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PRINCIPAL INVESTIGATOR: Zhiyuan Shen, M.D., Ph.D.

CONTRACTING ORGANIZATION: University of Illinois
Chicago, Illinois 60612-7205

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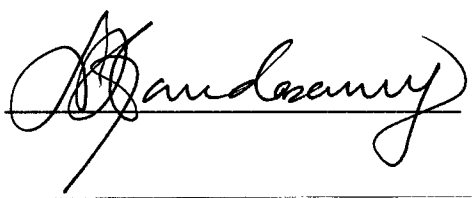

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Zhiyuan Shen, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Illinois
Chicago, Illinois 60612-7205

E-MAIL:
zshen@salud.unm.edu

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13. ABSTRACT ***(Maximum 200 Words)***

The major objectives of this IDEA award are: 1) to identify new proteins that may interact with BRCA2 in order to elucidate novel cellular function of BRCA2, and 2) to confirm that BRCA2 plays roles in DNA homologous recombination and cellular sensitivity to DNA damage. Progress has been made in the last year to achieve these objectives. We have identified 2 new protein interaction partners of BRCA2. One of the new partners is a novel protein, BCCIPα. BCCIPα gene is located on chromosome 10q26.1, a region susceptible for many human cancers. Another partner is a known protein that involved in cytoskeleton structure. The identification of these new BRCA2 interaction partners could reveal more functional roles of BRCA2 in mammary tumorigenesis. We are in the process of establishing BRCA2 dominant negative expression system to study the role of BRCA2 in homologous recombination and cellular sensitivity to DNA damage.

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Introduction:

Inactivation of BRCA2 is responsible for a significant portion of hereditary breast cancers. The original IDEA proposal has 3 major objectives. One objective is to identify new proteins that may interact with BRCA2 (task 5). We proposed to use yeast two hybrid system to identify new proteins that may interact with BRCA2. The second objective (tasks 1-4) is to determine whether modulation of BRCA2 function would affect homologous recombination and cellular sensitivity to DNA damage. We proposed to establish a cell system that can efficiently affect BRCA2 function, and then investigate whether the change of BRCA2 function would affect DNA homologous recombination. The last objective is to summarize data, write manuscripts, and apply for additional grant to continue the project developed from this IDEA award.

Body of progress report:

Objective 1 (task 5).

The original objective for task 5 is to identify and confirm interaction of BRCA2 with new proteins within the first 34 months. We used a conserved region of BRCA2 as the bait in a yeast two-hybrid system, and identified two proteins that interact with BRCA2. One of the identified candidates is a novel gene. We have named this gene as BCCIPα, for BRCA2, Cip1, Ca-interacting protein. We have completely achieved this objective by now. BCCIPα is a novel nuclear protein that is evolutionary conserved. These works are summarized and written in a manuscript submitted to Oncogene(Appendix 2). We have found that BCCIPα gene is located at chromosome 10q26.1, a cancer susceptibility region for many human tumors, including advanced brain tumors, endometrial tumors, advanced prostate cancer, and some breast cancer. Preliminary data suggest that BCCIPα is a candidate for a tumor suppressor located at 10q26.1. Please see appendices for details. We will focus on the effect of BCCIPα on breast cancer cell lines in the next year.

In addition to BCCIPα, we have also found that the acting binding protein, ABP280 forms stabe complex with BRCA2 in vitro. Further, we found that lack of ABP280 renders cells to hypersensitivity to radiation at the same scale as BRCA2 mutation. In the next year we will further analyze the role of ABP280/BRCA2 interaction in cellular sensitivity to DNA damage.

Objective 2 (tasks 1, 2, 3 and 4):

The objective of tasks 1-4 is to investigate the effect of the expression of BRCA2 dominant negative fragments on DNA homologous recombination and cellular sensitivity to DNA damage agents. As planned in the 1999 annual report, we have cloned 4 dominant negative fragments of BRCA2 that interact with RAD51 into a mammalian expression vector that was tagged with EGFP. Transfection of these fragments into HT1080-1853 cells failed to produce detectable level of dominant negative BRCA2 fragments. We will use a retrovirus-based approach to stably express BRCA2 fragments in HT1080 cells. We plan to clone the fragments into pLXSN retroviral vector. Retrovirus that express dominant negative BRCA2 fragments will be introduced into HT1080-1853 cells. Preliminary data suggest that HT1080-1853 cells is highly susceptible to pLXSN based virus infection. We anticipate this will enable us to effectively modulate BRCA2 function in the cells and to study the effect of BRCA2 function in homologous recombination.

The objective in task 5 has been accomplished ahead of schedule (please see above). This will enable us to invest more resource on the objectives in tasks 1-4.

The PI has moved from University of Illinois at Chicago (UIC) to the Department of Molecular Genetics at University of New Mexico Health Sciences Center. This move has some positive impact on the project outlined in tasks 1, 2, 3, and 4. As outlined in the 1999 Progress Report, we decided to use HT1080-1853 cells provided by Dr. Mark Brenneman as the alterative system to achieve the goal set by tasks 1-4. Dr. Brenneman is currently a postdoc fellow in Dr. Jac Nickoloff's lab in the same department as the PI. Dr. Brenneman has a DOD Breast Cancer Research Postdoc Fellowship award to study the roles of BRCA2 in genetic stability. We will have more close interaction with Dr. Brenneman's project, which will facilitate the progress of this study.

Objective 3 (task 6).

The objective of task 6 is to summarize data, and write new grant application based on the IDEA project. We have summarized some of the work in a manuscript (enclosed as appendix). We anticipate that we will have sufficient preliminary data on BCCIPα function for a new grant application in the next funding period.

Key Research Accomplishments

- Isolation of a new BRCA2 interacting gene.
- Discovery that ABP280 forms complex with BRCA2.
- Submitted one manuscript for publication.

Reportable Outcomes

1. Manuscripts and abstracts, presentation:

- 1 Manuscript has been submitted for publication (appendix 2).
- 1 Abstract was presented at the Era of Hope Breast Cancer Research meeting held in Atlanta (appendix 1).

2. Patents and Licences applied for and/issued:

None.

3. Degree obtained:

None.

4. Development of cell lines, tissue or serum repositories:

None.

5. Informatics such as databases and animal models:

None.

6. Funding applied for based on work supported by this award.

None.

7. Employment or research opportunities.

Not applicable.

Conclusions

We have confirmed the interaction of BRCA2 with BCCIPα and ABP280. These findings are potentially important in the study of mammary carcinogenesis. It may provide new insights to BRCA2's role in breast cancer. In the future, we will attempt to secure more research funding to investigate the physiological role of BRCA2/BCCIPα and BRCA2/ABP280 interactions. We are still in the stage of establishing expression system for dominant negative expression of BRCA2 in an effort to confirm its role in homologous recombination and cellular response to DNA damages.

References

None.

Appendices

1 Abstract:
Zhiyuan Shen, Yuan Yuan, Jingmei Liu,Bahar Hesabi, and Jun Huan. 2000. Identification of a novel BRCA2 associated protein. Era of Hope Department of Defense Breast Cancer Research Program Meeting, Atlanta, June, 2000. Proceedings page 87.

2. Manuscript:
Liu, J. Yuan, Y. Huan, J. and Shen, Z. 2001. BRCA2 interacts with an evolutionary conserved nuclear protein encoded by chromosome 10q25.3-26.2, BCCIP. Oncogene, submitted.

Appendix 1

Identification of a Novel BRCA2 Associated Protein.

Zhiyuan Shen, Yuan Yuan, Jingmei Liu, Bahar Hesabi, Jun Huan.

Dept. of Mol. Genetics, University of Illinois at Chicago,
900 S. Ashland Ave.,Chicago, IL 60607.

zshen@uic.edu

The breast cancer susceptibility gene BRCA2 has been implicated in the same DNA damage repair pathway that RAD51 is involved in. It is possible that other proteins may interact with BRCA2 protein. In an effort to identify such protein, we used a conserved region of BRCA2 protein as the bait in a yeast two-hybrid screen. We identified a novel protein that interacts with BRCA2. This protein is named BRAP5, for BRCA2–Associated Protein, clone-5. The BRCA2/BRAP5 interaction is confirmed by *in vitro* interaction assays using recombinant BRCA2 and BRAP5 proteins. BRAP5 protein does not have sequence homology with any known protein, nor does it contain any known protein motif. However, highly conserved open-reading frames are identified from databases of mouse EST, *Caenorhabditis elegans* genomic DNA, *Arabidopsis thaliana* genomic DNA, and *Saccharomyces cerevisiae* genomic DNA, suggesting a conservative function of BRAP5 in the evolution. Using an antibody against recombinant BRAP5, we have found that BRAP5 is a nuclear protein, expressed in a variety of human tissues, with the highest protein level in the testis. No fluctuation of BRAP5 protein level was observed in different phases of cell cycle in MCF7 cells. BRAP5 gene is located at chromosome 10q26.1.

The U.S. Army Medical Research and Materiel Command under DAMD 17-98-1-8198 supported this work.

Appendix 2

(submitted to Oncogene)

BRCA2 interacts with an evolutionary conserved nuclear protein encoded by chromosome 10q25.3-26.2, BCCIP.

Jingmei Liu [1], Yuan Yuan [1,2], Juan Huan[2], Zhiyuan Shen [1,3]

[1]Dept. of Molecular Genetics and Microbiology, University of New Mexico Health Sciences Center; 915 Camino de Salud, NE. Albuquerque, NM 87131.

[2]Graduate Program of Molecular Genetics, College of Medicine, University of Illinois at Chicago, 900 S. Ashland Ave. Chicago, IL 60607.

[3]Correspondence to:

Zhiyuan Shen, Ph.D, MD.
Dept. of Molecular Genetics and Microbiology
University of New Mexico Health Sciences Center.
915 Camino de Salud, NE
Albuquerque, NM 87131
Phone: 505-272-4291
FAX: 505-272-6029
Email: zshen@salud.unm.edu

Key words: BRCA2, Calcium-binding protein, BCCIP.

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Abbreviations are: GST: Glutathion S-Transferase; BCCIP: Brca2, Ca, and Cip1 Interacting Protein; DAPI: 4', 6-diamidino-2-phenylindole; NAD: N-terminus Acidic Domain; ICD: Internal Conserved Domain; CVD: C-terminus Variable Domain.

Abstract

BRCA2 is a tumor suppressor gene involved in mammary tumorigenesis. Although important functions have been assigned to a few conserved domains of BRCA2, little is known about the longest internal conserved domain encoded by exons 14-24. In this study, we identified a novel nuclear protein, designated BCCIP that interacts with part of the internal conserved region of human BRCA2. Human BCCIP represents a new family of proteins that are evolutionarily conserved. Members of the BCCIP protein family contain three distinctive sequence domains: a N-terminus acidic domain (NAD) of 30-60 amino acids, an internal conserved domain (ICD) of 180-220 amino acid, and a C-terminus variable domain (CVD) of 30-60 amino acids. The N-terminus half of the human BCCIP ICD shares moderate homology with calmodulin and the calcium binding domain of M-calpain, suggesting that BCCIP might be a Ca-associated protein. A second isoform of this protein that differs in the CVD was identified in human. Similar to BRCA2 protein, BCCIP is a nuclear protein highly expressed in testis. *BCCIP* gene is located at chromosome 10q25.3-26.2, a region frequently altered in brain and other cancers.

Introduction

The human tumor suppressor gene *BRCA2* encodes for a large protein of 3418 amino acids. Mutations of *BRCA2* contribute to a significant portion of hereditary breast cancers. BRCA2 protein has no significant homology with any protein of known function. The overall homology between human and mouse BRCA2 is moderate at about 59%. Highly conserved regions (>75% homology) have been identified in human and mouse BRCA2. It is expected that important functions of BRCA2 reside in these conservative domains. Several models based on the functional analysis of the conserved BRCA2 domains have been proposed to interpret the role of BRCA2 in tumorigenesis.

An N-terminus conserved domain in exon 3 (amino acids 48-105) has been indicated in transcriptional regulation of gene expression (Milner *et al.*, 1997; Nordling *et al.*, 1998). Deletion of this region has been identified in breast cancers (Nordling *et al.*, 1998). Therefore, the transcription activity itself is directly relevant to tumorigenesis.

Although the overall sequence in exon 11 shows only moderate homology between mouse and human, eight internal BRC repeats in exon 11 are highly conserved (Bignell *et al.*, 1997). Each of the repeats is about 90 amino-acid long, and some of these repeats interact with RAD51 (Katagiri *et al.*, 1998; Marmorstein *et al.*, 1998; Wong *et al.*, 1997). A conserved C-terminal BRCA2 domain (amino acids 3196-3232 of mouse BRCA2) mediates mouse BRCA2/RAD51 interaction (Sharan *et al.*, 1997), and corresponds to a human BRCA2 C-terminus domain that is deleted in most truncated forms of *BRCA2*. This region of mouse BRCA2 has 72% amino acid identity with human BRCA2. Functional analysis of these conserved domains in BRCA2 suggests that human BRCA2 may participate in RAD51-dependent DNA homologous recombination, suggesting that BRCA2 serves as a caretaker to

maintain genome stability. Mutation of *BRCA2* that directly affects these RAD51-interaction domains could result in genome instability, and promote tumorigenesis (Chen *et al.*, 1999; Yuan *et al.*, 1999).

A third model involves the cellular localization of BRCA2 proteins. The functional nuclear localization signals (NLS) for BRCA2 have been identified at the C-terminus (Spain *et al.*, 1999; Yano *et al.*, 2000). Most of the BRCA2 mutations identified in breast cancers are truncations, resulting in deletion of the C-terminal NLS and the RAD51 interaction domain. It is possible that the lack of NLS in BRCA2 mutants results in abnormal cellular localization of BRCA2, preventing BRCA2 function (such as transcription and genome stability control), and subsequently responsible for tumorigenesis associated with BRCA2 mutation. This model may explain cases that involve the deletion of NLS in BRCA2 patients. However, some internal mutations, as exemplified by deletion of the transcriptional domain (Koul *et al.*, 1999), would not affect the cellular location of BRCA2. These may still be responsible for tumorigenesis.

Another conserved domain precedes the C-terminus RAD51 interaction domain. This domain is the longest conserved domain in BRCA2. It covers exons 14-24 (Gayther & Ponder, 1998). The role of this region in tumorigenesis is unclear. It is anticipated that this conserved domain carries essential, yet to be identified, functions of BRCA2. BRCA2 may be a protein with multiple functional domains. It may possess cellular functions other than RAD51-associated DNA homologous recombination. Identification of novel proteins associating with BRCA2 would provide clues for additional *BRCA2* functions. We used part of the long internal conserved domain covering exon 14-24 as "bait" in a yeast two hybrid screen, and identified a novel nuclear protein, designated BCCIP, that interacts with a part of this conserved region coded by exons 21-24.

Material and Methods

Plasmids. A fragment of BRCA2 cDNA covering exons 21-24 was isolated using RT-PCR, and confirmed by sequencing. This fragment was linked with BamHI and SalI sites, and fused to the Gal4-binding domain in yeast two-hybrid vector pAS2-1, resulting in pAS2-1/BRCA2H. PCR primers tagged with BamHI/SalI sites were used to amplify regions of BRCA2 cDNA, and cloned into pGEX-5X (Pharmacia) to generate vectors that express glutathion S-transferase (GST) fused protein fragments of BRCA2. The same regions of BRCA2 were also cloned into the two hybrid vector pGBT9 to express Gal4-DNA binding domain fused protein in yeast. BCCIP cDNA was cloned into pET28 (Novagen), creating pET28/BCCIP that expresses histidine tagged BCCIP protein in BL21(DE3) cells. BCCIP cDNA was also cloned into pGEX-5x to generate pGEX/BCCIP expressing recombinant GST-BCCIP fusion protein in BL21 cells. All plasmid constructs were confirmed by DNA sequencing.

Recombinant proteins and BCCIP antibodies. Recombinant protein expression and purification were performed as previously described (Shen *et al.*, 1996b). Briefly, pET28/BCCIP was transfected into BL21(DE3) cells, $(His)_6$ –tagged BCCIP protein (His-BCCIP) was expressed, and purified. GST-BCCIP and GST-BRCA2 fusion proteins were expressed and purified in BL21 cells using pGEX/BCCIP, and pGEX/BRCA2 vectors. His-BCCIP was injected into rabbits to produce polyclonal antibodies, and GST-BCCIP was used to for affinity purification of polyclonal anti-BCCIP antibodies.

***BCCIP* DNA sequence and human EST database analysis.** Plasmids from positive clones of the two hybrid screen were sequenced and data were analyzed with DNA Star sequence analysis software. GenBank nucleic acid and human EST data bases were searched to identify

matching ESTs. The complete cDNA sequence of BCCIP was constructed from the cDNA sequence of our clones and matching ESTs.

Protein interaction assays. pAS2-1/BRCA2H was used to screen a cDNA library expressing Gal4-DNA activation domain fused proteins (Clonetech, Palo Alto, CA) as described (Shen *et al.*, 1996a). MV103 cells hosting LacZ and Uracil, and Histidine reporters were used for the screen (Vidal, 1997). To confirm the two hybrid interaction, an independent two hybrid assay and in vitro GST pull-down assays were performed as described (Shen *et al.*, 1996b). Briefly, various regions of BRCA2 cDNA was cloned into pGBT9. These vectors were co-transfected with pACT/BCCIP, and assayed for LacZ activity in the host cell strain SFY526 (Clontech, Palo Alto, CA). GST-tagged BRCA2 fragments were bound to glutathione beads, and incubated with His-tagged BCCIP protein. After extensive washing, His-BCCIP proteins bound to GST-fusion protein were analyzed by Western blot using anti-BCCIP antibodies.

Immunoprecipitation and Immunoblotting. Cells were collected, treated with lysis buffer (150mM NaCl, 1mM EDTA, 50mM Tris.Cl, pH 7.5, 1% NP-40) for 30 min in ice, and sonicated, resulting in whole cell extract. One mg of whole-cell extract was incubated with 50µl of anti-HA Affinity Matrix (Roche Molecular Biochemicals) at 4°C on a rocker for 2 hours. The matrix was washed six times in 1 ml of cold lysis buffer. Proteins bound to the beads were eluted in 2xSDS sample buffer by boiling 5 minutes, resolved on SDS-PAGE and transferred to PVDF membrane(Osmonics). Immunoblotting using appropriate antibodies was as described before (Li *et al.*, 2000). To detect BRCA2, a rabbit anti-BRCA2 antibody (Oncogene Researcher Products) was used.

Immunostaining and confocal microscopy. Cells were cultured on a cover slip, and fixed with cold 3% paraformaldehyde solution buffered with PBS for 1 hr. Fixed slides were

treated with permeablization buffer (0.5% triton X-100, 50mM NaCl, 3mM $MgCl_2$, 300mM sucrose, and 20mM HEPES, pH7.4) for 20 min. Slides were blocked with 2% BSA in PBS for 30 min at room temperature, and incubated with 1 μg/ml of primary anti-BCCIP antibodies overnight at 4°C. After the primary antibody incubation, the slides were washed 5 times with PBS, and incubated with rhodamin-conjugated anti-rabbit IgG antibodies (Pierce) for 30 min at room temperature. Slides were washed with PBS 5 times, and mounted with Vectashield mounting solution containing 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA). The slides were stored in the dark at 4°C. A Zeiss LSM510 confocal microscope with Ar/Kr, and UV laser sources was used to observe the stained slides, and images were digitally recorded.

Other procedures. Western blots were performed as described previously (Li *et al.*, 2000). Fluorescent *in situ* hybridization (FISH) was performed as described before (Shen *et al.*, 1996a) using *BCCIP* cDNA as the probe. Tumor cell lines were purchased from ATCC.

Results and Discussion

Identification and nomenclature of a BRCA2-interacting protein, BCCIP.

Amino acids 2883-3053 of BRCA2 (termed BRCA2H) is a part of the longest internal conserved region encoded by exons 14-24. BRCA2H is 78% identical between mouse and human, compared to the overall homology of 59%. Using Gal4-DB/BRCA2H as bait, we screened $4x10^5$ independent clones of a human cDNA library, and identified 14 interacting clones. DNA sequencing identified 5 clones derived from the same gene. Clone number 5 contained the longest cDNA, and encodes an open reading frame with a stop codon at the 3'-end, but no translation start codon at the 5'-end. Since this protein may also associate with Ca and

Cip1(p21) (see latter discussion for detail), it is designated BCCIP as BRCA2, Ca, and Cip1 – Interacting Protein as recommended by HUGO Gene Nomenclature Committee.

To obtain the complete cDNA sequence of BCCIP, we searched the human EST database and identified more than 40 ESTs that matched the sequence of BCCIP, we concluded that our clone was missing 42bp at the 5'-end. The full-length cDNA transcript of BCCIP encodes a protein of 322 amino acids. The missing 42 bp at the 5'-end was synthesized and added to our cDNA clone to generate the full-length cDNA of BCCIP (data not shown). Interesting, the EST database contained additional ESTs that matched the 5' end of the clone, but had a completely different sequence at the 3'-end. Using this information, we composed a complete cDNA sequence that codes for an open reading frame of 314 amino acids (data not shown). Since the ESTs were identified from various tissues and by independent investigators, it is likely that they are true transcripts from human tissues. Therefore, the BCCIP with 322 amino acids was named BCCIP-α, and the one with 314 amino acids was named BCCIPβ. BCCIPα and BCCIPβ have identical amino acids N-terminus 258 amino acids, but differ in their C-terminus. Searching of GenBank identified two new cDNA entries that code for identical amino acids as BCCIPα and BCCIPβ (see GenBank Entry Nos. NM_016567 and AB040451). The author in these GenBank entries stated that these proteins interact with cell cycle control protein CDK inhibitor, p21(Cip1).

In addition, our further amino acid sequence analysis has found that amino acids 45-100 of BCCIP shares 29% identity and 58% similarity to the Ca-binding domain of M-calpain (Figure 1) (Pontremoli *et al.*, 1999). The same region also shares homology with the N-terminus Ca-binding site of calmodulin. Therefore, amino acid 45-100 of human BCCIP contains a putative Ca-binding site. Calmodulin is composed of three distinct domains (Yjandra *et al.*, 1999), an N-terminus calcium binding domain, a C-terminus calcium binding domain, and an

internal helix domain. In addition to the Ca-binding site, amino acids 100-150 of human BCCIP also share homology with the internal helix of calmodulin. Amino acids 50-150 of human BCCIP shares 26% identity and 45% similarity with amino acids 1-90 of the calcium binding regulator protein calmodulin (Yjandra *et al.*, 1999) (Figure 1). Therefore, BCCIP is a putative Ca-associated protein.

Considering the three known properties of this newly isolated protein, i.e. association with BRCA2, Cip1(p21) and/or Ca, we suggest this new protein be named as: BCCIP for BRCA2, Ca, or Cip1 – Interacting Protein.

BCCIP is evolutionary conserved.

By searching databases with mouse EST, and genomes of *C. elegans, S. cerevisiae*, and *A. thaliana*, we identified homologous genes from these species. Their anticipated protein sequences were compared with BCCIPα and BCCIPβ. They share a common structural profile. They all have an N-terminus acidic domain (NAD) rich in residues DE (aspartate and glutamate), an internal conserved domain (ICD), and a C-terminus variable domain (CVD) (Figure 2).

The DE-rich NAD domain shares moderate homology with many proteins with acidic domains. The CVD shares no homology among BCCIP family members, except that mouse BCCIP and human BCCIPβ are ~70% identical. The ICD is evolutionary conserved among the species analyzed. For example, human and mouse BCCIP share 97% similarity, while the yeast and plant BCCIP share about 70% similarity to human BCCIP (Figure 2A). The putative Ca-binding domain resides in the ICD. The location of putative Ca-binding domain is illustrated in Figure 2B.

Expression of BCCIP in human tissues.

We subIconed the BCCIPα into pET28 and purified His tagged BCCIP recombinant protein from bacteria. GST tagged BCCIPα proteins were also purified. The His-BCCIPα was used to make rabbit polyclonal antibodies against BCCIP, and GST-BCCIPα was used to affinity purify the anti-BCCIP antibodies. This antibodies recognized both the full length BCCIPα, as well as the N-terminal common region between BCCIPα and BCCIPβ (data not shown).

These antibodies are reactive to two species of proteins from human skin fibroblasts (HSF) and HT1080 cells (Figure 3A). Since BCCIPα shares significant homology to BCCIPβ, and the antibodies recognize the N-terminus common region of BCCIPα and BCCIPβ, we predict that the top band (~50 kDa) is BCCIPα (322 amino acids), and the lower band (~46 kDA) is BCCIPβ (314 amino acids).

A human tissue protein membrane was purchased from DNA Technology Inc. Although the resolution of the pre-made membrane was compromised, and it is hard to distinguish the bands for BCCIPα and BCCIPβ in tissues (Figure 3B), it is interesting that the BCCIP proteins are highly expressed in testis, as is BRCA2 (Sharan & Bradley, 1997).

Nuclear localization of BCCIP

To determine the cellular localization of BCCIP, we stained the breast cancer cell line MCF-7 with the affinity purified polyclonal BCCIP antibodies. As shown in Figure 4, BCCIP protein is predominantly expressed in the nucleus. Under the same condition, pre-immuno serum did not react to nucleus(data not shown). It is interesting that BCCIP reactive proteins do not colocalize with condensed chromosome DNA in mitosis (Figure 4A-4C). Confocal microscopy of HeLa cells and human skin fibroblasts showed the same cellular distribution as

MCF-7 cells (data not shown). We also expressed EGFP-tagged BCCIP protein in HeLa cells, and it confirmed that majority of the BCCIP protein localizes to the nucleus (data not shown).

In vivo interaction between BCCIPα and BRCA2.

In order to confirm the complex formation between BRCA2 and BCCIP in vivo, proteins were expressed in 293 human kidney cells, and co-immunoprecipitation experiments were performed.

In the first experiment, two BRCA2 fragments, BRCA2F (amino acids 2883-3418) and BRCA2B (amino acids 2883-3194) were cloned into pHA-CMV vector to express HA-fused BRCA2 fragments. The BCCIPα cDNA were tagged with a FLAG-coding sequence by PCR, and cloned into pCMV (Clonetech) to express Flag-tagged BCCIPα. The HA-tagged BRCA2 and Flag-tagged BCCIP-α were co-expressed in 293 cells by transient transfection. The HA-tagged BRCA2 proteins were precipitated with an anti-HA affinity matrix, and co-precipitated BCCIPα was detected by anti BCCIP antibodies. As demonstrated in Figure 5A, BCCIPα was co-precipitated with both HA-BRCA2F and HA-BRCA2B that contain the fragment H (amino acid 2883-3053).

In a second experiment, HA-tagged BCCIPα protein was transiently expressed in 293 cells, and immunoprecipitated with the anti-HA affinity Matrix. Co-immunoprecipitated endogenous BRCA2 proteins were detected by a rabbit anti-BRCA2 antibodies. As demonstrated on Figure 5B, immunoprecipitation of HA-BCCIPα co-precipitates the full-length endogenous BRCA2 protein.

These data suggest a stable complex formation between fullength BRCA2 and BCCIPα in human cells.

A small region of BRCA2 is responsible for BCCIP interaction.

To further characterize the interaction between BCCIPα and BRCA2, we fused a series of BRCA2 fragments with the Gal4-DNA binding domain, and tested their interaction with Gal4-DNA activation domain fused BCCIP protein using LacZ as reporter in the independent yeast strain SF526. As shown in Figure 6A, BRCA2H indeed interacts with BCCIP; the minimum interacting region of BRCA2 is in amino acids 2973-3001.

To confirm this, a set of GST-fusion proteins of BRCA2 fragments was purified. GST-BRCA2 proteins were incubated with His-BCCIP protein, and pulled down with glutathion beads. Bound His-BCCIP proteins were analyzed with Western Blot. As shown in Figure 6B, GST protein and resin alone could not pull down His-BCCIP. However, BRCA2 proteins containing region 2973-3001 pulled down His-BCCIP, suggesting an interacting between His-BCCIP and this BRCA2 region. This region is 71% identical between mouse and human BRCA2, which is significantly above the average homology between human and mouse BRCA2. Since purified proteins were used for the binding assay, this data demonstrates that the BRCA2 and BCCIP interaction is a direct protein-protein binding.

***BCCIP* is located on chromosome 10 at q25.3-26.2.**

We used *BCCIP* cDNA as a probe for a FISH analysis. Among 100 mitotic cells analyzed, 67 of them have paired FISH signals, and all of them were present near the end of chromosome 10q (Figure 7A). Further analysis mapped this gene to 10q25.3-26.2 (Figure 7B). To confirm this finding, we used the BCCIP sequence as probe, and searched a human chromosome marker database, and identified a marker (SGC33832) at chromosome 10q26.1 that has identical

sequence to part of the *BCCIP*α cDNA. Therefore, this result confirmed that *BCCIP* is present at 10q25.3-26.2.

Abnormalities in region of chromosome 10q25.2-26.2 have been observed in many tumors, including brain tumors (Maier *et al.*, 1997; Rasheed *et al.*, 1995), endometrial tumors (Peiffer *et al.*, 1995), small cell lung cancers (Kim *et al.*, 1998; Petersen *et al.*, 1998), melanoma (Robertson *et al.*, 1999; Walker *et al.*, 1995), and other tumors (Ittmann, 1996). It has also been reported that the deletion of this region is responsible for developmental retardation in children with 10q deletions (Tanabe *et al.*, 1999; Taysi *et al.*, 1982; Waggoner *et al.*, 1999).

It has been demonstrated that at least one tumor suppressor is located in 10q25.3-26.2 (Petersen *et al.*, 1998; Rasheed & Bigner, 1991; Rasheed *et al.*, 1995). This tumor suppressor has been suggested to be responsible for all grades of brain tumors. A candidate tumor suppressor (DMBT1) was identified in this region (Mollenhauer *et al.*, 1997). However, studies have shown that the status of DMBT1 in brain tumors has no connection with tumor phenotypes, suggesting that another gene in the region is responsible for brain tumors (Steck *et al.*, 1999).

We have measured the BCCIP protein expression in a few brain tumor cell lines. We found that one of the tested cell lines lacks the expression of BCCIPα but retained BCCIPβ expression (Shen, unpublished data). Considering the interaction of BCCIP with known tumor suppressor BRCA2, the similar tissue expression pattern of BCCIP with BRCA2, and the *BCCIP* chromosome location to a region that is frequently altered in various tumors, it is conceivable to suggest that *BCCIP* might be a candidate tumor suppressor. However, more extensive studies are required to test this hypothesis. These will include at least, but not last, the following studies: extensive expression and mutation analysis of BCCIP in primary tumor specimens, suppression of tumor phenotypes in tumor cells by introducing wild type BCCIP into tumor cells with *BCCIP*

mutation, and promotion of tumorigenesis/carcinogenesis by inhibition of *BCCIP* gene function in normal cells.

Searching the yeast genome database, we have identified a yeast strain that has the *BCCIP* open reading frame deleted. It is interesting that the *BCCIP* null mutation is lethal to yeast, suggesting an essential role of yeast *BCCIP*. Further analysis of the yeast *BCCIP* mutant will be perform to provide clues to human BCCIP function.

To summarize, we identified a new protein, designed BCCIP, that interacts with BRCA2. At least two isoforms of this protein are expressed in human tissues. BCCIP is evolutionarily conserved with several distinctive structural domains. A part of the internal conserved domain of BCCIP shares a moderate degree of homology with the Ca-binding domains of M-calpain and calmodulin. Further studies will be focused on its expression in human tumors and its functional role in tumorigenesis.

Acknowledgments

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Figure legends.

Figure 1. Sequence comparison of BCCIP with the Ca-binding regions of calmodulin and M-calpain. The top panel is an amino acid alignment of BCCIP with human calmodulin. The bottom panel is an amino acid alignment of BCCIP with human M-calpain. The numbers indicate the amino acid residue number from the N-terminus of each protein.

Figure 2. Domain analysis of BCCIP proteins (drawing not in scale).

A. Domain comparison of human BCCIP with its homologues in other species. According to the sequence conservation, the human BCCIP was arbitrary divided into 3 domains: a N-terminus Acidic Domain (NAD), an Internal Conserved Domain (ICD), and a C-terminus Variable Domain (CVD). Numbers in the ICD of BCCIPs indicate the percentages of sequence identity and similarity of the specific BCCIP with human BCCIPα.

B. Sequence similarity of the putative Ca-binding domain in the internal conserved domain (ICD) of human BCCIP with human calmodulin and the Ca-binding region of M-Calpain. Numbers in the parenthesis above the Ca-binding domains of calmodulin and M-calpain indicate their percentage of sequence identity and similarity to the putative Ca-binding site of human BCCIP.

Figure 3. BCCIP Protein expression in human tissues.

A. Two distinctive species of BCCIP are reactive to BCCIP antibodies in normal human skin fibroblasts (HSF), and HT1080 cells. The higher molecular weight band is designated

BCCIP-α (approximately 50 kDa) , and the lower molecular weight band is designated BCCIP-β (approximately 46 kDa).

B. BCCIPs are expressed in several human tissues, with the highest expression level in testis. The double band pattern is not readily visible in the tissues in this illustration due to the compromised resolution of the pre-made membrane provided by DNA Technology. However, the same double band pattern as in Figure 3A was observed with a lighter exposure (data not shown) in all the tissues.

Figure 4. Nuclear localization of BCCIP proteins. BCCIP localizes to cellular nuclear in MCF-7 breast cancer cells. Red signals from panel A are the anti-BCCIP antibodies reactive proteins. Three mitotic cells with condensed DNA are indicated by arrows. The blue signals from the panel B are DAPI stained cellular DNA, showing nucleus or condensed chromosome DNA (arrows). Panel C shows both the DNA signal and BCCIP signals.

Figure 5. *In vivo* protein complex formation between BRCA2 and BCCIPα.

A. Lanes 1-3 are whole protein extract from 293 cells transfected with various plasmids. Lanes 4-5 are the anti-HA matrix precipitated proteins from the whole cell extract. Lanes 1 and 4 derived from co-expression of Flag-BCCIPα and a control vector (pHA-CMV), lanes 2 and 5 derived from co-expression of Flag-BCCIPα and HA-BRCA2B (amino acid 2883-3149), and lanes 3 and 6 are proteins from cells expressing Flag-BCCIPα and HA-BRCA2F (amino acid 2883-3418). Bottom panel is blotted with anti-HA antibodies, demonstrating that HA-BRCA2B and HA-BRCA2F are expressed in the total cells extracts (lanes 2 and 3) and precipitated with anti-HA matrix (lanes 4 and 5). The top

panel was blotted with anti-BCCIP antibodies, demonstrating that BCCIPα can be co-precipitated with the HA-BRCA2 and BRCA2F (lanes 5 and 6). This data suggest that the c-terminus of BRCA2 containing amino acids 2883-3053 form complex with full length BCCIPα.

B. Full length BCCIP were fused with HA, and expressed in 293 cells. Lane 1 is the whole protein extract from 293 cells transfected with pHA-CMV control vector. Lane 2 is the whole cell protein extract from 293 cells transfected with pHA-CMV/BCCIP expressing HA-BCCIPα protein. Lane 3 is the whole protein extracts from MCF-7 breast cancer cells (used as control for Western blot). Lane 4 is the precipitated protein from the same extract as lane 1. Lane 5 is the precipitated protein from the same protein as lane 2. The bottom panel is blotted with anti-HA antibodies, showing that the HA-BCCIP is expressed in transfected cells (lane 2) and precipitated with anti-HA matrix (lane 5). The top panel demonstrates the endogenous BRCA2 protein exists in 293 cells transfected with both control vector (lane 1) and pHA-CMV/BCCIP (lane 2), but was co-precipitated only with the HA-BCCIP (lane 5). This data suggest a stable complex can be formed between the full-length endogenous BRCA2 and full length BCCIP protein.

Figure 6. Mapping of BRCA2 regions that interact with BCCIP.

A. Results from two hybrid analysis. "+" on the right side of the illustration indicate a positive interaction of the BRCA2 fragment (left side) with BCCIP in yeast two hybrid system (drawing not in scale). The amino acid border of various BRCA2 fragments are listed in the parenthesis under the name of the BRCA2 fragment. The shaded region is the minimum amino acids of BRCA2 for interacting with BCCIP.

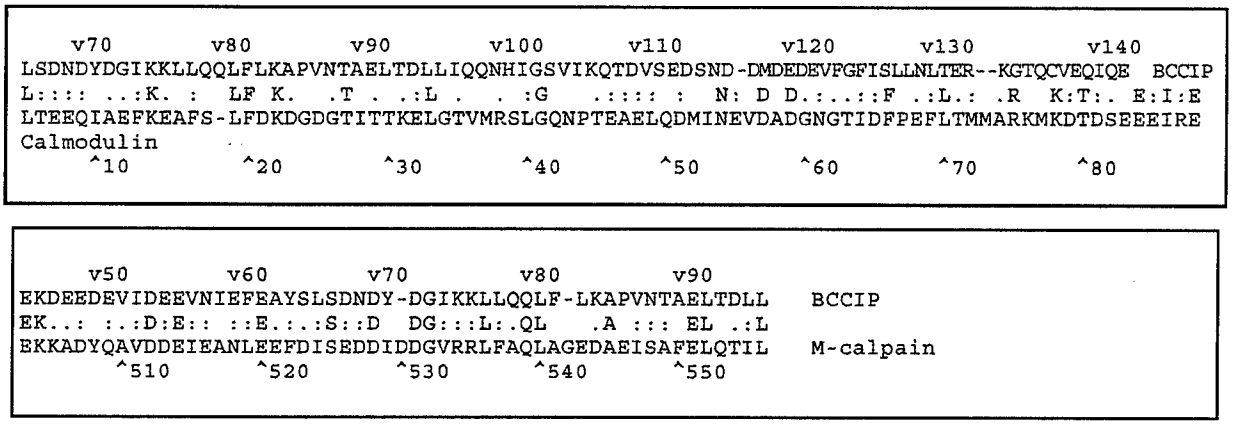
B. Results from *in vitro* protein binding. Label on the top indicate the BRCA2 fragments used with GST (the same BRCA2 fragment nomenclature as Figure 6A is used in this figure).

Figure 7. Chromosome location of *BCCIP* gene.

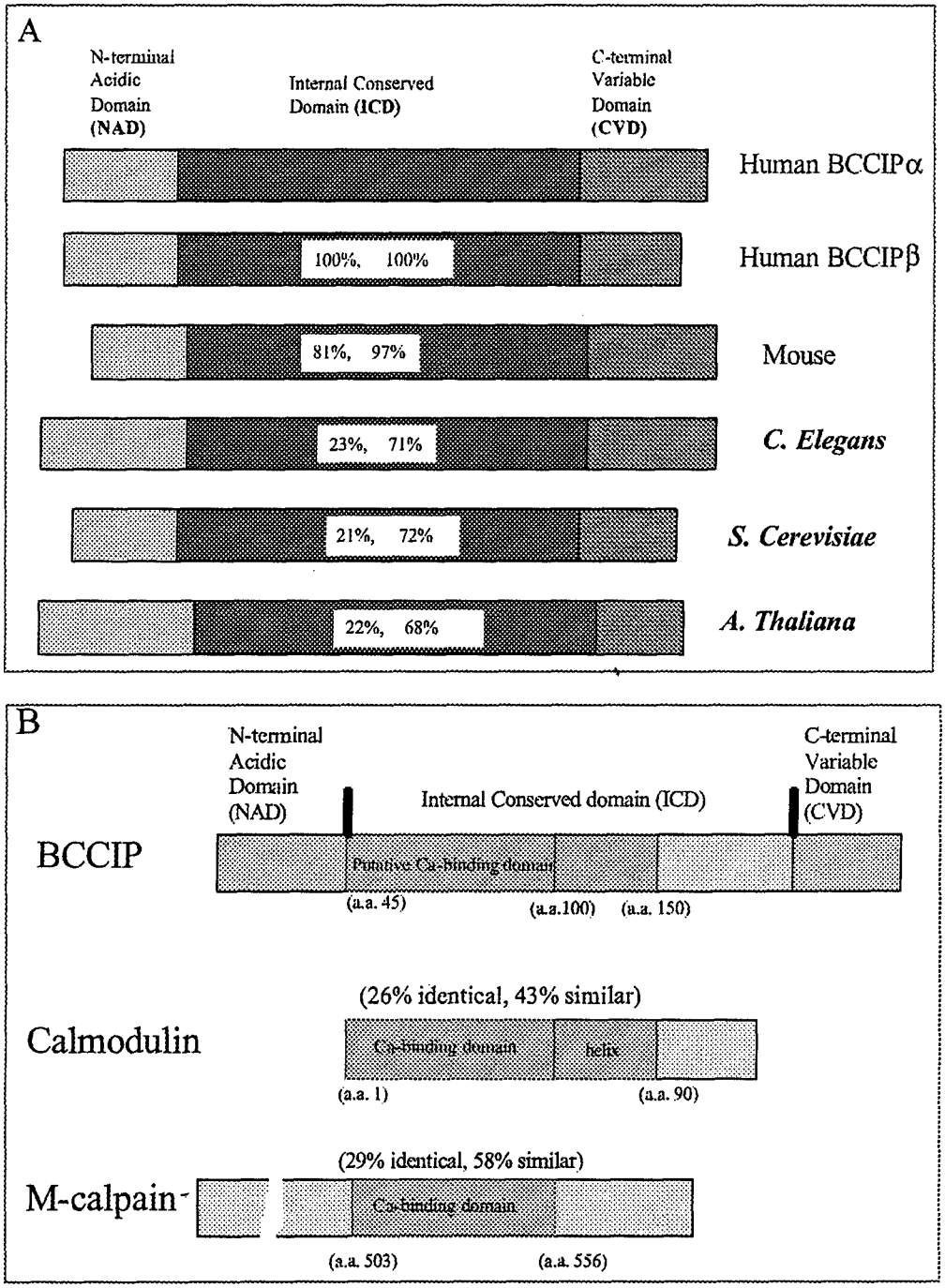
A. FISH analysis of *BCCIP* cDNA. The bright signal (arrow on the left panel indicate the location of BCCIP gene). The right panel is DAPI staining of the same mitotic cell showing chromosome 10.

B. The defined location of BCCIP on chromosome 10q25.3-26.2 (results from detailed band analysis of 10 individual cells).

J. Liu, et al. Figure 1.



J. Liu, et al. Figure 2.



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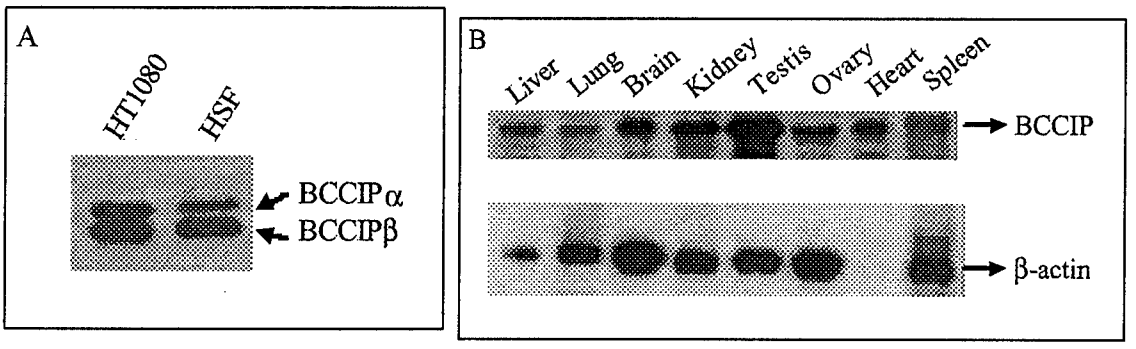

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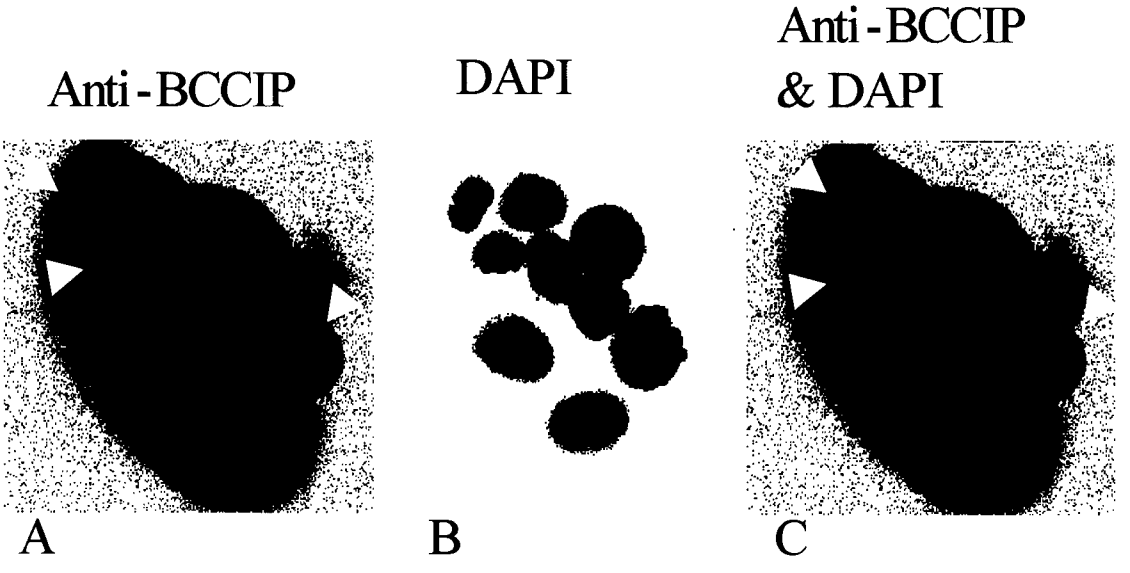

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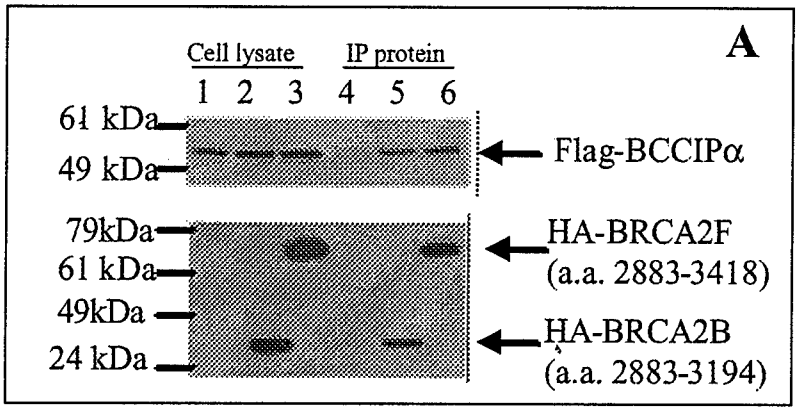

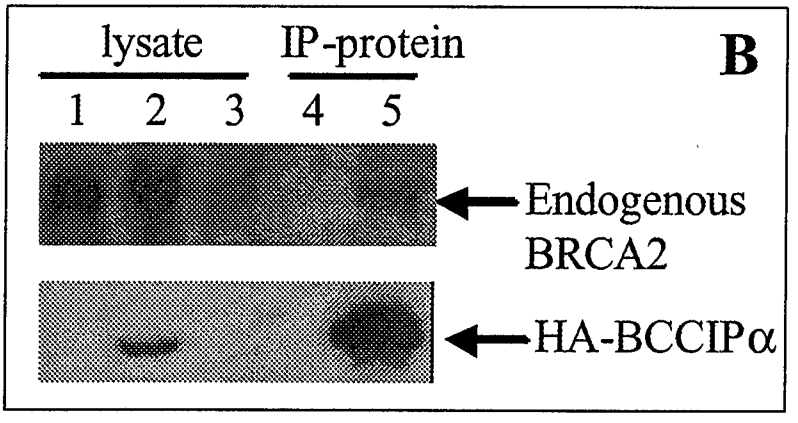

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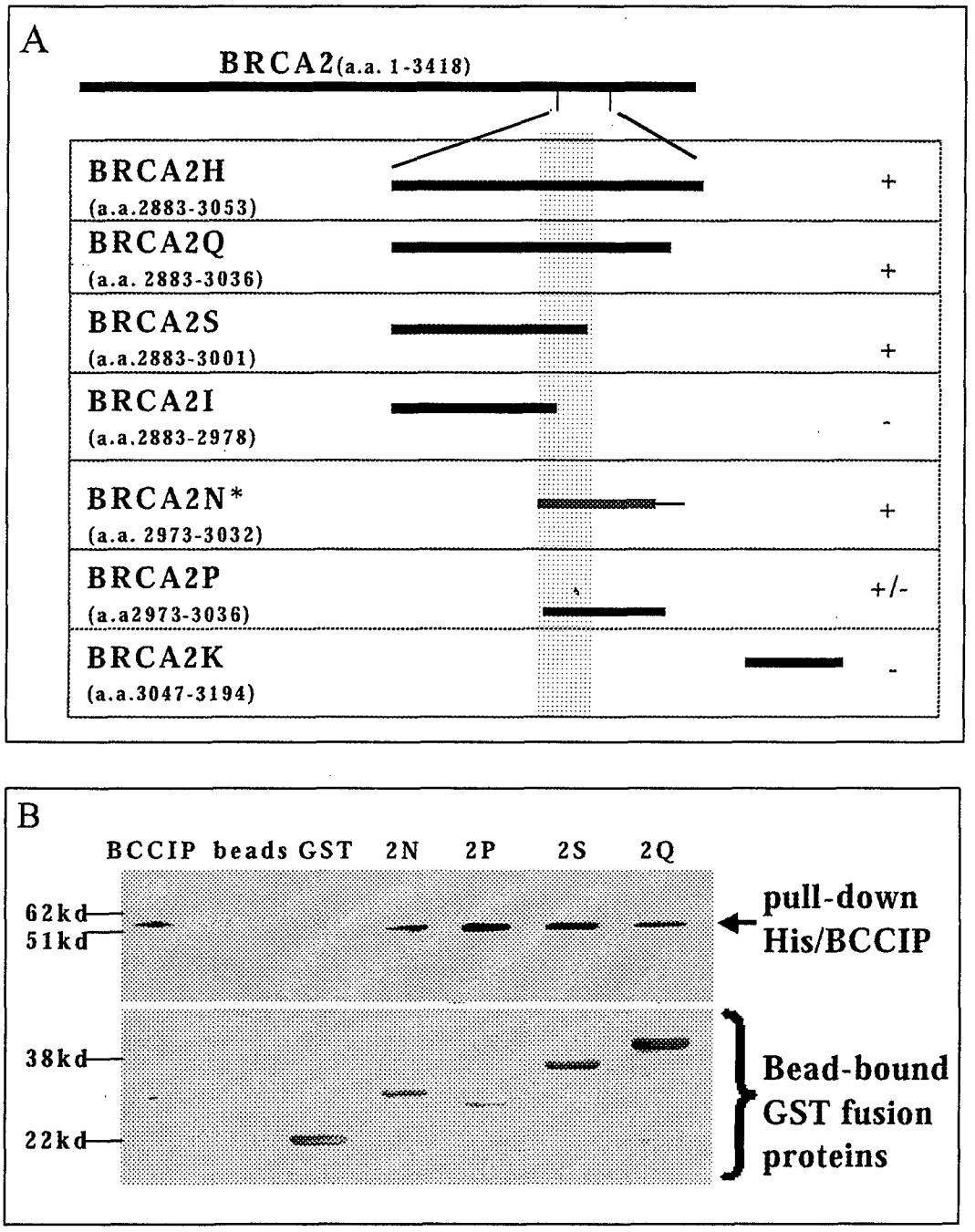

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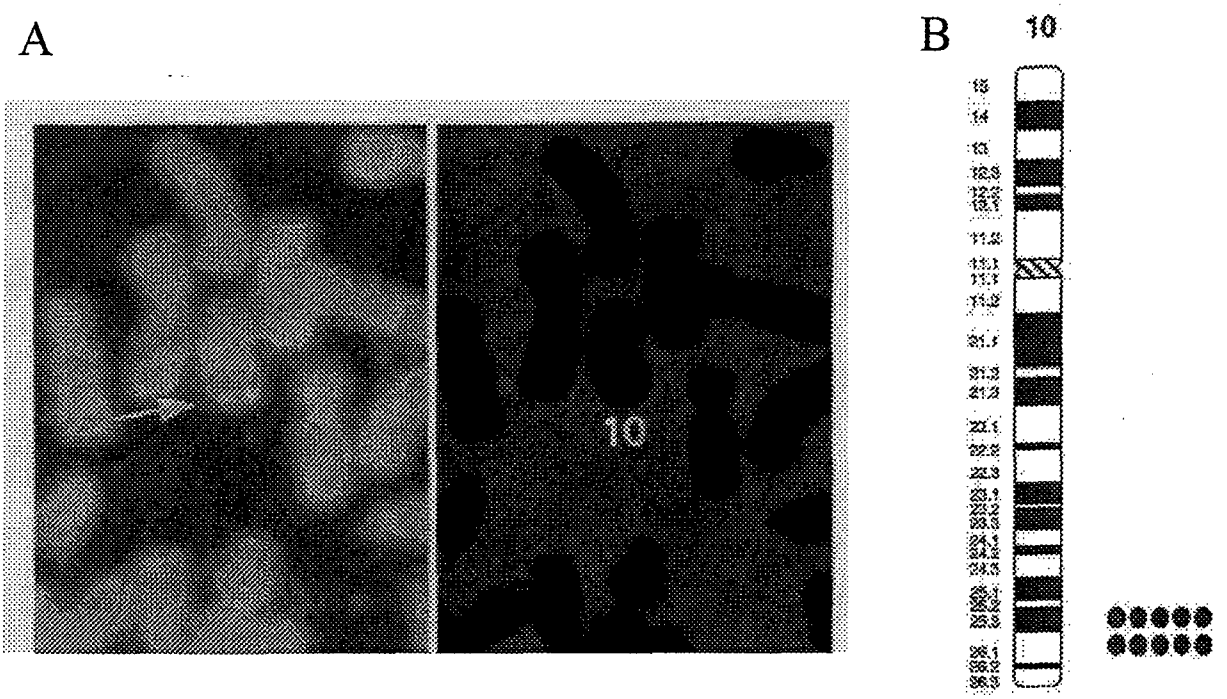

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