000), and alkaline phosphatase staining was performed with NBT/BCIP solution. Finally, the embryos were photographed under a dissecting microscope (Carl Zeiss, Oberkochen, Germany).

Cell culture and transfection

HeLa, SK-N-SH, and A172 cells were obtained from ATCC (Rockville, IN, USA). HeLa cells were cultured in DMEM (Gibco, Rockville, IN, USA) supplemented with 10% fetal calf serum (Gibco), 100 U/ml penicillin, and 100 μg/ml streptomycin; HEK293FT cells were cultured according to a standard protocol (Invitrogen); human neuroblastoma SK-N-SH cells were cultured in EMEM (Gibco) supplemented with 10% fetal calf serum and 0.1 mM non-essential amino acids (Gibco); and human glioblastoma A172 cells were cultured in DMEM supplemented with 10% fetal calf serum and 4 mM glutamine. All cells were incubated in a humidified atmosphere at 37°C with 5% CO$_2$. HeLa, SK-N-SH, and A172 cells were transfected using Lipofectamine™ 2000 according to the manufacturer's instructions (Invitrogen), and HEK293FT cells were transfected using routine calcium phosphate precipitation method [20]. Cells were used for further study 48 h after transfection.

Immunofluorescent staining

Cells cultured on glass coverslips were fixed with 4% PFA/PBS. Immunofluorescent staining of full-length and truncated human ZCCHC12 was performed as described previously [21]. Rabbit polyclonal anti-Myc antibody (Sigma) and Alexa Fluor® 488 goat anti-rabbit antibody (Invitrogen) were used as primary (1:1000) and secondary antibody (1:1000), respectively. Hoechst 33258 (Sigma) was used to stain the nuclei. Fluorescent signals were analyzed using a fluorescence microscope (Carl Zeiss).

Luciferase reporter assays

Reporter plasmids pAP1-Luc and pCRE-Luc (Clontech) contain multiple copies of AP-1 response element and cAMP response element fused TATA-like promoter region, respectively. They were used to investigate the potential roles of human *ZCCHC12* in the regulation of transcription factors AP-1 and CREB in HeLa and HEK293FT cells. For the GAL4-based transcription regulatory assays, HEK293FT cells were transiently transfected with pL8G5-Luc reporter and other indicated vectors. The luciferase activity assay was performed using luciferase assay system (Promega, Madison, WI, USA) according to the manufacturer's instruction. Relative luciferase activities were normalized with

transfection efficiency through co-transfection with pCMV-LacZ and spectrophotometry analysis. Each experiment was performed in triplicate.

Co-immunoprecipitation

HEK293FT cells were transfected with pCMV-Myc-ZCCHC12. Twenty-four hours later, cells were harvested and lysed in RIPA buffer [50 mM Tris–HCl (pH 7.2), 150 mM NaCl, 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, and 0.1% (w/v) SDS] with protease inhibitors. Immunoprecipitation was performed using mouse monoclonal anti-Myc antibody (Santa Cruz Biotech, Santa Cruz, CA, USA) or pre-immune serum (negative control). Co-precipitated proteins were subjected to electrophoresis on 10% SDS–polyacrylamide gel, and then analyzed by western blot analysis using 1:200 dilution of rabbit polyclonal anti-c-Jun antibody (Santa Cruz Biotech). The immunoprecipitated Myc-ZCCHC12 was detected using anti-Myc polyclonal antibody (Sigma) after stripping the membrane with

Restore™ Western blot Stripping Buffer (Pierce, Rockford, IL, USA).

Results

Cloning and sequence analysis of ZCCHC12

The full-length human *ZCCHC12* was isolated by RT–PCR from a human brain cDNA library, and comparative sequence analysis was performed based on the information published in NCBI human genome database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene&cmd=search&term=ZCCHC12). Human *ZCCHC12* gene locates on chromosome Xq24 and contains four exons encoding 402 amino acids with a predicted molecular mass of 45.4 kDa. This protein contains one typical Cys-X2-Cys-X4-His-X4-Cys (CCHC) type zinc finger motif in the C-terminal and two NLSs [**Fig. 1(A)**], and it does not contain any other identified motifs or domains.

Up to now, only three *ZCCHC12* sequences (*H. sapiens*, *R. norvegicus*, and *M. musculus*) and two predicted *ZCCHC12* sequences (*B. taurus* and *E.*
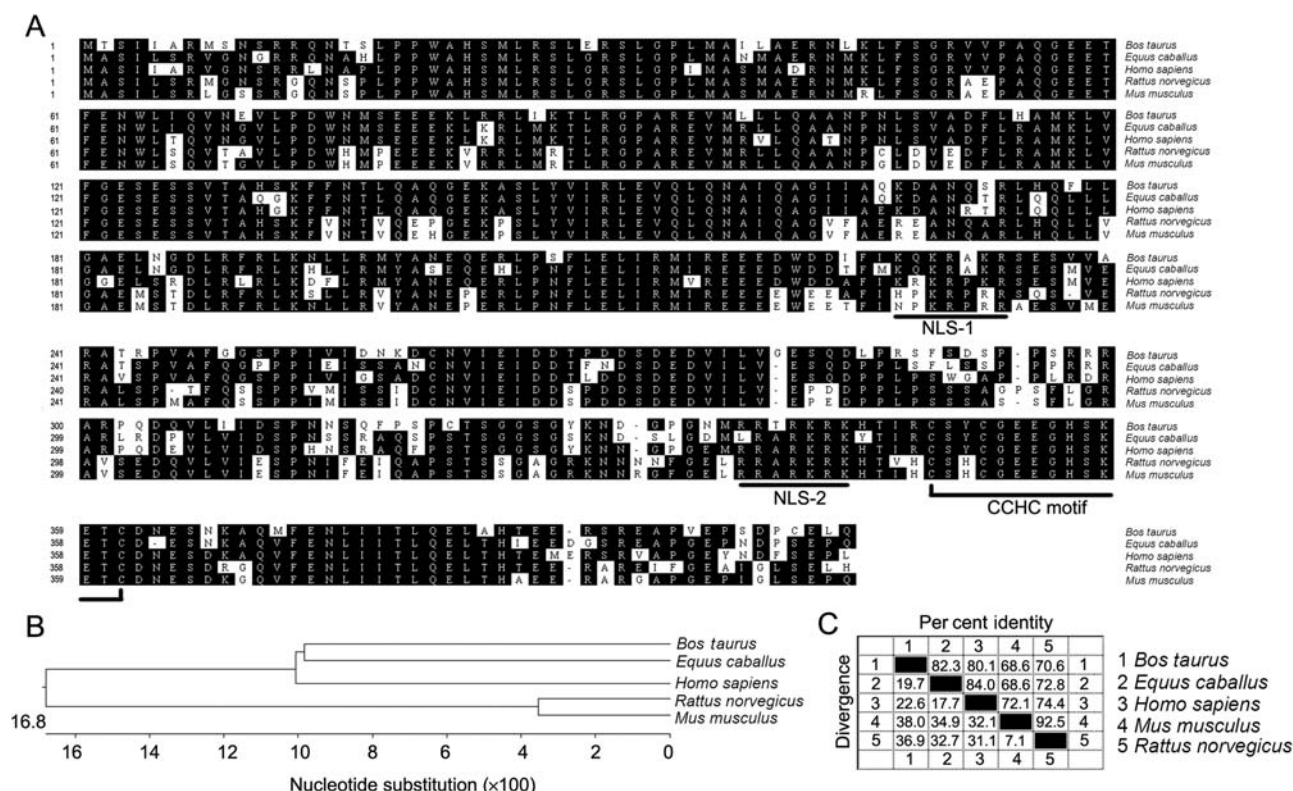

Figure 1 Sequence analysis of ZCCHC12 (A) Alignment analysis of amino acid sequences of ZCCHC12 from *B. taurus*, *E. caballus*, *H. sapiens*, *M. musculus*, and *R. norvegicus*. Identical amino acid residues were shaded dark, and gaps between amino acids were filled with dashes. CCHC zinc finger motif and NLS were underlined. (**B**) The evolutionary relationship among these five ZCCHC12 proteins was shown by phylogenetic tree analysis. (**C**) The identity percentage between any two ZCCHC12 proteins from different species.

caballus) have been reported in NCBI database. Using the DNAStar program, these five *ZCCHC12* genes were aligned [**Fig. 1(A)**]. An evolutionary relationship among these proteins was also examined by phylogenetic tree analysis [**Fig. 1(B)**]. The identity percentage between any two ZCCHC12 proteins from different species was shown in **Fig. 1(C).** ZCCHC12 protein of *H. sapiens* shares 74.4% identity with that of *M. musculus*, and 84% identity with that of *E. caballus*. Although sequences of *ZCCHC12* from other species are unavailable yet, it seems that *ZCCHC12* could be conserved. No homologous genes were found in genomes of bacteria, fungi, plants, invertebrate, and even vertebrate such as *Danio rerio*, suggesting that *ZCCHC12* may specifically express in higher animal.

Expression profile analysis of *ZCCHC12*

To examine the tissue expression profile of human *ZCCHC12*, human multiple-tissue northern blot analysis was performed and the result was shown in **Fig. 2(A).** A 2.4 kb transcript responsible for *ZCCHC12* strongly expressed in human brain, but no signals were detected in heart, liver, lung, spleen, kidney, testis, and skeletal muscle, indicating that *ZCCHC12* is mainly present in human brain.

The expression of *ZCCHC12* in various human cell lines was also tested by quantitative RT−PCR analysis. As shown in **Fig. 2(B),** human *ZCCHC12* was expressed not only in brain-derived cell lines but also in other tissue-derived cell lines. The expression of *ZCCHC12* was relatively high in neuroblastoma SK-N-SH cell, moderate in glioblastoma A172 cell and other tissue-derived cells (cervical carcinoma HeLa cells, breast cancer MCF7 and MDA-MB-231 cells, permanent embryonal kidney HEK293FT cells, and fibrosarcoma HT1080 cells), and low in hepatocellular carcinoma HepG2 cell. *ZCCHC12* as a brain-specific gene was expressed in many other tissue-derived tumor cell lines, implying that *ZCCHC12* may be related to tumorigenesis.

To further check the expression pattern of *ZCCHC12* in embryos, the RNA whole-mount *in situ* hybridization of E10.5 mouse embryos was carried out. Strong signals were detected in neuroepithelium of forebrain, midbrain, and diencephalon [**b** and **c** in **Fig. 2(C)**] using anti-sense ZCCHC12 RNA as a probe, whereas the negative control (corresponding sense probe) did not give out any signal [**a** in **Fig. 2(C)**]. This again demonstrated that *ZCCHC12* plays a role in the brain development during early embryogenesis.
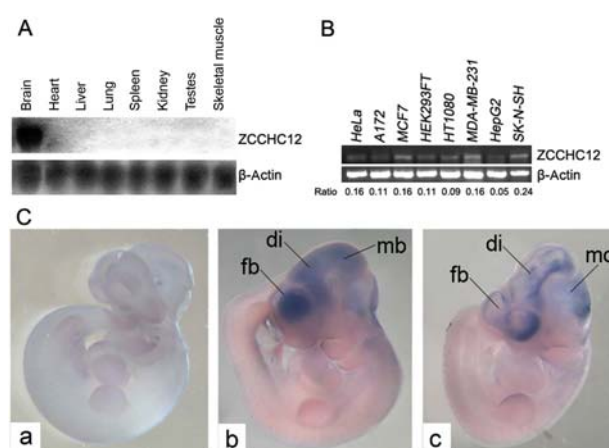

Figure 2 Expression profile of ZCCHC12 (A) Multiple-tissue northern blot analysis of human ZCCHC12 mRNAs. The membrane was probed with α-^{32}P-ATP-labeled human ZCCHC12 cDNA (top), and stripped and reprobed with α-^{32}P-ATP-labeled β-actin cDNA probe for loading control (bottom). A 2.4 kb transcript of ZCCHC12 was only detected in brain. (B) Quantitative RT−PCR analysis of ZCCHC12 transcript in various human cell lines. The gel was representative of three independent experiments. β-Actin (bottom) was used to verify equal loadings, and densitometric analysis of ZCCHC12/β-actin ratios was performed with Gel-Pro Analyzer 4.0 software. (C) Whole-mount *in situ* hybridization of ZCCHC12 mRNAs in E10.5 mouse embryos. **a,** embryos hybridized with negative control sense probe, no signal was detected. **b** and **c,** embryos hybridized with antisense probe, strong signals were detected in neuroepithelium of forebrain (fb), midbrain (mb), and diencephalon brain (di).

ZCCHC12 is a nuclear protein and activates the transcriptional activities of AP-1 and CREB

As human ZCCHC12 contains two putative NLSs, to examine if it localizes in the nuclei, expression vector pCMV-Myc-ZCCHC12 was transfected into HeLa, SK-N-SH, and A172 cells. Immunofluorescent staining in **Fig. 3(A)** showed a nuclear punctate distribution of ZCCHC12 in HeLa (**a**−**c**), SK-N-SH (**d**−**f**), and A172 (**g**−**i**) cells. This implies that ZCCHC12 is a nuclear protein, and probably bind to chromatin and activate transcription of target genes [22].

To identify which putative NLS directs the nuclear localization, four truncated ZCCHC12 were constructed [**Fig. 3(B)**]. Immunofluorescent staining of HeLa cells transfected with the truncated form of 2-N showed very clear nuclear foci similar to the full-length ZCCHC12 protein [**Fig. 3(C)**]. However, truncated forms of 1-N, 1-C, and 2-C were found to spread in the entire cell. 1-C localized predominantly within the nucleus with very few foci; in contrast, 1-N and 2-C localized predominantly in the cell cytoplasm. The results indicated that
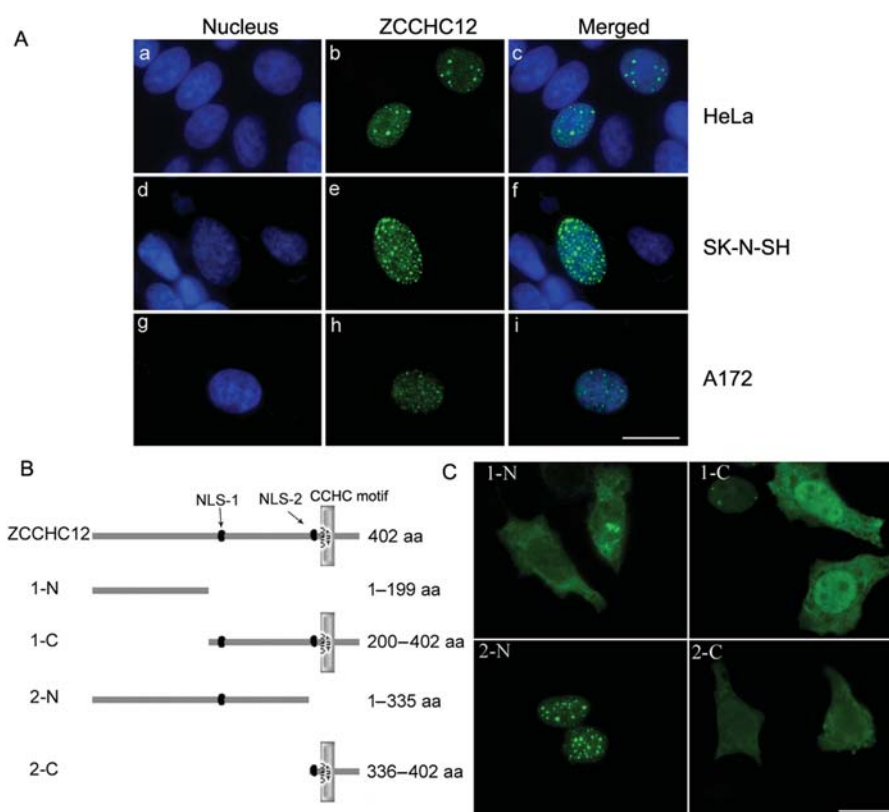
Figure 3 Subcellular localization of full-length and truncated human ZCCHC12 (A) Nuclear localization of full-length ZCCHC12 in human cell lines. The Myc-ZCCHC12 expression vector was transfected into HeLa, SK-N-SH, and A172 cells. The nuclei were stained with Hochest 33258 (**a, d,** and **g**). The Myc-ZCCHC12 fusion protein was detected by anti-Myc antibody (**b, e,** and **h**). Scale bar = 10 μm. (**B**) Schematic diagram of full length and truncated human ZCCHC12 protein. Human ZCCHC12 protein contains two NLSs and one CCHC zinc finger motif. (**C**) The first NLS and N-terminal of human ZCCHC12 are necessary for its nuclear localization. Immunofluorescent staining was performed using anti-Myc polyclonal antibody in HeLa cells transfected with truncated Myc-tagged ZCCHC12. Scale bar = 10 μm.

the first NLS and the N-terminal of ZCCHC12 are necessary for its nuclear localization.

To investigate the role of human ZCCHC12 in the nuclei, we examined its effects on regulation of AP-1 and CREB signal pathways in HeLa cells. When pAP1-Luc or pCRE-Luc was cotransfected with pCMV-Myc-ZCCHC12 in HeLa cells, the relative luciferase activities were statistically significantly up-regulated compared with vector control [**Fig. 4(A)**]. Luciferase reporter assays of ZCCHC12 truncated forms showed that 2-N activated AP-1 and CREB signaling pathways most significantly, which is consistent with the punctate nuclear distribution (data not shown). To exclude the cell-specific effects, the experiment was also performed in HEK293FT cell line. As shown in **Fig. 4(B),** similar results were obtained. These results indicated that ZCCHC12 activates the transcriptional activities of AP-1 and CREB.
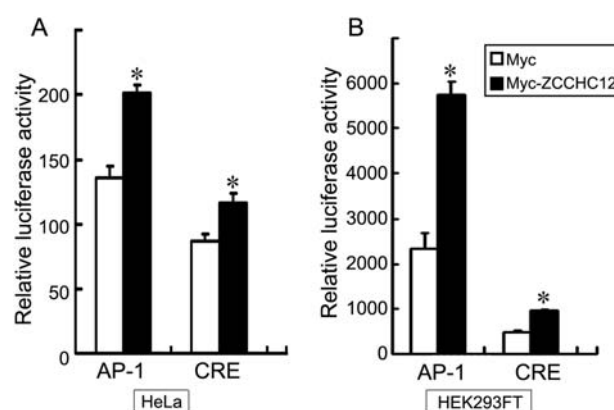

Figure 4 ZCCHC12 activates the transcriptional activities of AP-1 and CREB (**A**) Luciferase reporter vectors (pAP1-Luc or pCRE-Luc) were co-transfected with pCMV-Myc-ZCCHC12 or pCMV-Myc into HeLa cells. Forty-eight hours later, cell lysates were subjected to luciferase assay. (**B**) The same experiment was performed in HEK293FT cells. Statistical comparisons were determined using t-test. *$P < 0.05$ compared with the respective vector control.

ZCCHC12 functions as a transcriptional co-activator of AP-1 and CREB, and interacts with c-Jun

To verify the mechanism of ZCCHC12 regulating the transcriptional activities of AP-1 and CREB, GAL4-based transcription regulatory assays were performed. Because transcription factor AP-1 is composed of c-Jun and c-Fos, pGal4DB-c-Jun, pGal4DB-c-Fos, and pGal4DB-CREB were constructed for this purpose. pGal4DB-c-Jun and pL8G5-Luc reporter construct were cotransfected into HEK293FT cells together with pCMV-Myc or pCMV-Myc-ZCCHC12. Only ZCCHC12 overexpression did not enhance the report activity. But as expected, pGal4DB-c-Jun showed strong transactivation of pL8G5-luc reporter [23], and cotransfection of pCMV-Myc-ZCCHC12 with pGal4DB-c-Jun further enhanced the reporter transcriptional activity [**Fig. 5(A)**]. ZCCHC12 overexpression also strongly increased Gal4DB-c-Fos and Gal4DB-CREB responsive L8G5-Luc reporter gene expression [**Fig. 5(B,C)**]. These results indicated that ZCCHC12 functions as a transcriptional co-activator of AP-1 and CREB, possibly through interaction with AP-1 and CREB.

To demonstrate the possible interaction between ZCCHC12 and c-Jun in mammalian cells, half endogenous co-immunoprecipitation of these two proteins was performed. First, HEK293FT cells were transiently transfected with expression vector pCMV-Myc-ZCCHC12. Twenty-four hours later, cells were harvested, and the lysates were immunoprecipitated with either control pre-immune serum or anti-Myc antibody. The co-immunoprecipitated proteins were examined for the presence of c-Jun by immunoblotting assay using anti-c-Jun polyclonal antibody. As shown in **Fig. 6**, c-Jun could be co-immunoprecipitated by Myc tagged ZCCHC12 (lane 2), but not by control pre-immune serum (lane 1). This result demonstrated that ZCCHC12 and c-Jun could be found in the same complex. Considering the punctate distribution of ZCCHC12 in nuclear, we speculate that ZCCHC12 might activate AP-1 through recruiting AP-1 to the transcription sites on the chromatin.

Discussion

In this study, we independently cloned human *ZCCHC12*, and did comparative sequence analysis of *ZCCHC12* from *M. musculus*, *H. sapiens*, *R. norvegicus*, *B. Taurus*, and *E. caballus* published in NCBI genome database. Previous study has reported one NLS near the
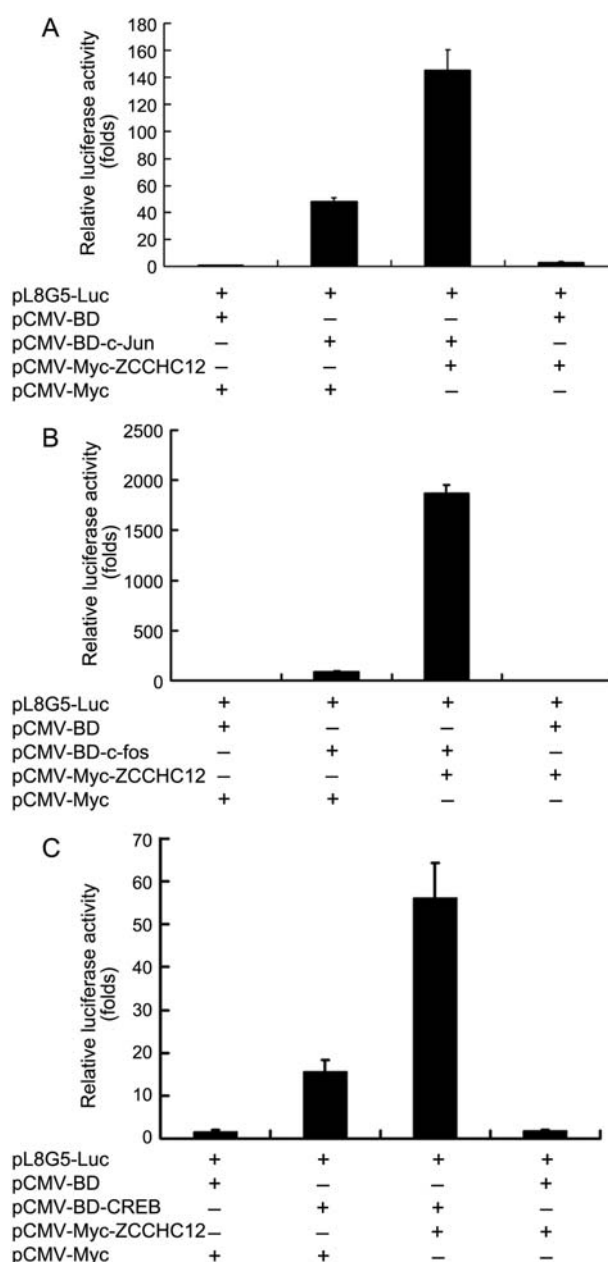

Figure 5 ZCCHC12 functions as a transcriptional co-activator of AP-1 and CREB (**A**) ZCCHC12 effectively enhanced c-Jun-mediated transactivation in GAL4-based transcription regulatory assays in HEK293FT cells. The pL8G5-Luc reporter vector was cotransfected with plasmids encoding Gal4-c-Jun, Myc or Myc-ZCCHC12. At 48 h post-transfection, cells were harvested for luciferase assay. (**B** and **C**) ZCCHC12 strongly increased c-Fos and CREB-mediated transactivation.

CCHC motif in the C-terminal of mouse ZCCHC12 [13]. Our alignment analysis showed that human ZCCHC12 contains another putative NLS in the middle of the amino acid sequence. Immunofluorescent staining of ZCCHC12 in various human cell lines revealed its
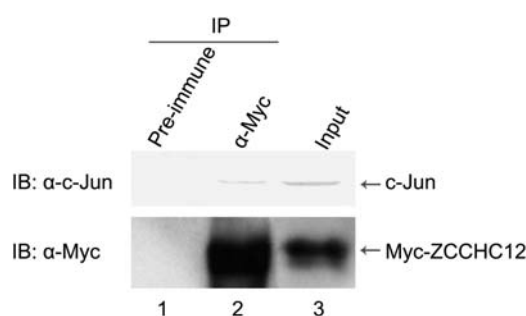
Figure 6 ZCCHC12 interacts with c-Jun in HEK293FT cells Cell lysates (800 μg) of HEK293FT cells transfected with expression vector pCMV-Myc-ZCCHC12 was precipitated with anti-Myc antibody for detection of c-Jun (lane 2). Immunoprecipitates with pre-immune serum were used as negative control (lane 1). A total of 50 μg of cell extract were used as positive control (lane 3, input). The co-immunoprecipitated c-Jun and the expression of c-Jun in cell extract were detected by anti-c-Jun polyclonal antibody. The immunoprecipitated Myc-ZCCHC12 and the expression of Myc-ZCCHC12 in cell extract were detected by anti-Myc polyclonal antibody. IP, immunoprecipitation; IB, immunoblot.

nuclear punctate distribution. Protein truncation assay further indicated that the newly identified NLS was responsible for the nuclear localization. However, without the N-terminal, human ZCCHC12 localized predominantly within the nucleus only with very few foci, which implies that some other nuclear proteins might help to recruit ZCCHC12 onto chromatin through binding its N-terminal. Informatics analysis of human ZCCHC12 could not characterize any other identified protein domains or motifs. More experiments might be required for elucidating this issue.

Similar to mouse *ZCCHC12*, human *ZCCHC12* was demonstrated by multiple-tissue northern blot assay to highly and specifically express in the brain. Yet, quantitative RT−PCR experiments detected *ZCCHC12* not only from brain-derived cell lines (SK-N-SH and A172) but also from other tissue-derived cell lines (HeLa, MCF7, MDA-MB-231, HEK293FT, HT1080, and HepG2). Because DNA methylation plays a key role in the regulation of gene expression in specific tissues and tumor cells [24], we used online software Methylator (http://www.bioinfo.tsinghua.edu.cn/~tigerchen/memo/form.html) to predict methylation sites and found many potential methylation sites present in the promoter region of *ZCCHC12*. Therefore, we speculate that expression of *ZCCHC12* in other tissue-derived cell lines is possibly caused by alteration of DNA methylation in *ZCCHC12* promoter region. Furthermore, as most of these cell lines are from tumor patients, even HEK293FT cell is also from transformed human embryonic kidney, *ZCCHC12*

detection in these cell lines implied that it may play important roles during tumorigenesis. In addition, the expression of *ZCCHC12* was relatively higher in neuroblastoma SK-N-SH cell than in glioblastoma A172 cell, suggesting that *ZCCHC12* expressed stronger in neurons than in glia cells.

Originally, mouse ZCCHC12 was identified from a regulator screen of dorsal−ventral patterning in forebrain of mouse early embryos, and the expression of *ZCCHC12* in E12.5 and E14.5 mice was restricted to the ventral forebrain [12]. However, our whole-mount *in situ* hybridization of mouse E10.5 embryo indicated that *ZCCHC12* was expressed in forebrain, midbrain, and diencephalon regions. The differences of the expression pattern of mouse *ZCCHC12* in the brain between E10.5 and E12.5 mice may be due to the different development stages. Moreover, microarray expression data indicated that in E16 and E18 brain, mouse *ZCCHC12* expressed stronger in a thin layer of cells in the lower cortical plate of the cingulate cortex than in septum, striatum, diencephalons, and amygdale [25]. Taking together, the expression pattern of *ZCCHC12* in brain is a timing event and developmentally regulated.

AP-1 and CREB are two important transcription factors. AP-1 regulates a wide range of cellular processes, including cell proliferation, death, survival, and differentiation [26]. CREB is a key mediator of stimuli-induced nuclear responses underlaid the development, function, and plasticity of the nervous system, and it is critical for synaptic function in neurons [16]. Mouse ZCCHC12 has been reported to activate BMP signaling by interacting with Smad1 and associating with CBP [12]. CBP has intrinsic histone acetyltransferase activity, and can interact with components of the basal transcription machinery and a variety of different DNA-binding factors to activate the transcription [27,28]. In addition, CBP is proven to be the co-activator and binding partner for AP-1 [29] and CREB [30,31]. Since human ZCCHC12 shares 74.4% identity with mouse ZCCHC12, analogously, human ZCCHC12 could be possibly associated with CBP too. In this study, we demonstrated that human ZCCHC12 acted as a co-activator, and positively regulated the transcriptional activity of AP-1 and CREB. Considering that ZCCHC12 expresses at high level in neurons, activates CREB signaling pathways, and is a candidate gene of X-linked mental retardation [13] or responsible for Orphan Parkinson loci [32], ZCCHC12 is probably involved in the long-term potentiation of neurons.

In conclusion, we characterized human *ZCCHC12* as a nuclear protein, specifically expressed in human brain

and mouse embryonic brain, functioned as a positive modulator of AP-1 and CREB signal. Moreover, we found a novel NLS in human ZCCHC12 necessary for the nuclear localization and punctate distribution. ZCCHC12 can bind to c-Jun to activate AP-1 transcription. However, the regulation mechanism of CREB by ZCCHC12 still needs further investigation in the future. The roles of *ZCCHC12* played in brain development also need to be defined.

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