Identification and Verification of Cdk5 Phosphorylated Deubiquitinating Enzyme BRCC3

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Research Article

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Abstract

Background: It is known that the expression of the deubiquitinating enzyme BRCA1-BRCA2-containing complex subunit 3(BRCC3) is increased, as well as Cyclin-dependent protein kinase 5(Cdk5) in Parkinson's Disease(PD), and both of them to be involved in neuroinflammatory response. But the regulatory mechanism of Cdk5 on the post-translational modification of BRCC3 remains unclear. This study aimed to investigate the phosphorylation of Cdk5 on the BRCC3.

Methods and Results: Cdk5 phosphorylation of BRCC3 was predicted by GPS5.0 software. His-BRCC3 plasmid was constructed by cloning the BRCC3 gene into pGEX-6P-1 vector, and then His-BRCC3 fusion protein was induced by IPTG and purified by His-tag purification agarose. The BRCC3 fusion protein was reacted with Cdk5 in vitro, and the phosphorylation was detected by mass spectrometry and Western blot. The results showed that multiple phosphorylation sites were predicted by GPS5.0, and the His-BRCC3 fusion protein was successfully induced and purified. In vitro kinase assay, Western blot and mass spectrometry identified that Cdk5 can phosphorylate BRCC3.

Conclusions: It has been demonstrated that the protein kinase Cdk5 can phosphorylate the deubiquitinating enzyme BRCC3 in vitro, which provide evidences for further study on the mechanism of neurodegeneration.

Introduction

Phosphorylation, methylation, ubiquitination, acetylation and glycosylation are conventional posttranslational modifications of proteins. Phosphorylation often occurs during development of some diseases and is an important regulatory mode in prokaryotes and eukaryotes[1]. This process is completed by a series of kinase reactions in the organism, and plays a significant role in enzyme activities and cascade reactions. Proteins undergo posttranslational modification during synthesis. Some specific modifications are closely related to certain diseases, and improper modifications may cause dysfunction of the body.

Cyclin-dependent kinase 5 (Cdk5), an important member of the Cdk family, is a proline-dependent serine/threonine protein kinase. As a protein kinase, many substrate proteins can be phosphorylated by Cdk5. These phosphorylation processes are involved in the molecular mechanism of disease and a variety of cellular signaling pathways, such as apoptosis, autophagy, DNA damage, etc. Cdk5 is highly expressed in the nervous system and is involved in neuronal differentiation, survival and synaptic occurrence, which is associated with the development of neurodegenerative diseases (eg. Parkinson's disease (PD), Alzheimer's disease (AD))[2-4]. Cdk5 protein kinase alone does not have enzymatic activity, and it needs to combine with the companion cytokines cyclins P35 or P39 to form a complex. P35 can be spliced by specific proteases to form P25, which changes the characteristics of Cdk5 and then participates in neurodegenerative diseases through phosphorylation[5].

Mouse-BRCC3 and its human homolog BRCC36, is a component of the BRCA1-A complex, which widely expressed in different tissues such as brain, muscle, kidney, etc. and regulates the abundance of Lys63-linked ubiquitin chains[6]. It has many connections with cell signal transduction, cell cycle regulation, DNA damage repair[7], and NF-kB signaling pathway[8, 9]. Studies have shown that BRCC3 can regulate the activity of NLRP3 inflammation through deubiquitylation[5, 10]. The activation signal can promote the deubiquitylation of NLRP3 through the lysine-specific deubiquitinating enzyme BRCC3 and then activate NLRP3 inflammation[5, 10]. The ubiquitin-dependent mechanism contributes to the development of potential inhibitor and activator therapeutic target drugs for inflammatory diseases.

We and others showed previously that Cdk5/P25 involved in the development of Parkinson's disease through phosphorylation[11-14]. Especially, Cdk5 can regulate the expression of BRCC3 in PD[5]. To gain further insight into the relationship between Cdk5 and BRCC36, we predicted the phosphorylation sites of Cdk5 modified BRCC3 using GPS5.0 software. And phosphorylation mass spectrometry indicated that BRCC36 was really the substrate of Cdk5 kinase. Besides this, we also confirmed that BRCC3 can be phosphorylated by Cdk5 using Western blot. Our further analyses of these suggest that Cdk5 is an upstream kinase of BRCC3 in vitro. The study provides a strong basis for further exploring the molecular mechanism of Cdk5-mediated BRCC3 involvement in inflammation in PD.

Materials And Methods

Main Reagents and Antibodies

Cdk5/p25 protein (active complex) was purchased from Millipore (cat#14-516, Birrica, Massachusetts,USA). The restriction enzyme BamHI, XholI and T4 ligase were purchased from NEB (cat#R0136，cat#R0146，cat#M0202，Ipsich, MA, USA). Competent cells of BL21 and DH5α were from Sangon Bioengineering (cat#B528414，cat#B528413，Shanghai,China). IPTG was from Beyotime Biotechnology (cat#ST098，Shanghai, China). Ni-NTA His-tag purification agarose was purchased from MedChemExpress (cat#HY-K0210，Monmouth Junction, NJ, USA). Anti-phospho-Ser/Thr-proline antibody was obtained from Cell Signaling Technology (cat#9381，Danvers, MA, USA). The antibody against His-tag was purchased from Proteintech (cat#66005-1，Rosemont, IL, USA). Antibodies were used according to the manufacturer's instructions. All the secondary antibodies (1:10,000) were procured from Jackson ImmunoResearch (Bar Harbor, ME, USA).

Prediction of bioinformatics software

Phosphorylation prediction software GPS5.0 (Group-based Prediction System Version 5 http://gps.biocuckoo.cn.) was used to estimate whether Cdk5 phosphorylating BRCC3, based on the amino acid of mouse-BRCC3 protein (NP_001159929.1). To compare, contrast and analyze the homology and conservatism of BRCC3 in common mammals, DNAMAN software was used to blast the amino acid sequences of human-BRCC3 (NP_001018065.1), mouse-BRCC3 (NP_001159929.1) and rat-BRCC3 (NP_001120772.1) derived from NCBI database.

Construction of pGEX-6P-1-His-BRCC3 plasmid

Primer design and synthesis

Based on gene bank BRCC3 (NM_001166457.1) and vector (pGEX-6P-1) sequences, a pair of primers of BRCC3 with BamHI/XhoI as the restriction sites were designed by Primer Premier 6.0 (BRCC3-F 5'-CGGGATCCATGGCGGTGCAGGTGGT-3' and BRCC3-R 5 '-CCGCTCGAGTTC TAGGGAAGACAGCTCTT -3') and synthesized by Thermo Fisher Scientific (Waltham, MA, USA). The product length is 876bp (full length of BRCC3 gene).

Target gene amplification and plasmid recombination

The plasmid pEGFP-BRCC3 generated previously by our research team was invoked as DNA template, and the target fragment was amplified by RT-PCR. The PCR product was run electrophoresis with 1% agarose gel. The target DNA gel was harvested according to the molecular weight of BRCC3 and extracted with DNA gel extraction kit. In the presence of T4 ligase, the purified DNA product of BRCC3 was ligated at room temperature for 1h with the pGEX-6P-1 vector which digested by BamH I and Xhol I. The ligation product was transformed into DH5α competent cells and monoclonal strains were cultured. The possible recombinant bacterial colony was randomly picked, extracted and then identified as positive colony by double enzyme digestion. The suspected positive plasmid was sent to Sangon Biotech (Shanghai, Co., Ltd) for sequencing to confirm the successful recombination.

Induction and purification of His-BRCC3 fusion protein

The pGEX-6P-1-BRCC3-His construct was transformed into BL21 competent cells, and cultured in LB medium for 14-16 h to observe the growth of the clone. Monoclones were selected and shaken at 37℃ for 16-18 h. Then some bacterial suspension was taken and added into 2×YTA medium (peptone 1.6g, yeast 0.8g, NaCl0.5g, ddH2O supplemented to 100 mL) at a ratio of 1:100, and continued shaking at 37℃ until OD600 to 0.8. At this time, IPTG (0.5 mM) was added into the bacterial suspension and incubated at 37℃. After 4h, the bacterial precipitation was collected by centrifugation. The supernatant was resuspended with lysis buffer (50mM NaH_2PO_4, 300mM NaCl, 10mM imidazole) and treated with ultrasonic homogenizer. Ni-NTA His-tag purification agarose was used to combine His-BRCC3 target protein according to the manufacture's instruction, then washed with washing buffer (50mM NaH_2PO_4, 300mM NaCl, 20mM imidazole), and His-BRCC3 protein was eluted with elution buffer (50mM NaH_2PO_4, 300mM NaCl, 250mM imidazole). Finally, SDS-PAGE gel was stained with Coomassie brilliant blue R250 to identify the induced His-BRCC3 fusion protein.

Kinase reaction in vitro

For in vitro kinase assays, active Cdk5/p25 complex protein was incubated with purified recombinant His-BRCC3 fusion protein in the kinase reaction buffer (8 mM MOPS/NaOH, 200 nM EDTA) plus 20 μM ATP at 30°C for 30 min. The reaction was stopped by adding SDT buffer (4%(w/v)SDS, 100mM Tris/HCl, 1Mm DTT, pH 7.6). The most of reaction product was analyzed by MALDI-MS/MS. The rest of it was boiled at 95°C for 5 min. The phosphorylation of substrate then was detected by autoradiography with phospho-Ser/Thr-proline antibody (1:1,000) following SDS-PAGE analysis. And total His-tagged proteins were re-probed with anti-His antibody (1:5,000).

Phosphorylation mass spectrometry

The sample of phosphorylated products were analyzed by Shanghai Applied Protein Technology Co. Ltd using LC-MS/MS(Nanolc-QE). Briefly, the chromatographic column was balanced with 95% liquid (solution of 0.1% formic acid), then the sample was loaded from the automatic sampler to the TRAP column for 1 hour. For mass spectral data collection, 20 fragment maps (MS2 scan) were collected after each full scan according to the mass charge ratio of polypeptides. The raw file of mass spectrometry test was retrieved from the database (R20190100160_ZJK.fasta) using Mascot2.2 software, and the identified protein results were obtained.

Results

Prediction of BRCC3 phosphorylation by Cdk5

In order to know that Cdk5 may be an upstream kinase of BRCC3, phosphorylation prediction software GPS5.0 was used to predict the phosphorylation between BRCC3 and Cdk5. The result showed that BRCC3 was the phosphorylated substrate of Cdk5. Five phosphorylation sites (Ser16, Ser28, Ser83, Ser93, Ser233) were predicted by GPS5.0 software (Table 1). Ser93, the highest score (16.846) among them, was the most likely phosphorylation site. Moreover, amino acid sequences of BRCC3 protein from human, mouse and rat were compared by DNAMAN software. The result showed that BRCC3 had a high conservatism and homology of 96.33% among different species of mammals (Fig. 1a, 1b).

pGEX-6P-1-BRCC3-His plasmid construction and His-BRCC3 fusion protein expression

To further verify the phosphorylation effect of Cdk5 on BRCC3, pGEX-6P-1-BRCC3 prokaryotic plasmid was constructed. The full-length target gene BRCC3 (876bp) was inserted into the pGEX-6P-1 vector (4984bp). The recombinant pGEX-6P-1-BRCC3 plasmid was detected through enzyme digestion of BamHI and XhoII. The agarose gel electrophoresis results showed two DNA bands around 5000bp and 900bp, which were identified as pGEX-6P-1 vector and target fragment BRCC3 respectively (Fig. 2a). Moreover, DNA sequencing was performed by Sangon Biotech (Shanghai) Co., Ltd. The sequence of the recombinant plasmid was exactly matched with the target sequence, which further demonstrated that pGEX-6P-1-BRCC3 plasmid was successfully constructed (Fig. 2b). Next, the recombinant His-BRCC3 construct was induced by IPTG and the fusion protein was identified by staining SDS-PAGE gel with Coomassie brilliant blue. The results showed that His-BRCC3 fusion protein was successfully expressed, and its molecular weight was about 36kD (Fig. 2c).

Phospho-mass Spectrometry of Kinase assays in vitro

The protein sample of phosphorylation product was analyzed using LC-MS/MS(nanoLC-QE). After getting the information of pep-tides, LC-MS/MS data were searched by Mascot mass spectrometry software to obtain the qualitative identification information of the target protein peptide molecules. The results of mass spectrometry showed that both BRCC3 and Cdk5 proteins were identified, and BRCC3 protein could be modified by oxidation and phosphorylation (Supplementary Table1). Eight phosphorylation sites (Ser227、Thr229、Ser233、Thr235、 Ser241、Thr244, Ser249 and Ser252) were screened out from 11 non-redundant phosphorylated peptides (Table2 and Supplementary Table1).

Identification of Phosphorylation with Western blot

To confirm the reliability of the mass spectrometry data, the product of kinase assays in vitro was verified for phosphorylation using Western blot. Detection of BRCC3 with Phospho-Ser/Thr-proline antibody also indicated that BRCC3 could be phosphorylated by Cdk5 (Fig. 3). These results indicate that BRCC3 was a substrate of Cdk5 in vitro, and the information obtained by using MS was reliable and might provide guidance for further study of the roles of these two proteins in disease.

Discussion

Since Cdk5 and BRCC3 expression were elevated in Parkinson's disease[5, 14], we speculated that Cdk5 might regulate the occurrence and progression of PD through phosphorylation of BRCC3. In order to prove the effect of Cdk5 on the deubiquitinating enzyme BRCC3, GPS5.0 software was used in this study to predict that Cdk5 might be the upstream kinase of BRCC3. The recombinant plasmid pGEX-6P-1-His-BRCC3 was constructed and successfully induced the His-BRCC3 fusion protein. After in vitro kinase reaction, the results of phosphorylation mass spectrometry and Western blot analysis showed that Cdk5 could phosphorylate BRCC3. This study provides a new perspective on the molecular mechanism of neurodevelopment and neurodegenerative diseases.

Cdk5 is a proline-dependent protein kinase that can phosphorylate serine and threonine residues, and its substrates contain (S/T) Px amino acid[15]. Qi et al. [16] found connexin 43 (CX43) protein regulated neuron migration and localization through astrocyte-neuron interactions during the early embryonic development of neurons. When Cdk5 phosphorylates CX43 at Ser279 and Ser282, the membrane localization and degradation of CX43 were modulated during neuron differentiation[16]. Moreover, Cdk5 also participates in the course of neurodegenerative disease by phosphorylating a large number of substrates, leading to their activation or inactivation. Wang et al.[17] found that Cdk5 phosphorylates Ser516 of GP78 in Parkinson's disease via the ubiquitin-proteasome pathway in vitro and in vivo, suggesting that the Cdk5-GP78 pathway provides a new pathway for neuroprotection in midbrain dopaminergic neurons. In Alzheimer's disease, Cdk5 hyperphosphorylates the Tau protein, causing a conformational change in the Tau protein that misfolds to form a double helix filament, eventually forming nerve fiber tangles[18]. Besides, Satow et al.[19] found that Cdk5 could regulate the expression of TPX2 by phosphorylating TPX2 Ser486, thereby promoting the proliferation and tumorigenicity of liver cells. In addition, Cdk5 can also regulate the molecular process of disease through phosphorylation of FAK, DRP1, PAK1, β-catenin, SRC, MAP1B, Nudel, Munc18, amphyphysin, signal molecules DAB1, Nudel, and so on[20]. Therefore, Cdk5 phosphorylation of BRCC3 in this study may also play an important pathway regulation role in nervous system diseases.

The deubiquitinating enzyme BRCC36 is a catalytic subunit of two multiprotein complexes, BRCA1-A and BRISC. In which, BRCA1-A protects genomic integrity by regulating the selection of DNA repair pathways, while BRISC serves cellular stress response and immune signaling function. BRCC3 promotes the repair of DNA double-strand breaks by removing H2AX (histone H2A)[6] and RAP80 (receptor associated protein 80)[21] ubiquitination, and participates in the repair of DNA cross-linking damage by removing the FANCG（Fanconi anemia，complementation group G）ubiquitination. BRCC3 also plays a role in cell cycle regulation by removing TNKs （tankyrase）and NUMA（nuclear mitotic appratus） ubiquitination[22, 23]. Studies have shown that BRCC3 can regulate the activation of NLRP3 inflammasome through deubiquitination, while deubiquitinating enzyme inhibitor G5 can inhibit the deubiquitination of NLRP3 inflammasome activation and the secretion of IL-1β[10]. Consistent with this, our previous studies also proved that BRCC3 was involved in the activation of NLRP3 inflammasome in Parkinson’s Disease[5].

Conclusion

In the present study, the phosphorylation of BRCC3 by Cdk5 was identified in vitro. Although the sites predicted by GPS5.0 was not completely consistent with the LC-MS/MS, the mechanisms of these kinase–substrate interactions need further investigation. The next study will focus on identifying the specific sites where BRCC3 is phosphorylated by Cdk5 and further prove its regulatory role in PD, so as to provide new support for the prevention and treatment of PD.

Declarations

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Conflict of Interest The authors declare that they have no conflicts of interest.

Data Availability The datasets used and/or analyzed in this manuscript have not been submitted to any other journal for simultaneous consideration and have never been published elsewhere in any form or language.

Code avaiablity Not applicable.

Author contributions Xiaoyun Shao and Shaoye Xu were responsible for the design and supervision of the whole project. Conghui Zhang performed the in vitro kinase assay and Western blot experiments. Kun Qin and Yu Han performed the plasmid construct and the expression of the fusion protein; Shaoye Xu

performed the prediction of phosphorylation software and the analysis of LC-MS/MS data. Conghui Zhang drafted the manuscript. Xiaoyun Shao and Shaoye revised the manuscript. All authors read and approved the final manuscript.

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent to publication Not applicable

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Tables

Table 1 Prediction of BRCC3 phosphorylation by Cdk5 with GPS5.0.

Position	Code	Kinase	Peptide	Score	Cutoff
16	S	CMGC/CDK/CDK5/CDK5	VQAVHLESDAFLVCL	10.507	8.808
28	S	CMGC/CDK/CDK5/CDK5	VCLNHALSTEKEEVM	9.41	8.808
83	S	CMGC/CDK/CDK5/CDK5	SVIILRRSDKRKDRV	9.326	8.808
93	S	CMGC/CDK/CDK5/CDK5	RKDRVEISPEQLSAA	16.846	8.808
233	S	CMGC/CDK/CDK5/CDK5	HSLTHLDSVTKIHNG	8.831	8.808

Notes of table header: Position: The position of the site which was predicted to be phosphorylated; Code: The residue which was predicted to be phosphorylated; Kinase: The regulatory kinase which was predicted to phosphorylate the site; Peptide: The predicted phosphopeptide with 7 amino acids upstream and 7 amino acids downstream around the modified residue; Score: The value calculated by GPS algorithm to evaluate the potential of phosphorylation. The higher value, the more potential the residue was phosphorylated; Cutoff: The cutoff value under the threshold. Different threshold meant different precision, sensitivity and specificity.

Table 2 A brief result of Phospho-mass Spectrometry of kinase assays in vitro.

	Reference	PepCount			
	FileScan	Sequence	Score	Reference	Modificati
$1-1	sp|P46737|BRCC3_MOUSE Lys-63-specific deubiquitinase BRCC36 OS=Mus musculus OX=10090 GN=Brcc3 PE=1 SV=1	430			
	R20190700933_CDK5,21762	K.IHNGSVFTKNLCSQM*S#AVSGPLLQWLEDR.L ! (S252) K.IHNGSVFTKNLCS#QM*SAVSGPLLQWLEDR.L ! (S249) K.IHNGSVFT#KNLCSQM*SAVSGPLLQWLEDR.L ! (T244) K.IHNGS#VFTKNLCSQM*SAVSGPLLQWLEDR.L(S241)	41.08	sp|P46737|BRCC3_MOUSE ! sp|P46737|BRCC3_MOUSE ! sp|P46737|BRCC3_MOUSE ! sp|P46737|BRCC3_MOUSE	15.994919 Oxidation (M); 79.966324 Phospho (ST)
	R20190700933_CDK5,23471	K.IHNGSVFTKNLCSQM*S#AVSGPLLQWLEDR.L ! (S252) K.IHNGSVFTKNLCS#QM*SAVSGPLLQWLEDR.L ! (S249) K.IHNGSVFT#KNLCSQM*SAVSGPLLQWLEDR.L ! (T244) K.IHNGS#VFTKNLCSQM*SAVSGPLLQWLEDR.L(S241)	84.36	sp|P46737|BRCC3_MOUSE ! sp|P46737|BRCC3_MOUSE ! sp|P46737|BRCC3_MOUSE ! sp|P46737|BRCC3_MOUSE	15.994919 Oxidation (M); 79.966324 Phospho (ST)
	R20190700933_CDK5,23909	R.IHSLTHLDSVTKIHNGSVFTKNLCS#QMS#AVSGPLLQWLEDR.L ! (S249, S252) R.IHSLTHLDSVTKIHNGSVFT#KNLCS#QMSAVSGPLLQWLEDR.L ! (T244, S249) R.IHSLTHLDSVTKIHNGS#VFT#KNLCSQMSAVSGPLLQWLEDR.L ! (S241, T244) R.IHSLTHLDSVT#KIHNGS#VFTKNLCSQMSAVSGPLLQWLEDR.L ! (T235, S241) R.IHSLTHLDS#VT#KIHNGSVFTKNLCSQMSAVSGPLLQWLEDR.L ! (S233, T235) R.IHSLT#HLDS#VTKIHNGSVFTKNLCSQMSAVSGPLLQWLEDR.L ! (T229, S233) R.IHS#LT#HLDSVTKIHNGSVFTKNLCSQMSAVSGPLLQWLEDR.L (S227, T229)	60.64	sp|P46737|BRCC3_MOUSE ! sp|P46737|BRCC3_MOUSE ! sp|P46737|BRCC3_MOUSE ! sp|P46737|BRCC3_MOUSE ! sp|P46737|BRCC3_MOUSE ! sp|P46737|BRCC3_MOUSE	79.966324 Phospho (ST)

Notes: (1) The protein information identified in row 1 (header) of the table. Reference: Protein registration number and description of other information in a database of proteins; PepCount: Total number of peptides detected. (2) The information of peptide corresponding to protein was shown in row 2 (header) of the table. FileScan: The corresponding scan number of the peptide in the MS file; Sequence: peptide Sequence. The amino acid that had been modified was marked with a symbol in the sequence. “#” represented phosphorylation modification; “*” represented oxidation modification.; Score: The Score of the peptide. The higher the score, the better the matching degree of secondary peptide chromatogram. Modification: Peptide Modification. It mainly includes oxidation of methionine (M) and phosphorylation of serine/threonine (ST). (3) The phosphorylated amino acids were marked in red in the sequence and the specific residues were shown in brackets.

Figures

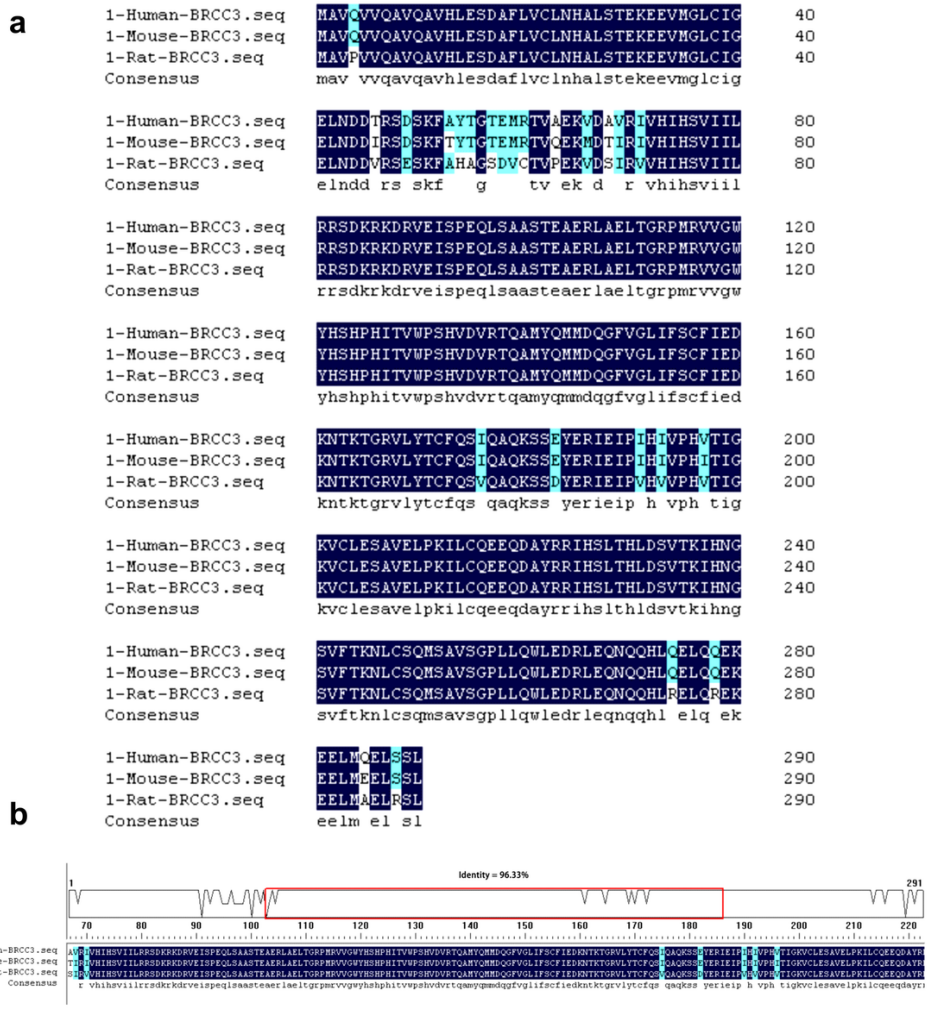

Figure 1

Comparison of conservatism and homology among different mammal species (a) Sequence alignment of BRCC3 protein among Human, Mouse and Rat. Protein sequences alignment of mouse-BRCC3 (NP_001159929.1), house-BRCC3 (NP_001018065.1) and rat-BRCC3 (NP_001120772.1) from NCBI were performed by using DNAMAN software. (b) The homology of BRCC3 protein was 96.33% among Human, Mouse and Rat.

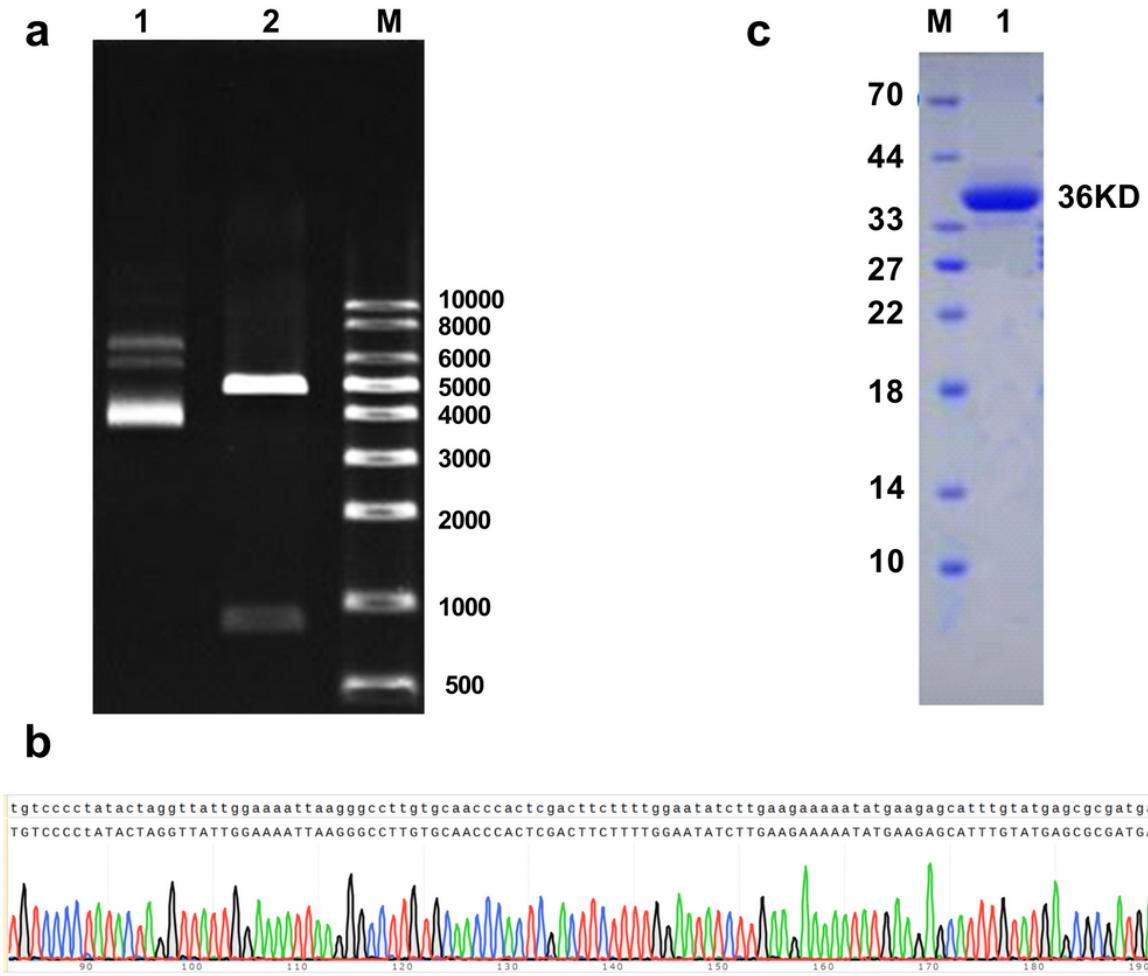

Figure 2

pGEX-6P-1-BRCC3-His plasmid construction and His-BRCC3 fusion protein expression (a) The DNA products of the recombinant plasmid pGEX-6P-1-BRCC3 were separated by using agarose gel electrophoresis after double enzyme digestion. Lane 1: The control DNA of recombinant plasmid pGEX-6P-1-BRCC3 without double enzyme; Lane 2: The recombinant plasmid DNA of pGEX-6P-1-BRCC3 digested by BamHI and XhoI；M：Marker; (b) A screenshot of partial sequencing result of pGEX-6P-1-BRCC3 recombinant construct. (c) The result of coomassie brilliant blue staining for His-BRCC3 fusion protein. M: Marker； Lane 1: Induced and purified His-BRCC3 fusion protein.

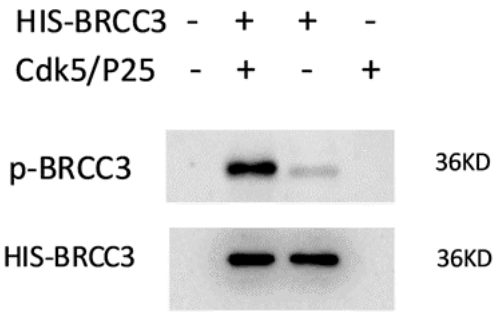

Figure 3

Identification of Phosphorylation with Western blot His-BRCC3 fusion protein reacted with active Cdk5/p25 kinase in vitro. The reaction product was performed by Western blot：the phosphorylation of BRCC3 was detected with phospho-Ser/Thr-proline antibody, and the expression of total BRCC3 was detected with His-tag antibody.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTable1.rar