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Award Number: DAMD17-02-1-0632

TITLE: Deciphering the Molecular Mechanisms of Breast Cancer

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REPORT DATE: March 2005

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*

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| **1. REPORT DATE** *(DD-MM-YYYY)* | **2. REPORT TYPE** | **3. DATES COVERED** *(From - To)* |
|---|---|---|
| 01-03-2005 | Final | 1 Mar 2002 – 28 Feb 2005 |

**4. TITLE AND SUBTITLE**

Deciphering the Molecular Mechanisms of Breast Cancer

**5a. CONTRACT NUMBER**

**5b. GRANT NUMBER**
DAMD17-02-1-0632

**5c. PROGRAM ELEMENT NUMBER**

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**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

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

The Wistar Institute
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**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSOR/MONITOR'S ACRONYM(S)**

**11. SPONSOR/MONITOR'S REPORT NUMBER(S)**

**12. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for Public Release; Distribution Unlimited

**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**
We have isolated a holoenzyme complex termed BRCC containing BRCA1, BRCA2, and RAD51. BRCC not only displays increased association with p53 following DNA damage but also ubiquitinates p53 in vitro. BRCC36 and BRCC45 are novel components of the complex with sequence homology to a subunit of the signalosome and proteasome complexes. Reconstitution of a recombinant foursubunit complex containing BRCA1/BARD1/BRCC45/BRCC36 revealed an enhanced E3 ligase activity compared to that of BRCA1/BARD1 heterodimer. In vivo, depletion of BRCC36 and BRCC45 by the small interfering RNAs (siRNAs) resulted in increased sensitivity to ionizing radiation and defects in G2/M checkpoint. BRCC36 shows aberrant expression in sporadic breast tumors. These findings identify BRCC as a ubiquitin E3 ligase complex that enhances cellular survival following DNA damage.

**15. SUBJECT TERMS**
**BRCA1, 2; BARD 1; RAD; BRCC; BRE; PCR; DNA; RNA**

| **16. SECURITY CLASSIFICATION OF:** | | | **17. LIMITATION OF ABSTRACT** | **18. NUMBER OF PAGES** | **19a. NAME OF RESPONSIBLE PERSON** |
|---|---|---|---|---|---|
| **a. REPORT** | **b. ABSTRACT** | **c. THIS PAGE** | | | USAMRMC |
| U | U | U | UU | 34 | **19b. TELEPHONE NUMBER** *(include area code)* |

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## INTRODUCTION:

Germline mutations in the tumor suppressor breast cancer genes 1 and 2 (BRCA1 and BRCA2) predispose individuals to breast and ovarian cancers. Although BRCA1 and BRCA2 have been implicated to play a role in transcription and/or DNA repair, the precise mode of their action is not yet understood. Using cell lines expressing a stable labeled breast cancer 1-associated RING domain protein, Flag-BARD1, we have isolated a multiprotein complex termed BRCC containing eight polypeptides including BRCA1, BRCA2 and the radiation-associated protein RAD51. The ubiquitin E3 ligase activity of BRCC is regulated by BRCC36, a novel component of the complex displaying sequence homology with a signalosome subunit. We show that the transient knock out of BRCC36 by small interfering RNA (siRNA) results in increased ionizing radiation sensitivity and disruption of DNA damage-induced G2/M cell cycle check point. These findings identify BRCC as a ubiquitin E3 ligase complex that regulates DNA damage check point.

## BODY:

**Task 1**. Isolate and define the molecular characteristics of the BRCA1-BARD1 complex, using affinity purification by BARD1 antibodies. (months 1-18). This task has been accomplished and was reported on in the progress report (2003).

**Task 2**. Analyze the polypeptide composition of BARD1-BRCA1 complex purified from cells carrying mutations in BRCA1 (months 18-30). This task has been accomplished and was reported on in the progress report (2004).

**Task 3**. Molecular cloning of BRCA1-associated polypeptides and isolation of the complex from BRCA1 mutant cells (months 24-36). This task was also accomplished and was reported on in the progress report (2004).

**Task 4**. Analyze the functional activity of BARD1-BRCA1 complex *in vivo* (18-36).

**Involvement of BRCC36 and BRE in response to ionizing radiation and checkpoint control.**

Number of studies has shown that cells carrying inactivating mutations of BRCA1 display increased sensitivity to ionizing radiation and defective control of G2/M checkpoint**.** To determine whether loss of BRCC36 and BRE resulted in similar DNA repair defects, HeLa cells were treated by siRNAs against BRCA1, BRCC36, Brain and reproductive organ expressed protein (BRE) or control siRNA and their responsiveness to ionizing radiation was measured (Fig. 1A). The experiments were performed in triplicates comparing the cells treated with siRNAs against BRCC36 or BRE, to those treated with siRNAs against BRCA1 and control siRNAs. BRCA1-, BRCC36- and BRE-depleted cells displayed a potent increased sensitivity to ionizing radiation at all doses examined (Fig. 1A).

We next asked whether depletion of BRCC36 and BRE would result in disruption of the G2/M checkpoint arrest. Consistent with a defect in G2/M checkpoint, analysis of mitotic cells following 2 and 4Gy of ionizing radiation indicated that approximately three fold more BRCC36- and BRCA1-depleted cells entered into mitosis (Fig. 1B). Depletion of BRE resulted in a more moderate defect in G2/M checkpoint arrest (Fig. 1B). Taken together these results demonstrate that BRCC36 and BRE are not only components of a multiprotein complex which modulate the enzymatic activity of BRCA1/BARD1 but also participate in a similar pathway of cellular responsiveness to ionizing radiation.

**Aberrant expression of BRCC36 in sporadic breast cancer.**

Quantitative real-time PCR (qPCR) was performed to evaluate the expression of *BRCC36* mRNA levels in multiple independent normal breast organoids (uncultured breast ducts composed of luminal and myoepithelial cells), primary epithelial cell cultures, non-tumorgenic breast epithelial cell lines, breast cancer cell lines, and human breast tissue specimens surgically obtained from patients with primary invasive carcinoma as described in the experimental procedure section. The expression levels of BRCC36 mRNA were elevated in 58% (11 of 19) of the breast tumors evaluated when compared to normal breast organoids (Fig. 2A). A subset of these tumors (6 of 19) showed very high levels of expression relative to both the organoids and primary epithelial cultures (Fig. 2A). The difference in *BRCC36* mRNA levels in the normal organoids or primary epithelial cell cultures may be due to differences in age, parity, or hormonal status of the patients from whom the organoids were derived or to the tissue culturing conditions of the primary epithelial cells. To further validate the expression of *BRCC36* in breast tumors, we performed qPCR analysis on laser captured micro-dissection (LCM)-purified normal mammary ductal epithelial cells (NE) and malignant epithelial (ME) cells. Two normal breast tissues and 9 invasive ductal carcinomas were micro-dissected and the RNA evaluated by RT-PCR (Fig. 2B). We found that 100% of these tumors (9 of 9) showed elevated levels of *BRCC36* mRNA relative to the normal mammary ductal epithelium (Fig. 2B). Seventy-seven percent of these tumors expressed very high levels of *BRCC36* (>20-fold) as compared to normal epithelial cells.

## KEY RESEARCH ACCOMPLISHMENTS:

1-Isolated the BRCA1-BARD1 complex from human cells.

2-Determine the polypeptide composition by MS/MS sequencing.

3-Characterized the ubiquitin ligase activity of BRCA1-BARD1 complex.

4-Cloning of BRCC36 and BRCC45.

5-Estabilishing stable lines expressing BRCC36 and BRCC45.

6-Analysis of polypeptide composition of BRCA1 complexes containing the truncated forms of BRCA1 or BARD1.

7-Laser capture microdissection of nine invasive ductal carcinomas yielded malignant cells with elevated levels (77% >20-fold) of BRCC36 in 9/9 cases as compared with levels in normal ductal cells from two women.

8-Discovery of the aberrant expression levels of BRCC36 in sporadic breast cancers.

## REPORTED OUTCOMES:

### Manuscripts:

Dong Y. Hakimi M.A., Chen X., Kumaraswamy E., Cooch N.S., Godwin A.K., Shiekhattar R. Regulation of BRCC, a Holoenzyme Complex Containing BRCA1 and BRCA2, by a Signalosome-like Subunit and its Role in DNA Repair. Molecular Cell 12; 1087-1099 (2003).

### Presentations/Abstracts:

-Oncogenes and Tumor Suppressors. Gordon Conference, Newport, RI; June 2002. Presentation title: Elucidation of BRCA1 function.

### Patents and licenses:

None

### Personnel supported:

Yuanshu Dong, Ph.D. Postdoctoral fellow (June 2001-Oct 2003). Present: Staff scientist, Hershey Medical Center, Penn State University.

### Cell lines:

Established lines expressing BRCC36 and BRCC45

**Data bases:**
NCBI Nucleotide Sequence
NCBI Protein

**Funding Applied For:**
NCI Grant 7R01CA090758-07 Functional elucidation of BRCA1-containing complexes

**CONCLUSIONS:** Two novel components of BRCC, BRCC36 and BRE were identified in this study. Using recombinant subunits produced in insect cells we were able to partially reconstitute a four-subunit complex containing BRCA1/BARD1/BRE/BRCC36. This complex displayed an increased ubiquitin E3 ligase activity compared to that of BRCA1/BARD1 heterodimer. Therefore, while BRCA1/BARD1 constitute a core-enzyme complex, BRCC represent a holoenzyme containing additional regulatory elements such as BRE and BRCC36. While BRE does not display sequence homology to any known protein, BRCC36 is a protein with close homology to a subunit of the COP9 signalosome (Jab1) and a subunit of the 19S proteasome (pad1). This homology consist of a recently described domain termed the Jab1/MPN or JAMM domain which is predicted to encode a protein with ubiquitin or Nedd 8 deconjugating activity. Although we were unable to show a deconjugating activity for BRCC36 using either ubiquitinated BRCA1 or synthetic ubiquitin chains, it may play such a role once the true substrate is identified. Through the use of siRNAs we delineated a role for both BRCC36 and BRE in responsiveness to ionizing radiation, and the progression through the G2/M checkpoint. Taken together, these results demonstrate a role for BRCC36 and BRE in the DNA damage response pathway.

Since germline mutations of BRCA1 and BRCA2 genes result in breast cancer, we were interested to know whether there were mutations in BRCC36 in breast tumors. Although our preliminary analysis of sporadic breast tumors did not yield any mutations in the BRCC36 gene (data not shown), we found a profound increase in BRCC36 expression in breast tumors. Mirco-dissection analysis of the mammary epithelial ducts from these tumors revealed increased BRCC36 expression in every tumor analyzed. The consequences of BRCC36 over expression are not clear. A possible scenario may involve the disruption of the normal function of BRCC by over-expressed BRCC36. Furthermore, since germline mutations in BRCA1 and BRCA2 account for only 15-20% of breast cancer that clusters in families and 5% of breast cancer overall, BRCC36 and other components of BRCC complex may represent long sought after breast cancer susceptibility genes.

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Dong Y, Hakimi MA, Chen X, Kumaraswamy E, Cooch NS, Godwin AK, Shiekhattar R (2003) Regulation of BRCC, a Holoenzyme Complex Containing BRCA1 and BRCA2, by a Signalosome-like Subunit and its Role in DNA Repair. Molecular Cell 12; 1087-1099.

**APPENDICIES:**

**CURRICULUM VITAE**
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**(May 15, 2007)**

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**POSTDOCTORAL TRAINING AND FELLOWSHIP APPOINTMENTS:**

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1993-1997 Postdoctoral Training (D. Reinberg, Advisor), UMDNJ, New Jersey

**PROFESSIONAL EXPERIENCE**:

2006-present Professor, CRG, Barcelona, Spain
2005-present Professor, The Wistar Institute, Philadelphia, PA
2001-2005 Associate Professor, The Wistar Institute, Philadelphia, PA
1998-present Adjunct Professor, Biochemistry and Biophysics Department, Univ. of Pennsylvania
1997-2001 Assistant Professor. The Wistar Institute, Philadelphia, PA
1998-2004 Member, Graduate Group in Biochemistry & Molecular Biophysics, Univ. of Pennsylvania
1998-present Member, Graduate Group in Genetics, Univ. of Pennsylvania

**HONORS and AWARDS:**

1993-1996 National Institute of Health National Research Service Award
1997-1998 American Cancer Society Institutional Research Grant
1999-2001 Scholar of V Foundation for Cancer Research
1998-2001 W.W. Smith Charitable Trust

**MEMBERSHIP IN PROFESSIONAL SOCIETIES**:

**American Association for the Advancement of Science**
**American Association for Cancer Research**

**Grant Reviews/Study Sections**

**2002,2003,2004 Cancer Research, UK**
2002 NCI, Special Review Panel (Program Project)
2003 NIH, CDF-1 Study Section, Ad hoc member
2004 Cancer Research, Netherlands

**Institutional Faculty Committees/Activities:**

Current: Member, Gene Expression and Regulation Program
Member, Molecular & Cellular Oncogenesis Program
Member, Faculty Recruitment Committee

**Periodic Manuscript Reviews:**

Cell, Science, Molecular Cell, Molecular and Cellular Biology, Nature Genetics, Nature Cell Biology, J. Biol. Chem., P.N.A.S, Journal of Neuroscience, Cancer Research.

**Invited Meetings and Seminars:**

**2007**

-Keystone symposium

**2006**

-Annual Opnion Leader Consortium on Novel and Targeted Therapies for Head and Neck Cancer, Sonesta Beach Resort in Key Biscayne, Florida; Feb 2006.
-University of Alabama Birmingham, Al; March 2006.
-National Institute of Health. Washington; April 2006.
-Fourth international RANi 2006-Boston, MA; May 2006.
-IFOM-IEO Meeting on Cancer, Milan; May 2006.
-The university of Edinburgh, Scotland; June 2006.
-UK chromatin meeting 2006, Manchester, UK; June 2006.
-Annual Rett Syndrome Research Foundation, Chicago; June 2006.
-7th EMBL Transcription Meeting, Heidelberg; Aug 2006.
-ASBMB Chromatin Structure and Function. South Carolina; Oct 2006.
-ABCAM meeting on chromatin, Dominican Republic; Nov 2006.

**2005**

-Fox Chase Cancer Center, Philadelphia, PA; March 2005.
-Chromatin modifications. Keystone meeting, Snowbird, Utah; April 2005
-University of Delaware, Delaware; April 2005.
-Saint Louis University; Department of Biochemistry; April 2005.
-1st International Biochemistry and Molecular Biology; Tehran, IRAN, Sept 2005.
-New York University, NY; Oct 2006.
-RNAi workshop, Lake Tahoe; Nov 2005.
-ABCAM meeting on chromatin, Bahamas; Nov 2005.

**2004**

-Marie Curie Research Institute at Oxford UK; May 2004.
-Chromatin Structure and Function. ASBMB at Lake Tahoe; Oct, 2004.
-Epigenetics and Cancer. AACR at Kona, Hawaii; Nov, 2004.

**2003**

- Transcription and Chromatin. Keystone meeting, Santa Fe; March 2003.
-Cancer Symposium, Ann Arbor, Michigan Sept 2003.
-Children's Hospital of Philadelphia, Philadelphia, Oct 2003.
- Department of Biology, Temple University; Philadelphia; Nov 2003.

**2002**

- Department of Biochemistry and Biophysics, Tehran University, March 2002.
-Laval University Cancer Research Center, Hotel-Dieu de Quebec; Canada; May 2002.
-Oncogenes and Tumor Suppressors. Gordon Conference, Newport, RI; June 2002.

**2001**
- Department of Biology, University of Toronto; Canada; March 2001.
-Cancer Symposium, Amory University; Atlanta; June 2001.

**2000**
-Saint Louis University; Department of Biochemistry; Feb, 2000.
-Salk Institute, San Diego; Nov 2000.
-Department of Molecular Medicine, Ottawa Health Research Institute; Ottawa, Canada; Dec 2000.

**1999**
-Regulation of Eukaryotic Transcription; ASBMB at Lake Tahoe; Oct 1999.

**Research Interests:**

* Mechanism of Tumor suppressor functions
* Analysis of the role of chromatin-remodeling complexes in transcription and DNA repair
* Mechanism of transcriptional silencers
* Role of small RNA in the formation of higher order chromatin structure

**Patents:** PCT/USO1/22684. Title: Role of BRCA1 in chromatin remodeling. Inventors: Ramin Shiekhattar.

Title: BRAF35: the DNA-binding component of the BRCA2 complex. Inventors: Ramin Shiekhattar, A provisional patent application has been filed.

C. Past and Present Training Record of Advisor

1- Daniel Bochar, Ph.D. Postdoctoral fellow (Sept 98-Aug 2003). Present: Assistant Professor, Department of Biological Chemistry, University of Michigan.

2- Lihua Marmorstein, Ph.D. Postdoctoral fellow (Oct 98-May 99). Present: Assistant Professor, Department of Ophthalmology, University of Arizona.

3- Alexander Kinev, Ph.D. Postdoctoral fellow (Oct 98-Oct 2001). Present: Research Associate, University of North Carolina at Chapel Hill.

4- Hideo Beniya, Ph.D. Ph.D. Postdoctoral fellow (July 99-June 2001). Present: Returned to Japan.

5- Mohammed-Ali Hakimi. Ph.D. Postdoctoral fellow (Dec 2000-Dec 2002). Present: Assistant Professor, Epigenetic and Parasites Group; Parasitology Department; CNRS; France

6- Orr Barak, Predoctoral fellow (Jan 2001-Nov 2003). Present: Medical Student; University of Pennsylvania Medical School.

7-Yuanshu Dong, Ph.D. Postdoctoral fellow (June 2001-Oct 2003). Present: Post-Doctoral fellow, Hershey Medical Center, Penn State University.

8- Easwari Kumaraswamy, Associate Staff Scientist (Aug 2001-Jan 2006). Senior staff scientist, Childern's Hospital Philadelpia, PA.

9- Amuthan Govindasamy, Ph.D. Staff Scientist (Oct 2002-Dec 2004). Present: Assistant Professor at Singapore University.

10- Jessica Norman, Predoctoral fellow (Jan 2002-2006).

11- Chris Wynder, Ph.D. Postdoctoral fellow (Oct 2003- Sept 2006). Present: Assistant Professor at McGill University, Montreal, Quebec, Canada.

12- Tim Chendrimada, Ph.D. Postdoctoral fellow (Oct 2003-Aug 2006). Present: Staff Scientist, Wyeth Pharmaceuticals

13- Richard Gregory, Ph.D. Postdoctoral fellow (Nov 2003- Sept 2006). Present: Assistant Professor, Childrens Hospital, Harvard University, Cambridge Mass.

14- Min Gyu Lee, Ph.D. Postdoctoral fellow (Dec 2003- Feb 2007) Present: Staff Scientist Wistar Inst.

15- Behzad Doratotaj, M.D. Postdoctoral fellow (Feb 2004-Dec 2004). Present: Internship at Hahnemann Medical Center, Philadelphia PA.

16- Kaiping Yan, Ph.D. Postdoctoral fellow (Feb 2004-present).

17- David Baillat, Ph.D. Postdoctoral fellow (Dec 2003-present).

18- Frederic Tort, Ph.D. Postdoctoral fellow (Oct 2006-present).

19- Laure Weill, Ph.D. Postdoctoral fellow (Jan 2007-present).

20- Fab Lai, Ph.D. Postdoctoral fellow (Jan 2007-present).

**Manuscripts in Preparation**

Kumaraswamy E., Shiekhattar R. BACH1 is a component of a distinct BRCA1/BRCA2-containing complex involved in S phase progression. (submitted).

Yan K., Gregory R.I., Shiekhattar R. Argonautes associate with the human RNA polymerase II to mediate post-transcriptional gene silencing. (submitted).

**Research Publications, Peer Reviewed**

Chendrimada T.P., Gregory R.I., Shiekhattar R. MicroRNAs inhibit translational through RISC requirement of 60S ribosome and eIF6. (Nature in press).

Lee M.G., Norman J.A., Shilatifard A., Shiekhattar R. Physical and functional association of a trimethyl H3K4 demethylase and Ring6a/MBLR, a polycomb-like protein. Cell 128; 877-887 (2007).

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Bochar D.A., Wang L., Beniya H., Kinev A., Xue Y., Lane W.S., Wang W., Kashanchi F., Shiekhattar R. BRCA1 is associated with a human SWI/SNF-related complex: Linking Chromatin Remodeling to Breast Cancer. Cell 102: 257-256 (2000).

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SUPPORTING DATA

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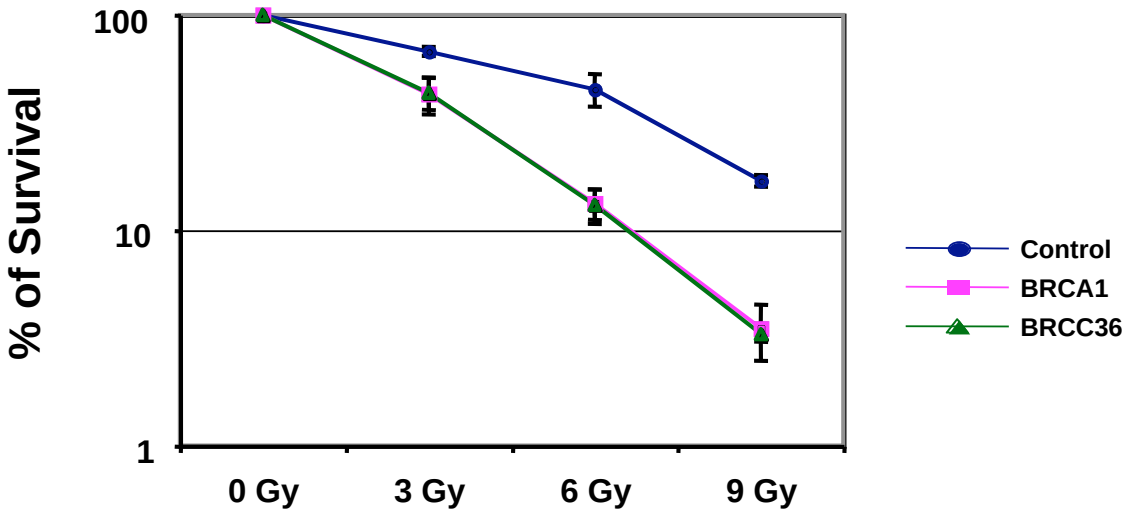


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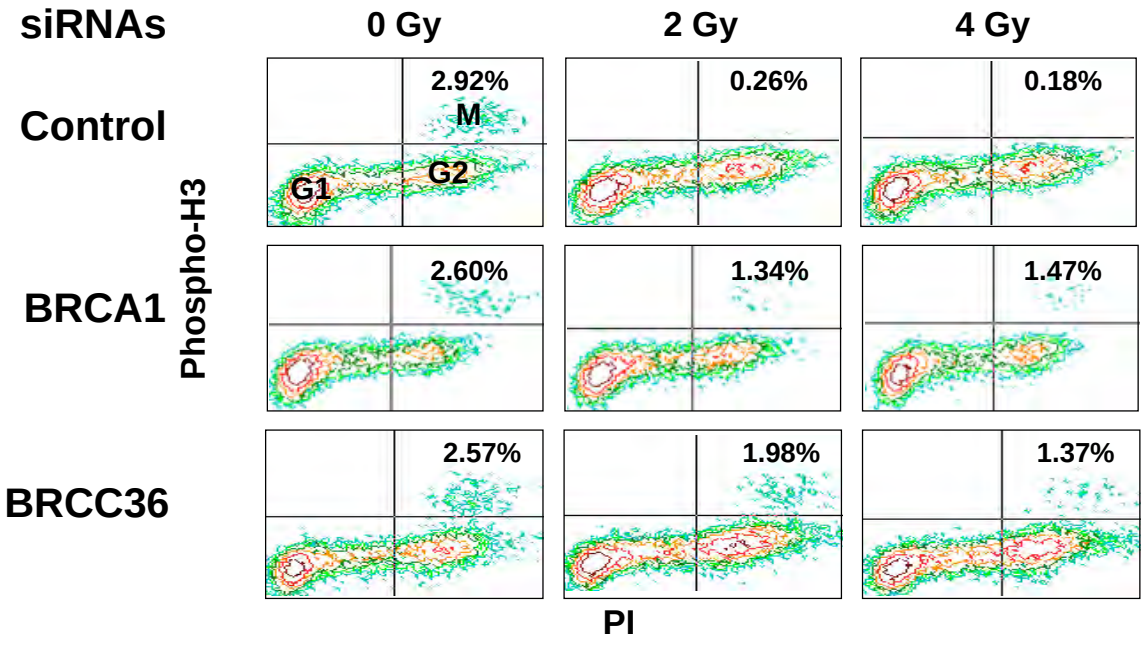


Figure 1

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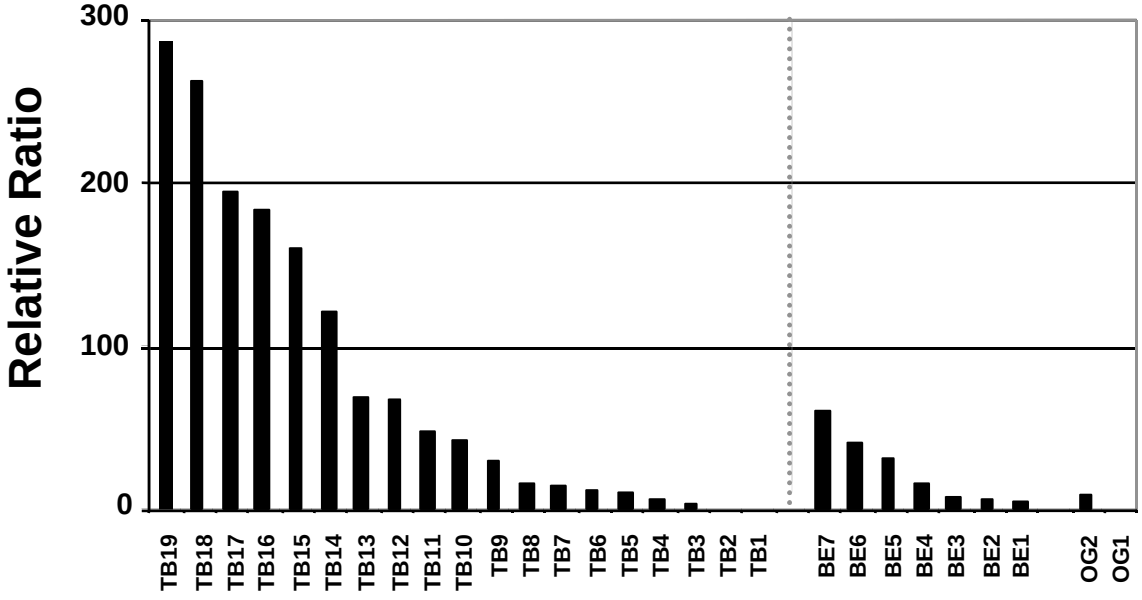
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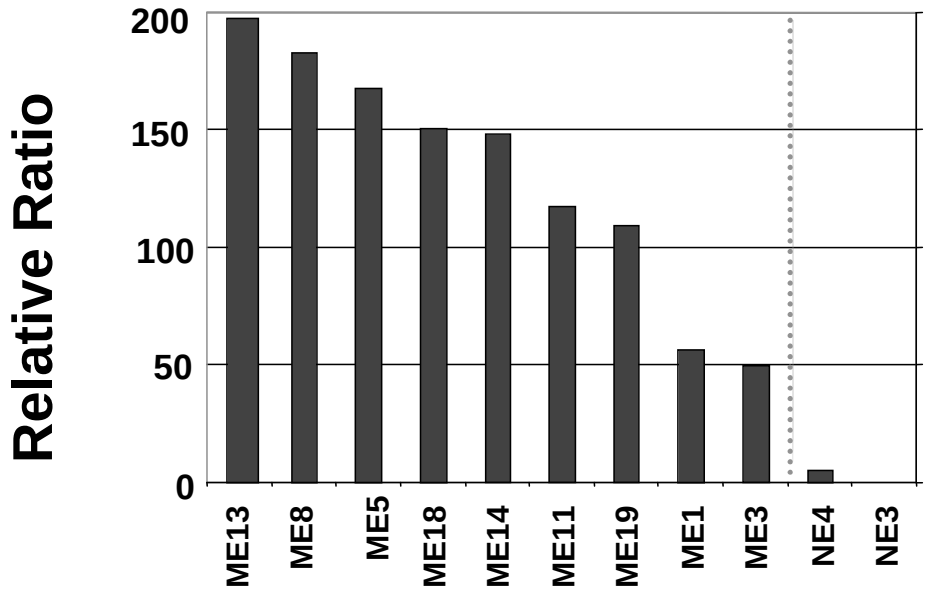
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Figure 2

**Figure Legends.**

**Figure 1. Depletion of BRCC36 and BRE result in increased sensitivity to ionizing radiation and defects in G2/M checkpoint arrest.**

(A) Treatment of HeLa cells with siRNA against BRCC36, BRE or BRCA1 results in enhanced sensitivity to ionizing radiation.

(B) HeLa cells were treated with siRNA against BRCA1, BRCC36, BRE or control siRNA and following treatment with ionizing radiation their cell cycle progression was measured by FACS.

**Figure 2. Aberrant expression of BRCC36 in breast tumors.**

(A) Real time PCR was used to analyze breast mammary organoids (OG), primary breast epithelial cells (BE), and breast tumors (TB). The expression levels of BRCC36 were adjusted for β-actin expression and the ratio for OG1 was set at 1, described in detail in Experimental Procedure.

(B) Quantitative real-time PCR was used to analyze normal mammary ductal epithelial cells (NE) and malignant epithelial (ME) cells captured by micro-dissection.

Molecular Cell, Vol. 12, 1087–1099, November, 2003, Copyright ©2003 by Cell Press

# Regulation of BRCC, a Holoenzyme Complex Containing BRCA1 and BRCA2, by a Signalosome-like Subunit and Its Role in DNA Repair

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## Summary

**We have isolated a holoenzyme complex termed BRCC containing BRCA1, BRCA2, and RAD51. BRCC not only displays increased association with p53 following DNA damage but also ubiquitinates p53 in vitro. BRCC36 and BRCC45 are novel components of the complex with sequence homology to a subunit of the signalosome and proteasome complexes. Reconstitution of a recombinant four-subunit complex containing BRCA1/BARD1/BRCC45/BRCC36 revealed an enhanced E3 ligase activity compared to that of BRCA1/BARD1 heterodimer. In vivo, depletion of BRCC36 and BRCC45 by the small interfering RNAs (siRNAs) resulted in increased sensitivity to ionizing radiation and defects in G2/M checkpoint. BRCC36 shows aberrant expression in sporadic breast tumors. These findings identify BRCC as a ubiquitin E3 ligase complex that enhances cellular survival following DNA damage.**

## Introduction

Germline mutations in *BRCA1* or *BRCA2* genes predispose women to early onset, familial breast cancer (Hall et al., 1990; Narod et al., 1991; Miki et al., 1994; Wooster et al., 1994, 1995; Tavtigian et al., 1996). Furthermore, deleterious alleles of *BRCA1* and *BRCA2* are responsible for almost all familial ovarian cancer, and deleterious alleles of *BRCA2* are also involved in hereditary male breast cancer (Wooster et al., 1995; Tavtigian et al., 1996; Easton et al., 1993; Miki et al., 1994).

Both BRCA1 and BRCA2 encode large proteins without extensive homology to other proteins in the database (Miki et al., 1994; Tavtigian et al., 1996). The primary sequence of BRCA1 contains two motifs characteristic of transcription factors (Miki et al., 1994). These include a RING finger motif and an acidic carboxyl terminus. Fusion of this carboxyl terminus to the DNA binding domain of the GAL4 protein endows the chimeric protein with transcriptional stimulatory activity (Chapman and Verma, 1996; Monteiro et al., 1996). BRCA1 was not only implicated to function as a coactivator for p53-mediated transcription (Ouchi et al., 1998; MacLachlan et al., 2002) but also to associate with RNA polymerase II (RNAPII) and the chromatin remodeling complex, SWI/SNF (Scully et al., 1997a; Bochar et al., 2000). Taken together, these observations led to the hypothesis that BRCA1 may function as a transcriptional regulator.

A host of other reports have implicated a role for BRCA1 and BRCA2 in DNA repair. BRCA1 was reported to interact with RAD51, BRCA2, FANCD2, and the RAD50 protein complex (Garcia-Higuera et al., 2001; Chen et al., 1998a; Zhong et al., 1999; Scully et al., 1997b, 1999; Sarkisian et al., 2001). BRCA1 mutant cells display sensitivity to DNA-damaging agents, and the BRCA1 protein was reported to control homology-directed DNA repair (Moynahan et al., 1999; Zhong et al., 2002). Truncation of BRCA1 exon 11 has also been shown to result in defective G2-M cell cycle checkpoint and an increased number of centrosomes (Xu et al., 1999). Moreover, a number of laboratories have reported that the BRCA2 protein interacts with RAD51 and plays a role in cell cycle regulation and homology-directed repair (Wong et al., 1997; Chen et al., 1998b; Mizuta et al., 1997; Marmorstein et al., 1998, 2001; Yu et al., 2000; Moynahan et al., 2001; Davies et al., 2001). These results were strengthened by a report showing that murine embryos with a targeted disruption of *BRCA2* displayed sensitivity to ionizing radiation (Sharan et al., 1997). Furthermore, mouse embryo fibroblasts (MEFs) with a targeted disruption of *BRCA2* exon 11 displayed increased sensitivity to ultraviolet light and methyl methanesulfonate (MMS) (Patel et al., 1998).

BRCA1 interacts with the BRCA1-associated RING domain (BARD1) protein to form a heterodimeric complex (Wu et al., 1996, Brzovic et al., 2001). Remarkably, BARD1 association with BRCA1 potentiates the newly discovered ubiquitin E3 ligase activity of the BRCA1 protein (Hashizume et al., 2001). Detailed analysis of the BRCA1 ubiquitin E3 ligase activity has identified the RING domain of BRCA1 as the catalytic determinant for ubiquitination (Lorick et al., 1999, Hashizume et al., 2001, Ruffner et al., 2001). Furthermore, a recent report describes the ability of BRCA1-BARD1 heterodimer to autoubiquitinate BRCA1 and BARD1 and transubiquitinate the histone H2A(X) (Chen et al., 2002).

To gain further insight into the molecular mechanism of the BARD1-BRCA1 complex, we generated stable cell lines expressing epitope-tagged BARD1. Using these cell lines, we have isolated a multiprotein complex containing BRCA1, BRCA2, and RAD51. Here, we describe two novel components of the complex, the BRCC36 and BRCC45 proteins. BRCC36 displays sequence homology to a 26S proteasome subunit and a subunit of the COP9 signalosome. These results demonstrate the stable association of BRCA1, BARD1, and BRCA2 in a ubiquitin ligase complex that is regulated through a direct interaction with novel regulatory subunits.

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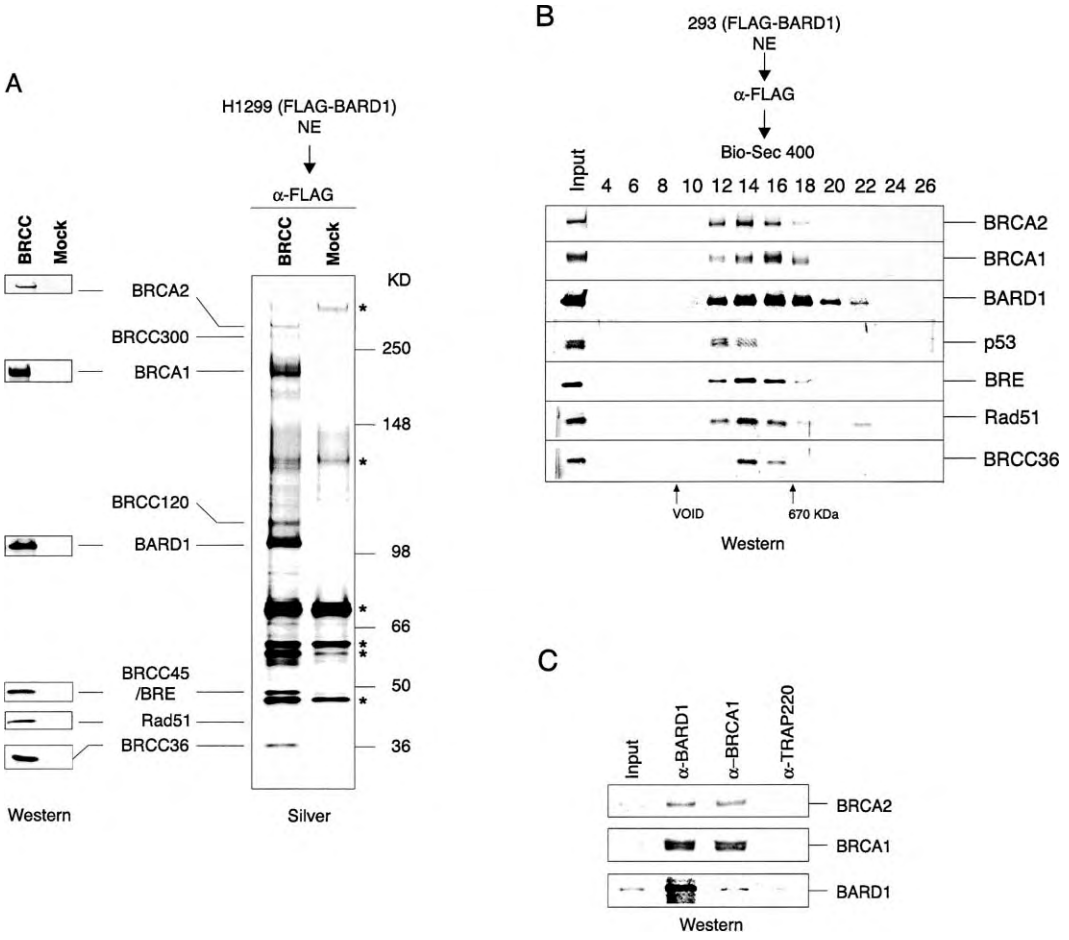


Figure 1. Purification of the BRCC Complex

(A) Analysis of anti-FLAG eluate by SDS-PAGE followed by silver staining and Western blot analysis using antibodies depicted. Asterisks denote nonspecific polypeptides.

(B) Purification scheme and Western blot analysis of Bio-Sec400 gel filtration column fractions using antibodies to the right of the figure.

(C) Immunoprecipitation followed by Western blot analysis using antibodies denoted in the figure.

## Results

### Isolation of BRCC

To isolate BARD1-containing complex(es), we developed H1299- and 293-derived cell lines expressing FLAG-tagged BARD1. Figure 1A depicts the purification of FLAG-BARD1 using anti-FLAG antibodies from H1299 cells. Nuclear extract from native H1299 cells was used as the control for anti-FLAG affinity purification. Analysis of the FLAG-BARD1 eluate by SDS-PAGE and silver staining revealed the specific association of BARD1 with polypeptides of 350, 300, 210, 120, 45, 40, and 36 kDa (Figure 1A). A combination of mass spectrometric sequencing and Western blot analysis identified the 350, 210, and 40 kDa bands as BRCA2, BRCA1, and RAD51, respectively (Figure 1A). We therefore named this complex BRCC for *BR*CA1-BRCA2-containing *c*omplex. Analysis of a number of preparations indicated that RAD51 is a substoichiometric component of this complex (Figure 1A). The 300, 120, 45, and 36 kDa polypeptides correspond to functionally uncharacterized genes.

To establish that BRCC represents a single complex and is not specific to H1299 cells, we isolated BRCC from a 293-derived cell line expressing FLAG-BARD1 and fractionated the complex by gel filtration chromatography using 500 mM KCl (Figure 1B). As Figure 1B indicates, BRCA2, BRCA1, BARD1, and RAD51 coelute as a large mutliprotein complex. Interestingly, a fraction of BRCA1 and BARD1 elute at a smaller molecular mass (peak fraction 16), indicating that a fraction of BRCA1/BARD1 can be resolved from the larger complex. Finally, immunoprecipitation experiments using anti-BARD1 and anti-BRCA1 antibodies demonstrate the association of BRCA1, BRCA2, and BARD1 from nuclear extract of native 293 cells (Figure 1C). Taken together, these results demonstrate the stable association of BRCA1, BRCA2, BARD1, and RAD51 in a multiprotein complex.

### BRCC Displays a Ubc5-Dependent E3 Ubiquitin Ligase Activity toward p53

Previous reports described the BRCA1-BARD1 heterodimer as an E3 ubiquitin ligase (Lorick et al., 1999, Ruff-

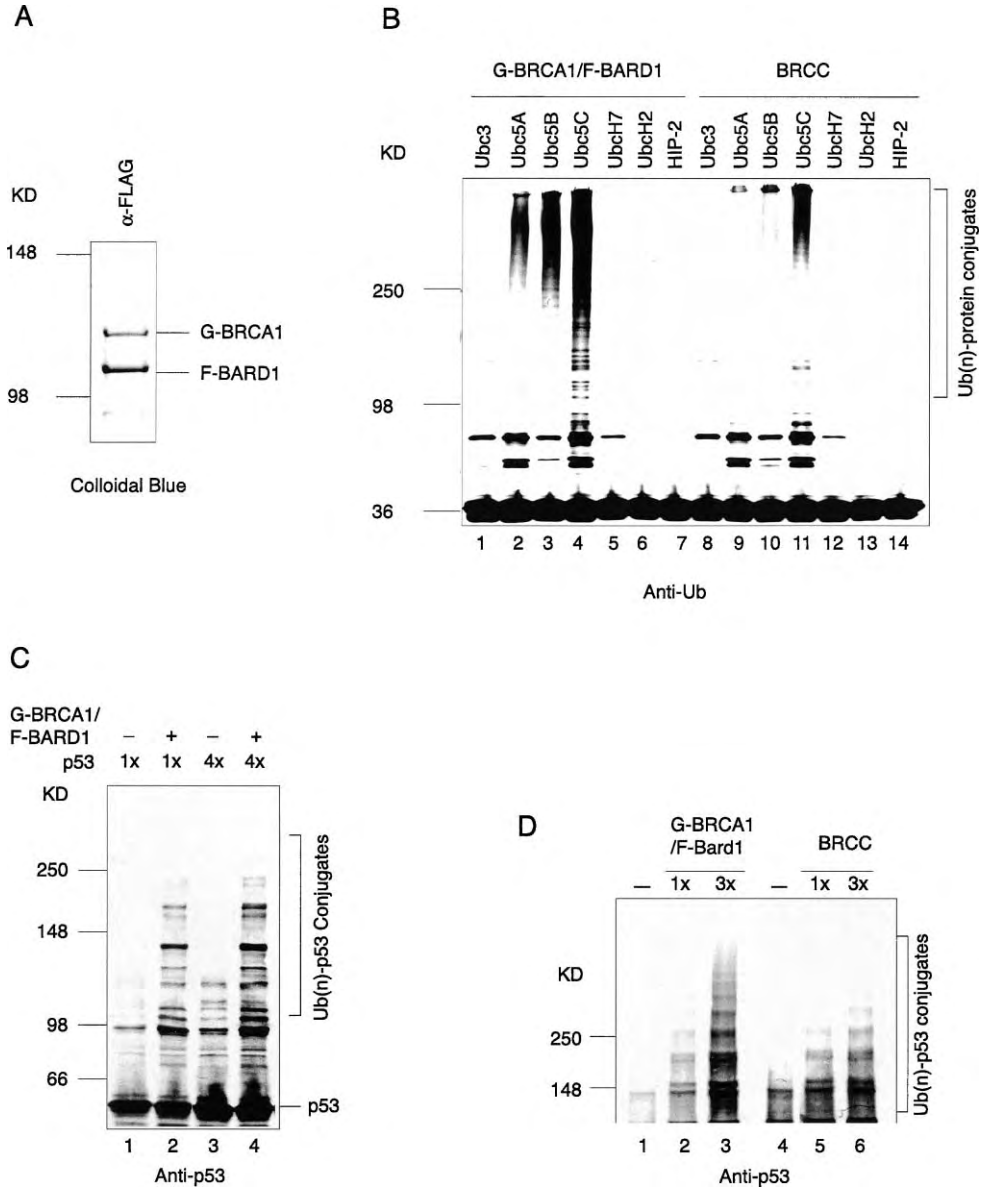


Figure 2. BRCC Is a Ubiquitin E3 Ligase

(A) SDS-PAGE followed by colloidal blue staining of recombinant GST-tagged *BRCA1* (1–639, G-BRCA1) and full-length FLAG-*BARD1* (F-BARD1) (150 ng) coexpressed in bacteria and purified through the FLAG epitope.

(B) Ubiquitin ligation assay using either recombinant G-BRCA1/F-BARD1 (10 ng) or BRCC (2.5 ng) as the source of E3. The concentrations refer to amounts of BRCA1-BARD1 heterodimer for each complex. Different E2s are denoted on the top of the figure.

(C) Ubiquitination of P53 (1x = 6 ng) by G-BRCA1/F-BARD1 (10 ng) using Ubc5c as E2.

(D) Ubiquitination of p53 (60 ng) by either G-BRCA1/F-BARD1 or BRCC (1x = 10 ng). Protein concentrations delineate equal amounts of BRCA1/BARD1 heterodimer.

ner et al., 2001, Hashizume et al., 2001). We therefore asked whether BRCC displays E3 ubiquitin ligase activity and whether its enzymatic activity is similar to that of recombinant BRCA1-BARD1. E1, E2, and ubiquitin were produced in bacteria and purified to near homogeneity. Additionally, recombinant G-BRCA1/F-BARD1 was generated by coexpressing GST-tagged BRCA1(1–639) and FLAG-tagged BARD1 in bacteria as previously described (Figure 2A; Chen et al., 2002). Both the recombinant G-BRCA1/F-BARD1 and BRCC demonstrated Ubc5-dependent ubiquitin E3 ligase activity (Figure 2B). Ubc5c displayed the most activity with either recombinant G-BRCA1/F-BARD1 or the BRCC complex as the E3 enzyme (Figure 2B). In Figure 2B, the concentrations of GST-BRCA1 and BARD1 were higher in the recombinant enzyme preparations. The two enzymes will be directly compared by partial reconstitution of BRCC using full-length recombinant BRCA1 in Figure 5.

We were next interested to examine the ubiquitin E3 ligase activity of the BRCC complex toward a substrate protein. A number of reports have described the physical and functional association of the BRCA1 and BRCA2

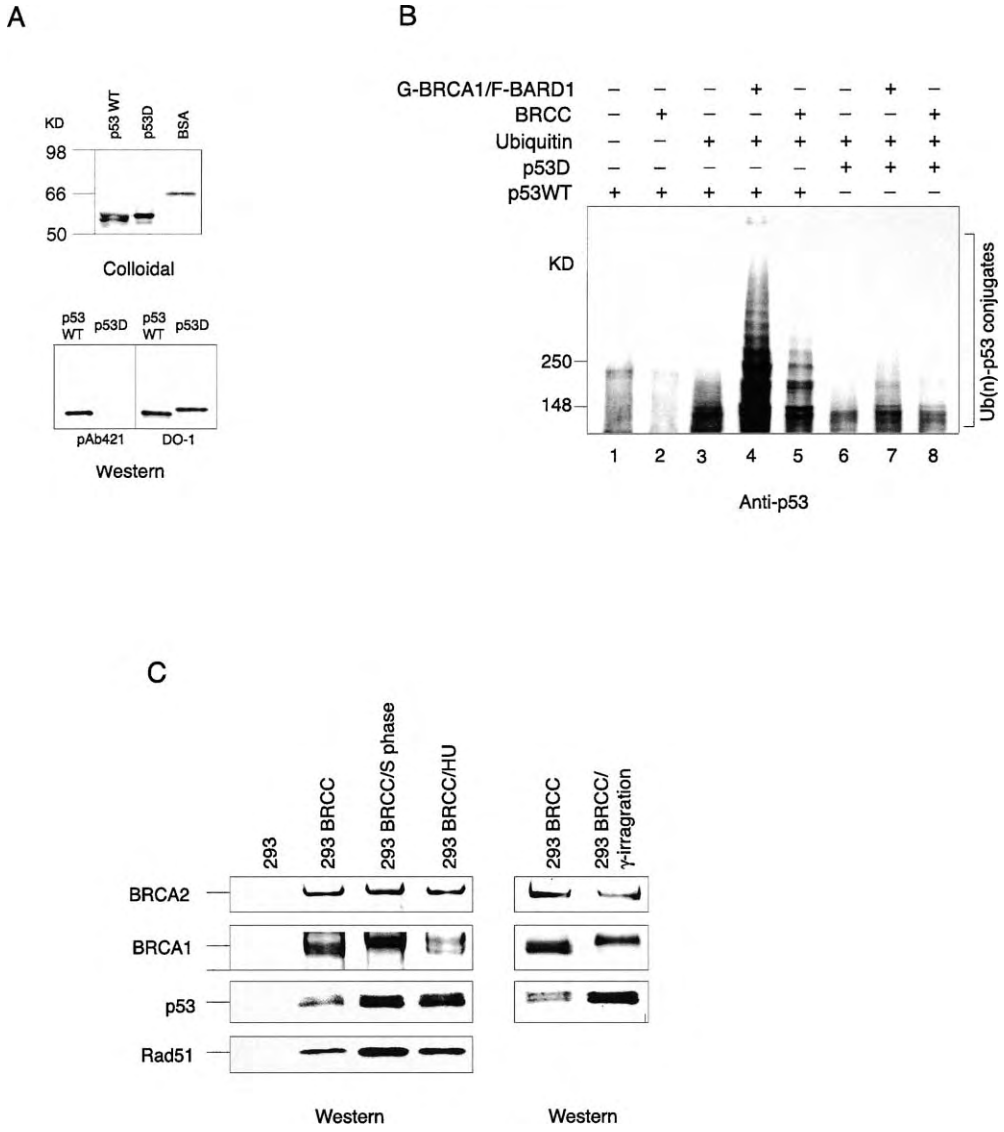


Figure 3. BRCC Ubiquitination of the C Terminus of p53 and Increased Association with p53 following DNA Damage

(A) Colloidal blue and Western blot analysis of recombinant p53 used as the substrate for ubiquitination assays.

(B) Wild-type or truncated p53 (20 ng) was used for ubiquitination experiments.

(C) Increased association of p53 and RAD51 with BRCC demonstrated by Western blot analysis of BRCC following treatment of cells with ionizing radiation, hydroxurea, or double thymidine block according to the Experimental Procedures using antibodies to the left of the figure. BRCC was purified from FLAG-*BARD1* containing cells using FLAG-affinity purification.

proteins with the cellular gate keeper p53 (Ouchi et al., 1998; Zhang et al., 1998; Marmorstein et al., 1998; Xu et al., 2001; MacLachlan et al., 2002). We therefore asked whether p53 can serve as an in vitro substrate for ubiquitination by BRCC. We first asked whether recombinant G-BRCA1/F-BARD1 ubiquitinates p53. As Figure 2C indicates, p53 protein displayed polyubiquitin chain formation following addition of the recombinant G-BRCA1/F-BARD1. Interestingly, addition of E1 and E2 proteins also led to a small amount of ubiquitin conjugate formation (Figure 2C, lanes 1 and 3). However, increasing concentrations of both the recombinant G-BRCA1/F-BARD1 and BRCC displayed a robust E3 ubiquitin ligase activity on recombinant p53 (Figure 2D, compare lanes 3 and 6 with 1 and 4, respectively).

### The Extreme C Terminus of p53 Is Ubiquitinated by BRCC

To examine the sites of ubiquitination of p53 by BRCC, we asked whether the last 30 amino acids of p53 which contain the lysine residues targeted by the MDM2 protein were also the sites for ubiquitination by BRCC (Rodriguez et al., 2000; Gu et al., 2001; Nakamura et al., 2000). Both recombinant full-length p53 (p53 WT) and p53 protein truncated for the last 30 amino acids (p53D) were produced in bacteria and purified to homogeneity (Figure 3A). The truncated p53 displayed a slight increase in molecular weight due to additional amino acids introduced by double tagging. To confirm that the truncated protein indeed lacked the last 30 amino acids, both proteins were subjected to Western blotting using

anti-p53 antibodies raised against the N or the C terminus of p53. Although anti-p53 antibodies raised against the N terminus of the protein (DO-1) recognized both the wild-type and the truncated protein, the antibodies against the C terminus (pAb421) only recognized the wild-type protein (Figure 3A).

We then used the wild-type or the truncated p53 as substrates for the E3 ubiquitin ligase assays. As Figure 3B indicates, while both recombinant G-BRCA1/F-BARD1 and BRCC specifically ubiquitinate the wild-type p53 protein, neither enzyme preparation could ubiquitinate similar amounts of the p53 protein lacking the last 30 amino acids, indicating that the lysine residues on the extreme C terminus of p53 are the sites of ubiquitination by the BRCC complex. It is also possible that the C terminus of p53 constitutes a binding site for BRCC.

### Analysis of BRCC following DNA Damage and at S Phase

Previous studies indicated that the BRCA1 protein is a phosphoprotein that is phosphorylated at S phase and following treatment of cells with DNA damaging agents (Scully et al., 1997c). Moreover, BRCA1 displays a nuclear dot pattern at the S phase of the cell cycle and that BRCA1 nuclear dots colocalize with those of RAD51 (Scully et al., 1997b). Therefore, we examined the changes in the BRCC polypeptide composition at the S phase of the cell cycle and following treatment of cells with hydroxyurea or ionizing radiation. While there was no detectable change with the E3 ubiquitin ligase activity of the complex or the BRCA2 protein, BRCA1 displayed the previously reported shift in electrophoretic mobility representing the phosphorylated form of the protein following all three treatments (Figure 3C). We then asked whether BRCC displays an increased association with RAD51 and the p53 protein. Interestingly, analysis of BRCC purified using FLAG-BARD1 cell lines following treatment of cells with DNA-damaging agents or cells synchronized at S phase revealed an increased association of both RAD51 and p53 with the BRCC complex (Figure 3C; also see Figure 1B). These results are not only the biochemical confirmation of the previous nuclear colocalization experiments but also extend these cell cycle and DNA damage-induced associations to the p53 protein.

### BRCC36 and BRCC45 Are Novel Subunits

Mass spectrometric sequence analysis of the 36 kDa band (BRCC36) identified this protein as the polypeptide encoded by the *c6.1A* gene (Figure 1A). The *BRCC36/c6.1A* gene is located at the Xq28 locus, a chromosomal break point in patients with prolymphocytic T cell leukemia (Fisch et al., 1993). BRCC36 displayed sequence homology with human Poh1/Pad1 subunit of the 26S proteasome and with the subunit 5 (Jab1) of the COP9 signalosome (Figure 4A). This homology is in the recently described Jab1/MPN or the JAMM domain (Verma et al., 2002; Cope et al., 2002; Yao and Cohen, 2002; see Figure 4A). Despite its homology to POH1 and Jab1, BRCC36 represents a distinct branch in the evolutionary tree. Interestingly, the COP9 complex has been shown to regulate the activity of the SCF ubiquitin ligase complex (Lyapina et al., 2001; Yang et al., 2002). Specifically, the Jab1 subunit of the COP9 complex is reported to modulate the degradation of p27 protein (Tomoda et al., 1999). BRCC45 protein correspond to the *b*rain- and *re*productive organs-specific gene, *BRE* (Gu et al., 1998). BRCC45/BRE does not display homology to any other protein in the human genome.

To rigorously establish the association of BRCC36, BRE, and BRCA1/BARD1, we developed a 293-derived cell line stably expressing FLAG-BRCC36. Isolation of FLAG-BRCC36 by FLAG-affinity purification demonstrated the stable association of BRCC36 with the other components of BRCC (Figure 4B; also see Figure 1B). It is noteworthy that we detected the endogenous BRCC36 in the FLAG-BRCC36 affinity eluate, indicating the presence of more than one BRCC36 protein per BRCC.

### Cancer-Causing Truncation of BRCA1 Abrogates the Association of BRCC36 and BRE

We next asked whether the truncation of BRCA1 protein, which may occur as a result of cancer-causing frameshift mutations in exon 11, will result in the loss of BRCC36 or BRE association. To obtain a truncated BRCA1 complex, we constructed a 293-derived cell line expressing a truncated (1–509) BRCA1. As a control we also constructed cell lines expressing a truncated form of BARD1 (1–398). Both truncated protein complexes were purified, and the resulting polypeptides were analyzed for protein composition. While truncation of BARD1 did not affect the association of any of the components of the complex, BRCA1 truncation completely abrogated the association of BRCC36 and BRE and reduced the association of both BRCA2 and RAD51 with BRCC (Figure 4C). In contrast, truncations of either BRCA1 or BARD1 did not affect the association of p53 and BRCC. These results correlate the loss of function of truncated BRCA1 protein with the loss of several key regulatory components of BRCC.

To determine whether BRCC36 can directly interact with BRCA1, six fragments of BRCA1 spanning the open reading frame were produced in bacteria and were tested for their association with recombinant BRCC36 also produced in bacteria (Figure 4D). To address whether BRCC36 association with BRCA1 also extended to other JAMM domain-containing proteins, the Jab1/CSN5 subunit of signalosome was also produced in recombinant form and was used in the protein-protein interaction assay. Consistent with the above results obtained from the truncating mutation of BRCA1, BRCC36 can specifically associate with fragments 3 and 4 located in exon 11 (Figure 4E). This association is specific for BRCC36 since Jab1/CSN5 did not associate with any fragments of BRCA1 protein (Figure 4E). Together these data indicate a direct interaction of BRCA1 and BRCC36 which is lost following cancer-causing truncations of BRCA1.

### BRCC36 and BRE Potentiate the E3 Ubiquitin Ligase Activity of BRCA1/BARD1

To directly address the role of BRE and BRCC36 in BRCC's ubiquitin E3 ligase activity, we reconstituted the complex using recombinant subunits expressed in insect cells. We produced either a 2 subunit BRCA1/BARD1 or a 4 subunit BRCA1/BARD1/BRE/BRCC36

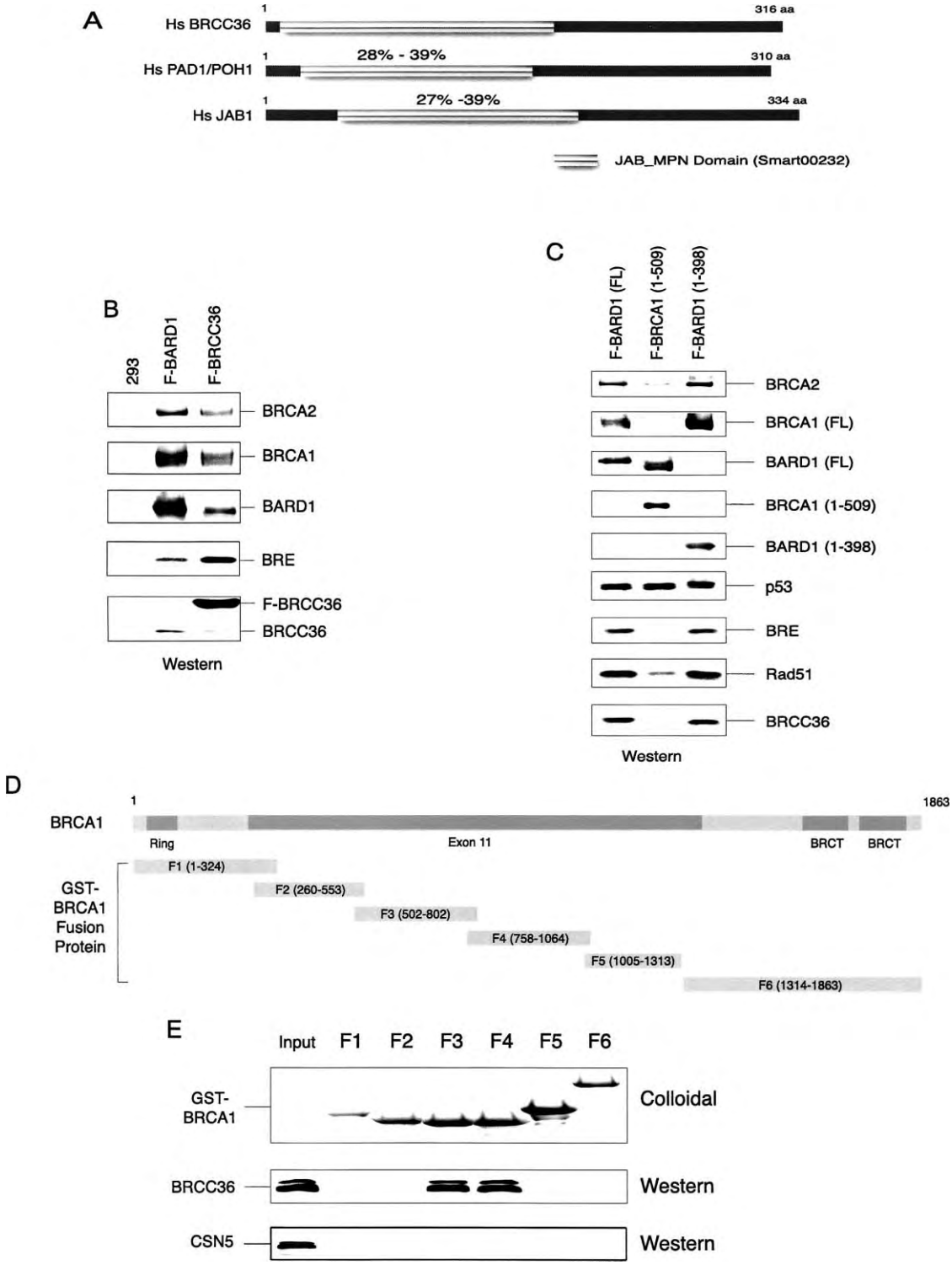


Figure 4. Cancer-Causing Truncations of BRCA1 Abolished the Association of BRCC36 and BRCC

(A) Diagrammatic alignment of human *BRCC36* (P46736), human *Poh1* (AAC51866), and human *Jab1* (NP_006828). The numbers represent amino acid sequences, and the shaded boxes reflect the homologous domain. The numbers above the shaded boxes represent percent identity and similarity of each protein to human *BRCC36*.

(B) Western blot analysis of the FLAG-*BRCC36* affinity eluate. Antibodies used are shown to the right of the panel.

(C) Western blot analysis of complexes purified from full-length FLAG-*BARD1*, truncated FLAG-*BARD1* (1–398), and truncated FLAG-*BRCA1* (1–509) stable cell lines using antibodies to the right of the panel.

(D) Diagrammatic depiction of the six GST fragments of BRCA1 used for the protein-protein interaction analysis shown in (E).

(E) GST pull-down experiments demonstrating the association of BRCC36 and not the Jab1 subunit of signalosome with fragments 3 and 4 of BRCA1 shown in (D).

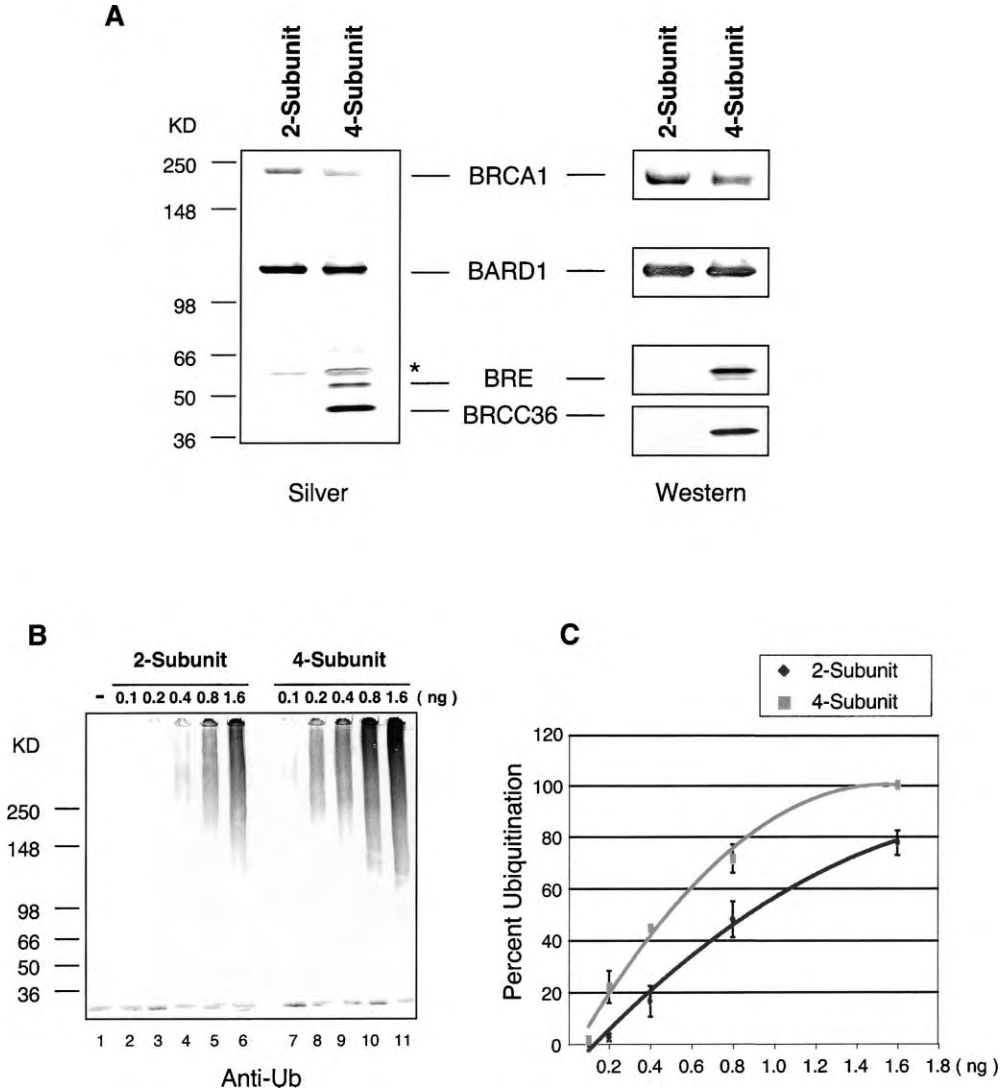


Figure 5. BRCC36 and BRE Play a Role in E3 Ubiquitin Ligase Activity of BRCC

(A) Analysis of reconstituted 2 subunit and 4 subunit complexes by SDS-PAGE followed by silver staining and Western blot analysis using the antibodies depicted. Asterisks denote nonspecific polypeptides.

(B) In vitro autoubiquitination assays using 2 subunit or 4 subunit complexes with the indicated amounts of E3.

(C) Quantification of (B). The amounts of ubiquitination product in each lane were quantified using ImageQuant 5.1. The highest amount of ubiquitination product for each experiment was set to 100%. The result is an average of three different experiments ± SEM.

complex. Insect cells were infected with viruses containing either FLAG-*BRCA1* and GST-*BARD1* or FLAG-*BRCA1*, FLAG-*BRCC36*, FLAG-*BRE*, and GST-*BARD1*. Each complex was purified initially through a FLAG-affinity column followed by GST-affinity purification. The two complexes purified to homogeneity (Figure 5A) were then utilized to assess the role of BRE and BRCC36 in the E3 ubiquitin ligase activity of BRCA1/BARD1. Analysis of the E3 ligase activity of an equal concentration of BRCA1/BARD1 for each complex determined by colloidal blue and quantitative Western blot analysis of the two complexes revealed an enhancement of ubiquitination by the 4 subunit complex (Figures 5B and 5C). Similar results were obtained when p53 was used as substrate (data not shown). These results indicate that the two new subunits, BRCC36 and BRE, potentiate the E3 ligase activity of BRCA1/BARD1.

### Involvement of BRCC36 and BRE in Response to Ionizing Radiation and Checkpoint Control

A number of studies have shown that cells carrying inactivating mutations of *BRCA1* display increased sensitivity to ionizing radiation and defective control of G2/M checkpoint. To determine whether loss of BRCC36 and BRE resulted in similar DNA repair defects, HeLa cells were treated by siRNAs against *BRCA1*, *BRCC36*, *BRE*, or control siRNA, and their responsiveness to ionizing radiation was measured (Figures 6A and 6B). The experiments were performed in triplicates comparing the cells treated with siRNAs against *BRCC36* or *BRE*, to those treated with siRNAs against *BRCA1* and control siRNAs. BRCA1-, BRCC36-, and BRE-depleted cells displayed a potent increased sensitivity to ionizing radiation at all doses examined (Figures 6C and 6D).

We next asked whether depletion of BRCC36 and BRE

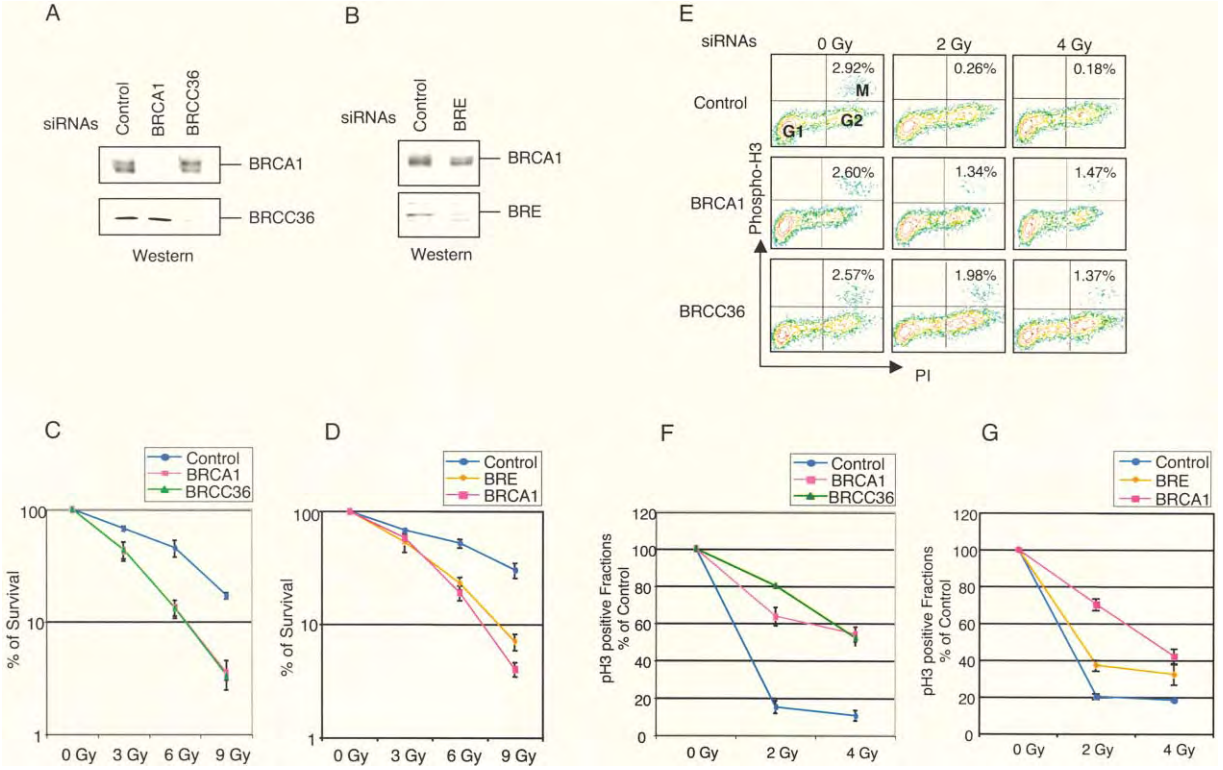


Figure 6. Depletion of BRCC36 and BRE Results in Increased Sensitivity to Ionizing Radiation and Defects in G2/M Checkpoint Arrest

(A and B) Western blot analysis of HeLa nuclear extract 72 hr following transfection of siRNA against *BRCC36*, *BRCA1*, *BRE*, or control siRNAs.

(C and D) Treatment of HeLa cells with siRNA against *BRCC36*, *BRE*, or *BRCA1* results in enhanced sensitivity to ionizing radiation. Cell survival was assessed as detailed in the Experimental Procedures.

(E–G) HeLa cells were treated with siRNA against *BRCA1*, *BRCC36*, *BRE*, or control siRNA, and following treatment with ionizing radiation their cell cycle progression was measured by FACS. Progression through mitosis was measured as detailed in the Experimental Procedures. (E) represents one such experiment using siRNA against *BRCC36* while (F) and (G) represent the quantification of at least three independent experiments.

would result in disruption of the G2/M checkpoint arrest. Consistent with a defect in G2/M checkpoint, analysis of mitotic cells following 2 and 4 Gy of ionizing radiation indicated that approximately 3-fold more BRCC36- and BRCA1-depleted cells entered into mitosis (Figures 6E and 6F). Depletion of BRE resulted in a more moderate defect in G2/M checkpoint arrest (Figure 6G). Taken together, these results demonstrate that BRCC36 and BRE are not only components of a multiprotein complex which modulate the enzymatic activity of BRCA1/BARD1 but also participate in a similar pathway of cellular responsiveness to ionizing radiation.

### Aberrant Expression of *BRCC36* in Sporadic Breast Cancer

Quantitative real-time PCR (qPCR) was performed to evaluate the expression of *BRCC36* mRNA levels in multiple independent normal breast organoids (uncultured breast ducts composed of luminal and myoepithelial cells), primary epithelial cell cultures, nontumorgenic breast epithelial cell lines, breast cancer cell lines, and human breast tissue specimens surgically obtained from patients with primary invasive carcinoma as described in the Experimental Procedures section. In comparison with nontumorigenic breast epithelial cell lines, the expression levels of BRCC36 mRNA were dramatically increased in about 75% of breast cancer cell lines (3 of 4) (Figure 7A). Furthermore, the expression levels of BRCC36 mRNA were elevated in 58% (11 of 19) of the breast tumors evaluated when compared to normal breast organoids (Figure 7B). A subset of these tumors (6 of 19) showed very high levels of expression relative to both the organoids and primary epithelial cultures (Figure 7B). The difference in *BRCC36* mRNA levels in the normal organoids or primary epithelial cell cultures may be due to differences in age, parity, or hormonal status of the patients from whom the organoids were derived or to the tissue-culturing conditions of the primary epithelial cells. To further validate the expression of *BRCC36* in breast tumors, we performed qPCR analysis on laser-captured microdissection (LCM)-purified normal mammary ductal epithelial cells (NE) and malignant epithelial (ME) cells. Two normal breast tissues and nine invasive ductal carcinomas were microdissected, and the RNA was evaluated by RT-PCR (Figures 7C and 7D). We found that 100% of these tumors (9 of 9) showed elevated levels of *BRCC36* mRNA relative to the normal mammary ductal epithelium (Figure 7D). Seventy-seven percent of these tumors expressed very high levels of *BRCC36* (>20-fold) as compared to normal epithelial cells.

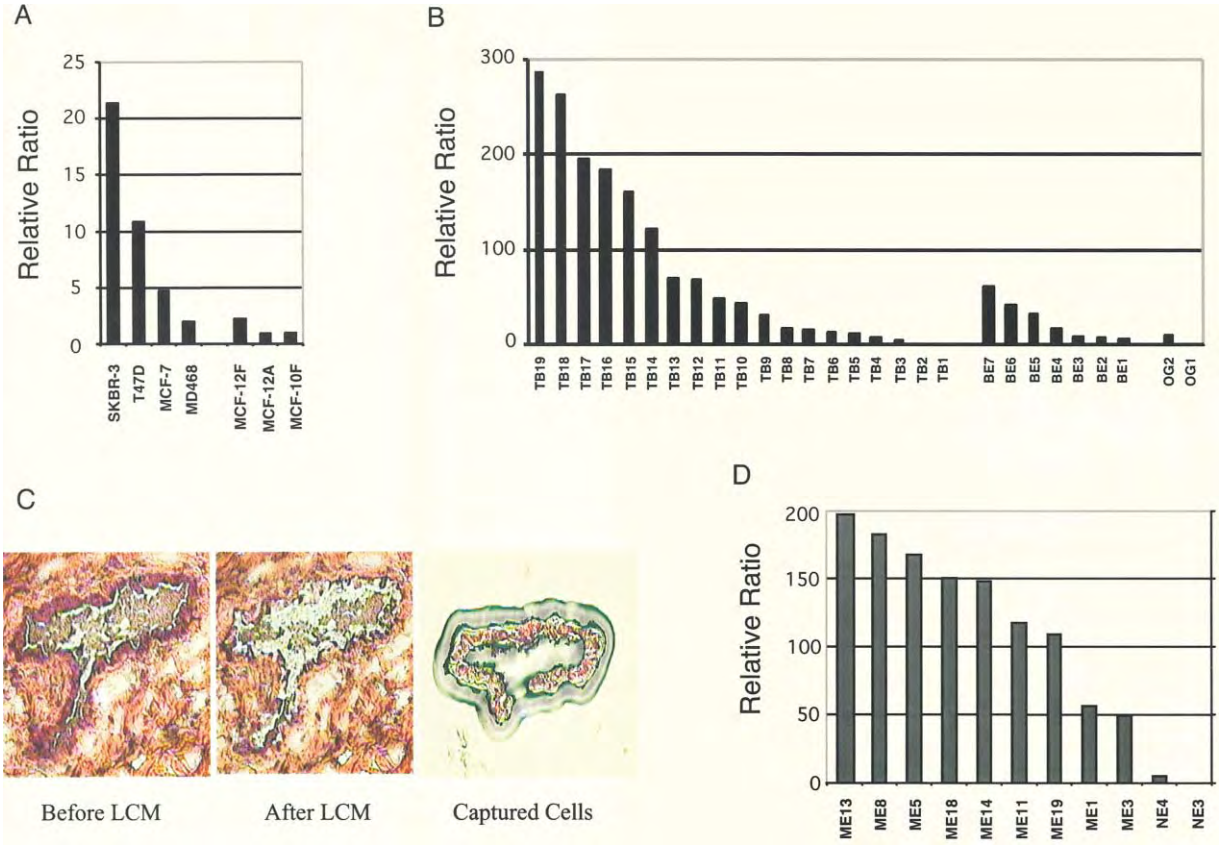


Figure 7. Aberrant Expression of *BRCC36* in Breast Tumors

(A) Analysis of breast tumor cell lines (SK-BR-3, T47D, MCF-7, and MDA-MB-468) and breast epithelial cell lines (MCF-12F, MCF-12A, and MCF-10F) for *BRCC36* mRNA expression using real-time PCR.

(B) Real-time PCR was used to analyze breast mammary organoids (OG), primary breast epithelial cells (BE), and breast tumors (TB). The expression levels of *BRCC36* were adjusted for β-actin expression, and the ratio for OG1 was set at 1, described in detail in the Experimental Procedures.

(C) Depiction of a tumor section before and after laser capture microdissection procedure as described in the Experimental Procedures.

(D) Quantitative real-time PCR was used to analyze normal mammary ductal epithelial cells (NE) and malignant epithelial (ME) cells captured by microdissection.

## Discussion

The key findings of this work lies in the following. First, it demonstrates a multiprotein E3 ubiquitin ligase complex containing BRCA1, BARD1, and BRCA2 termed BRCC. Second, it presents the increased association of RAD51 and p53 with BRCC following treatment of cells with DNA-damaging agents. Third, it shows that BRCC ubiquitinates p53 in vitro and this ubiqutination maps to the C-terminal regulatory domain of p53. Fourth, it identifies BRCC36 and BRE as a bona fide subunit of the BRCC. Fifth, through reconstitution of the BRCA1/BARD1/BRCC36/BRE complex it demonstrates a role for BRCC36 and BRE in modulation of BRCA1/BARD1 E3 ubiquitin ligase activity. Sixth, it provides, through the use of siRNAs, support for a role for BRCC36 and BRE in cellular responsiveness to ionizing radiation and G2/M checkpoint progression. Seventh, it presents evidence for aberrant expression of *BRCC36* in sporadic breast cancer.

BRCC represents a BRCA1/BRCA2/BARD1-containing complex that displays an E3 ubiquitin ligase activity. Although BRCC contains BRCA1, BRCA2, and BARD1, there are a number of differences in the polypeptide composition of BRCC and complexes reported by others. Importantly, with the exception of substoichiometric amounts of RAD51, BRCC does not stably associate with any known DNA repair factors. Indeed, fractionation of BRCC by ion-exchange chromatography results in the separation of the bulk of RAD51 from the other components of the complex (data not shown), suggesting a weaker interaction of RAD51 with the core subunits of BRCC. We therefore favor the contention that BRCC may not be playing a direct role in DNA repair, but through its E3 ubiquitin ligase activity BRCC can regulate factors involved in DNA repair.

Both BRCA1 and BRCA2 have also been implicated in DNA repair (Chen et al., 1998b; Moynahan et al., 1999, 2001; Scully et al., 1999; Yu et al., 2000; Zhong et al., 2002). Moreover, both BRCA1 and BRCA2 were shown to associate with RAD51 (Scully et al., 1997b; Marmorstein et al., 1998; Wong et al., 1997; Chen et al., 1998b; Mizuta et al., 1997; Davies et al., 2001). Here we show the increased association of RAD51with BRCC

following DNA damage. Through this increased association with RAD51, the BRCC complex may be targeted to DNA damage sites. Once at sites of DNA damage, the complex may modulate DNA repair by ubiquitinating either chromatin or the components of the DNA repair machinery. We have also observed an increased association of p53 protein and BRCC following DNA damage. Moreover, the C-terminal domain of p53 could be readily ubiquitinated by BRCC in vitro. However, since the same domain of p53 is also the target of ubiquitination by MDM2 protein, further in vivo experiments are required to demonstrate the biological relevance of p53 ubiquitination by BRCC.

### Two Novel Components of BRCC, BRCC36, and BRE Were Identified in This Study

Using recombinant subunits produced in insect cells, we were able to partially reconstitute a 4 subunit complex containing BRCA1/BARD1/BRE/BRCC36. This complex displayed an increased ubiquitin E3 ligase activity compared to that of BRCA1/BARD1 heterodimer. Therefore, while BRCA1/BARD1 constitute a core enzyme complex, BRCC represents a holoenzyme containing additional regulatory elements such as BRE and BRCC36. While BRE does not display sequence homology to any known protein, BRCC36 is a protein with close homology to a subunit of the COP9 signalosome (Jab1) and a subunit of the 19S proteasome (pad1). This homology consists of a recently described domain termed the Jab1/MPN or JAMM domain which is predicted to encode a protein with ubiquitin or Nedd 8 deconjugating activity (Verma et al., 2002; Cope et al., 2002; Yao and Cohen, 2002). Although we were unable to show a deconjugating activity for BRCC36 using either ubiquitinated BRCA1 or synthetic ubiquitin chains, it may play such a role once the true substrate is identified. Through the use of siRNAs we delineated a role for both BRCC36 and BRE in responsiveness to ionizing radiation and the progression through the G2/M checkpoint. Taken together, these results demonstrate a role for BRCC36 and BRE in the DNA damage response pathway.

We show that BRCC36 directly interacts with a fragment encoded by exon 11 of the BRCA1 gene. Isolation of BRCC from cell lines expressing BRCA1 with a truncation in this region (a C-terminal truncation), which mimics the cancer-causing mutations of BRCA1, resulted in the loss of BRCC36 from the BRCC. Interestingly, C-terminal truncations of BARD1 did not have a destabilizing activity on the components of BRCC. This may indicate that, while cells may be able to tolerate the truncating mutations of *BARD1*, similar mutations in *BRCA1* lead to a disruption of BRCC integrity and a concomitant deregulation of growth stimulatory pathways.

Since germline mutations of *BRCA1* and *BRCA2* genes result in breast cancer, we were interested to know whether there were mutations in *BRCC36* in breast tumors. Although our preliminary analysis of sporadic breast tumors did not yield any mutations in the *BRCC36* gene (data not shown), we found a profound increase in *BRCC36* expression in breast tumors. Mircodissection analysis of the mammary epithelial ducts from these tumors revealed increased *BRCC36* expression in every tumor analyzed. The consequences of *BRCC36* overexpression are not clear. A possible scenario may involve the disruption of the normal function of BRCC by overexpressed BRCC36. Furthermore, since germline mutations in *BRCA1* and *BRCA2* account for only 15%–20% of breast cancer that clusters in families and 5% of breast cancer overall (Nathanson et al., 2001), *BRCC36* and other components of the BRCC complex may represent long sought after breast cancer susceptibility genes.

## Experimental Procedures

#### Affinity Purification of FLAG-BARD1

FLAG-*BARD1* and a selectable marker for puromycin resistance were cotransfected into 293 human embryonic kidney cells or H1299 human lung cancer cells. Transfected cells were grown in the presence of 5 μg/ml puromycin, and individual colonies were isolated and analyzed for FLAG-BARD1 expression. To purify the BRCC complex, nuclear extract from the FLAG-BARD1 cell line was incubated with anti-FLAG M2 affinity gel (Sigma). After extensive washing with buffer A (20 mM tris-HCl [pH 7.9], 0.5 M KCl, 10% glycerol, 1 mM EDTA, 5 mM DTT, 0.5% NP40), the affinity column was eluted with buffer A containing FLAG peptide (400 μg/ml) according to the manufacturer's instructions (Sigma). Other FLAG-containing complexes were purified using a procedure similar to that described for full-length *BARD1*. Analysis of BRCC on gel filtration was similar to that previously described (Bochar et al., 2000). 293-derived cells were also treated with either 12 Gy of ionizing radiation, hydroxyurea, or thymidine as described (Scully et al., 1997c), and BRCC was isolated as delineated above. Protein identification using LC-MS/MS was performed as detailed in Bochar et al. (2000) and Marmorstein et al. (2001).

#### Immunoblot Analysis

Anti-BRCC36 and anti-BRE antibodies were developed to peptides corresponding to the C-terminal last 20 amino acids of BRCC36 and BRE, respectively. Anti-Rad51 antibodies were obtained from Upstate Biotechnology (NY). Anti-BARD1 antibodies were a gift of Junjie Chen.

#### Plasmid, siRNA, and Transfection

*BARD1* and *BRE* were cloned in pFLAG-CMV2 (Sigma) vector, while *BRCC36* were cloned in pCMV-5A (Sigma) vector using standard PCR techniques. Baculoviral transfer vectors for expression of *BRCC36* and *BRE* were constructed by inserting FLAG-tagged cDNA sequences encoding *BRCC36* and *BRE* into the pBlueBac4.5/V5-His and pFastBacHTb plasmids (Invitrogen), respectively.

The siRNAs were synthesized by Dharmacon, Inc. The sequence of *BRCA1* siRNA was AA-CUUAGGUGAAGCAGCAUCU, the *BRCC36* siRNA was AA-GAGGAAGGACCGAGUAGAA, the *BRE* siRNA was AA-GGUGCAGUACGUGAUUCAA, and the control siRNA was AA-GUUACUCAGCCAAGAACGA. siRNA transfection was performed with Lipofectamine 2000 (Life Technologies, Inc.) according to the manufacturer's instructions. In brief, cells were plated in 10 cm dish to 40% confluence. For each dish, 1.6 nmole siRNA was mixed with 20 μl Lipofectamine 2000 in 3 ml Opti-MEM. The mixture was added to cells and incubated for 6 hr. Twenty-four hours later, a second transfection was performed in the same way. Cells were treated or harvested as indicated 72 hr after the initial transfection.

#### Purification of Recombinant Proteins and Protein-Protein Interaction

(His)6-E1 and E2 proteins were purified form bacteria as described by Kamura et al. (1999). Bacterial expressed BRCA1 and BARD1 was purified as described (Chen et al., 2002). For in vitro interaction studies, whole-cell extracts from BL21 (GST-*BRCA1* fragments 1–6) were prepared. Five micrograms of purified (His)6-FLAG-BRCC36 protein was incubated with 10 μg of purified GST-BRCA1 fragment in binding buffer (20 mM Tris-HCl [pH 7.9], 0.1 M KCl, 10% glycerol, 1 mM EDTA, 2 mM MgCl, 2.5 mM DTT, 0.1% NP40) for 2 hr at 4°C with inversion. Glutathione-sepharose beads (Pharmacia), previously equilibrated in binding buffer, were added to the samples

and inverted for an additional 2 hr at 4°C. Beads were collected by centrifugation at 2000 × g for 30 s, washed several times in binding buffer containing 500 mM KCl and 0.1% NP-40, and resuspended in 2× SDS loading buffer. Proteins were resolved by SDS-PAGE, and Western blot analysis was performed using anti-FLAG antibodies (Sigma).

### Reconstitution of 2 Subunit and 4 Subunit Complexes

Recombinant viruses encoding FLAG-*BRCC36* and FLAG-*BRE*-(His)6 were obtained by transfecting Sf9 cells with the FLAG-*BRCC36*/pBlueBac4.5 and FLAG-*BRE*/pFastBacHTb vectors, respectively, using BAC-N-BLUE linear transfection kit (Invitrogen). Sf9 cells were coinfected with FLAG-*BRCA1* and GST-*BARD1* recombinant baculoviruses (kindly provided by Dr. Richard Baer) for reconstitution of 2 subunit complex, and FLAG-*BRCA1*, GST-*BARD1*, FLAG-*BRCC36*, and FLAG-*BRE* for 4 subunit complex. Cells were harvested 72 hr after infection, and the cell pellet was resuspended in lysis buffer (50 mM HEPES [pH 7.9], 250 mM NaCl, 0.1% Nonidet P-40, 10 mM 2-mercaptoethanol, 10% glycerol) with protease inhibitors (Roche Diagnostics). Cells were broken by sonicating, and the cell debris was removed by centrifugation at 30 K for 30 min at 4°C. The supernatants were incubated with anti-FLAG affinity resin (Sigma) for 2 hr at 4°C, washed three times with wash buffer (20 mM Tris-HCl [pH 7.9], 10 mM 2-mercaptoethanol, 10% glycerol, protease inhibitors) containing 750 mM KCl, and eluted with lysis buffer containing 500 μg/ml FLAG peptide. The eluates were incubated with glutathione-agarose beads (Sigma) for 3 hr at 4°C and washed twice with wash buffer containing 500 mM Kcl and twice with elution buffer (20 mM Tris-HCl [pH 7.9], 50 mM NaCl, 1 mM dithiothreitol, 10% glycerol, protease inhibitors). The beads were then eluted with elution buffer containing 20 mM glutathione. The concentration of 2 subunit and 4 subunit complexes was determined by comparing the intensity of colloidal staining of the BRCA1 subunit with that of 100 ng BSA loaded on the same gel.

### In Vitro Ubiquitination Assays

Ubiquitination assays were conducted in a final volume of 20 μl, containing 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 2 mM ATP, 0.6 mM dithiothreitol, 1 μg of FLAG-ubiquitin (Sigma), 25 ng of E1, 50 ng of the specified E2, and the indicated amount and type of E3 as specified in the figures. p53 was added as a substrate when indicated. The reactions were incubated at 37°C for 20 min and stopped with 5 μl of 5× SDS loading buffer. Samples were boiled for 3 min prior to SDS-PAGE analysis followed by Western blot analysis.

### Ionizing Radiation Sensitivity Assay

Seventy-two hours after siRNA transfection, $2 \times 10^4$ cells were exposed to various doses of IR or not and returned to culture for 4 days. Cells were then trypsinized and stained with trypan blue, and the numbers of live cells were counted. The percent of survival is obtained by comparing numbers of live cells in irradiated samples to that in nonirradiated sample treated using the same siRNA. Each experiment includes three repeats for each dosage and cell line.

### Flow Cytometric Analysis of Phosphorylated Histone H3 Staining

Seventy-two hours after siRNA transfection, cells were γ irradiated at different dosage or not, then incubated at 37°C for 1 hr before fixation in 70% ethanol. Cells were stained in 200 μl PBS containing 1% BSA and 1 μg of polyclonal antibody against phosphorylated H3 (Upstate Biotechnology, Lake Placid, NY) at 4°C overnight and then FITC-conjugated anti-rabbit IgG (Vector Laboratoies, Inc. Burlingame, CA) at room temperature for 1 hr. After washing with PBS, cells were suspended in 5 μg/ml PI (Sigma, St. Louis, MO), and the cellular fluorescence was measured by a flow cytometer.

### Breast Cancer Cell Lines, Tumor Specimens, Laser Capture Microdissection, and Quantitative Real-Time PCR

#### *Cell Lines and Cell Cultures*

Breast organoids were isolated from reduction mammoplasty specimens as previously described with minor modifications (Rudland et al., 1989). Primary cultures of human breast epithelial cell lines were established and cultured as previously described (Ethier et al., 1993). Nontumorigenic epithelial cell lines, MCF-10F, 12A, and 12F, were cultured in DMEM/F12 with reduced $Ca^{2+}$ (0.04 mM final), 20 ng/ml epidermal growth factor, 100 ng/ml cholera toxin, 0.01 mg/ml insulin, 500 ng/ml hydrocortisone, and 5% Chelex-treated horse serum.

#### *Surgical Specimens*

Nineteen human breast tissue specimens were surgically obtained from patients with primary invasive carcinoma at Fox Chase Cancer Center from year 2000 to the present. Grading of histological malignancy of each specimen was assessed according to the system reported previously (Bloom and Richardson, 1957; Elston and Ellis, 1991). Lymphonodal metastatic statuses were determined by histopathological examination in each case according to the pTNM classification as proposed by the International Union Against Cancer. All the samples were snap-frozen in liquid nitrogen and kept at −80°C until used.

#### *Laser Capture Microdissection*

LCM was preformed as previously described (Cvetkovic et al., 2003).

#### *Quantitative Real-Time PCR Analysis*

0.6 microliters of the cDNA mixture was used in a real-time PCR reaction (25 μl total volume) performed with Smart Cycle TD (Cepheid, Sunnyvale, CA) following the methods recommended by the manufacturer. Optimal conditions were defined as: Step 1, 95°C for 10 min; Step 2, 95°C for 15 s, 60°C for 60 s with Optics; repeated for 50 cycles. The relative mRNA expressions of BRCC36 were adjusted with ACTB. The primer and probe sets used for real-time PCR were as follows: BRCC36, forward primer, 5′-AATTTCTCCAGAG CAGCTGTCTG-3′; reverse primer, 5′-CATGGCTTGTGTGCGAACAT; Taqman probe, (FAM) 5′-AACTGACAGGCCGCCCCATGAG-3′ (BHQ1); ACTB, forward primer, 5′-GCCAGGTCATCACCATTGG-3′; reverse primer, 5′-GCGTACAGGTCTTTGC-GGAT; Taqman probe, (Cal red) 5′-CGGTTCCGCTGCCCTGAGGC-3′ (BHQ2).

### Acknowledgments

Thanks to Drs. Joan W. Conaway for plasmids encoding E2s and Junjie Chen and David Livingston for the BARD1 antibodies. Also, many thanks to Z.Q. Pan for the gift of plasmids to BRCA1 and BARD1 and Ricard Baer for the gift of viruses encoding BRCA1 and BARD1. We thank T. Beer of the Wistar Proteomics Facility for expertise in the microcapillary HPLC/mass spectrometry. Thanks to J. Norman, D. Bochar, O. Barak, and T. Halazonetis for critical comments. Also, thanks to D. Cvetkovic for the great help in LCM. R.S. was supported by grants from NIH (CA90758-02), ACS, USAMRMC, and the Breast Cancer Research Foundation (BCRF). A.K.G. was supported in part by a grant from the NIH (Ovarian Cancer SPORE P50 CA83638) and the USAMRMC (DAMD17-01-1-0521). X.C. was supported by a postdoctoral fellowship from the Eileen-Stein Jacoby Fund. M.-A.H. was supported by a postdoctoral fellowship from the Association pour la Recherche Sur le Cancer (France).

Received: July 2, 2002
Revised: October 14, 2003
Accepted: October 17, 2003
Published: November 20, 2003

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## Accession Numbers

Accession number AY438030 corresponds to BRCA1-BRCA2-containing complex subunit 36 (BRCC36) mRNA. Accession number AY438031 corresponds to BRCC45 (BRCA1-BRCA2-containing complex subunit 45)/BRE (brain and reproductive-organs-specific gene) mRNA.