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PRINCIPAL INVESTIGATOR: Wen-Hwa Lee, Ph.D.

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

The objective of the program is to train highly qualified doctoral students in the genetic, cellular, and molecular basis of Breast Cancer. The training program, conducted within the Molecular Medicine Ph.D. Program, was administered by a select group of faculty whose research projects were intimately involved in breast cancer. An additional goal of the program was to promote synergistic interactions between the various laboratories engaged in breast cancer research. Breast cancer meetings, Molecular Medicine Distinguished Seminar Series were integral parts of the training program for students supported by the Breast Cancer Training Program. The major strengths of the program were the high quality of the Program faculty, and the interactive nature of the Breast Cancer research community in San Antonio. The program faculty, organized into three subprograms, encompassed scientists and physicians studying different aspects of breast cancer and cancer therapy, as well as fundamental mechanisms of DNA repair, cell growth and cell differentiation. Key research accomplishments during the 1999-00 reporting period were: **1)** publication of 30 peer-reviewed articles by students in the program; **2)** two DOD BCRP pre-doctoral grants awarded to students in the program; **3)** the addition of five new highly qualified faculty; and **4)** four faculty awarded DOD BCRP Idea grants; **5)** five Ph.D. students supported by the program graduated.

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## INTRODUCTION

### Brief Description of the Training Program and Its Objectives

The goal of the program is to establish at the University of Texas Health Science Center in San Antonio an in-depth training program in the Molecular Genetics of Breast Cancer. The most important goal of the program is to train highly qualified Ph.D. students in the genetic, cellular, and molecular basis of Breast Cancer. Toward these ends, the program has been extremely successful. Based on the publication record of our trainees, our expectation for significant discoveries is being realized. During the reporting period, students supported by the training program achieved a total of 30 publications relevant to breast cancer.

The training program was conducted within the Molecular Medicine Ph.D. Program by a select group of faculty whose research projects are relevant to breast cancer. An additional goal of the program was to promote synergistic interactions between the various laboratories engaged in breast cancer research. An important meeting was the Annual Breast Cancer Symposium held in San Antonio. All students supported by the program were required to attend. Finally, an outstanding Molecular Medicine Seminar Series sponsored by the Department of Molecular Medicine was also a requirement for all trainees. The following seminars in this series were pertinent to breast cancer:

- Spring Semester: 1999

| | |
|---|---|
| *Hugo J. Bellen* | "Genetic dissection of neurotransmitter release and endocytosis" |
| *Riccardo Dalla-Favera* | "Molecular genetics of B-cell lymphoma" |
| *Cynthia McMurray* | "Mechanism DNA expansion in human disease" |
| *Stewart Shuman* | "Mechanisms of DNA cleavage and rejoining" |
| *Yue Xiong* | "The regulation of p53 and pRb tumor suppression pathways" |
| *Chein Ho* | "From tracking cell movement to detecting organ rejection by MRI" |
| *David S. Papermaster* | "Retinal Degeneration induced in transgenic frogs" |
| *John Petrini* | "The Mre11/Rad50 protein complex mediates diverse function in the DNA damage response" |
| *Michael Karin* | "Protein kinase cascades that control AP-1 and NF-κB: regulation and function" |
| *C.C. Wang* | "Distinctive mechanisms regulating the proteasomes in a protozoan species, the Trypanosomes" |
| *Francis Barany* | "New methods of dectecting genetic diseases and cancers" |
| *Steven L. McKnight* | New PAS domain proteins, new places, new biology" |
| *Robert A. Scalafani* | The role of Cdc7 and Cdk protein kinases in the cell cycle of yeast and human cancer cells" |
| *Stephen A. Johnston* | "Genetic immunization and other technologies that my revolutionize the ability to manipulate the immune response" |
| *James C. Garrison* | "The diversity of the G protein βγ subunits and their role in cell signaling" |

| | |
|---|---|
| *Richard W. Carthew* | "The role of transcription repressors in photoreceptor cell development" |

- Fall Semester 1999:

| | |
|---|---|
| *Antonio Giordano* | "The Rb Family and Cdks in the Cell Cycle and Cancer" |
| *Deepak Srivastava* | "Molecular Pathways Regulating Cardiac Development and Congenital Heart Disease" |
| *James Wang* | "DNA Topoisomerase II: A Molecular Machine that Moves One DNA Double Helix through Another" |
| *William F. Morgan* | "DNA Damage and Genomic Instability" October 12, 1999 |
| *Alfred L. Goldberg* | "Role of the Proteasome in Protein Degradation and Immune Surveillance" |
| *Leroy E. Liu* | "Novel Roles of Topoisomerases in Tumor Cell Apoptosis" |
| *Susan L. Forsburg* | "Licensed to Replicate, Analysis of MCM Protein Function in Fission Yeast" |
| *Mani Ramaswami* | "Mechanisms and Regulation of Synaptic Vesicle Cycling: Insights into Tumor Suppression, Memory and Life from Studies on the Fruit Fly" |
| *Nicholas R. Cozzarelli* | "Mechanisms of DNA Unlinking and Chromosome Segregation" |
| *Terumi Kohwi-Shigematsu* | "The Roles of SATB1 and MARs in Gene Regulation, Replication and Apoptosis in T Cell Nuclei" |
| *David M. Gilbert* | "Establishing a Spatial and Temporal Program for Mammalian Chromosome Replication" |
| *Li-Huei Tsai* | "Function and Dysfunction of Cyclin-dependent Kinase 5 in Brain Development and Degeneration" |
| *Richard S. Lewis* | "Store-operated Calcium Channels and the Control of Gene Expression in T Lymphocytes" |

- Spring 2000:

| | |
|---|---|
| *W. Jonathan Lederer* | "Calcium Flux Through Sodium Channels: Slip-Mode Conductance" |
| *Ming-Jer Tsai* | "Roles of bHLH Transcription Factor BETA2/NeuroD in Pancreatic and Neuronal Development" |
| *Malcolm C. Pike* | "Estrogen and Progesterone as Major Determinants of Breast Cancer Risk: Implications for Design of Hormonal Contraceptives and Hormone Replacement Therapy" |
| *Gilbert Chu* | "How Cells Recognize and Respond to DNA Damage" |
| *Ann Marie Pendergast* | "Role of Tyrosine Kinases and Adaptor Proteins in Cell Migration" |
| *Harold R. Garner* | "Technology (software and instrumentation) for Genome Analysis" |
| *Raymond N. DuBois, Jr.* | "Targets for Cancer Prevention" |
| *Tomas Lindahl* | "DNA Repair: From Protein Structures to Gene Knockout Mice" |
| *John A. Tainer* | "Defining the Structural Biochemistry for DNA Base Damage Recognition, Excision, and Repair Pathway Coordination |

| | |
|---|---|
| *Craig L. Peterson* | "Chromatin Remodeling Machines: Control of Gene Expression in Mitosis" |
| *Margaret A. Shupnik* | "Estrogen Action in the Pituitary: Specific Roles for Estrogen Receptor Isoforms" |
| *Roger A. Schult* | "Mouse Models for Bloom Syndrome: Chromosome Instability and Cancer Predisposition" |
| *Chuxia Den* | "Tumor Suppressor Brca1 in Genetic Stability and Tumorigenesis" |
| *Hong Yan* | "Functional Analysis of Werner Syndrome Protein in Xenopus Egg Extracts" |
| *Anne Villeneuve* | "Chromosome Pairing and Recombination During C. elegans Meiosis" |
| *Frank J. Rauscher, III* | "The KRAB-Zinc Finger Superfamily of Transcriptional Repressors: A Potential Link to Heterochromatin-Mediated Gene Silencing in Mammals" |

*Breast Cancer Research Programs and Faculty.*

One of the major strengths of the program is the high quality of the Program faculty, and the interactive nature of the Breast Cancer research community in San Antonio, which encompass scientists and physicians studying different aspects of breast cancer and cancer therapy, as well as fundamental mechanisms of to maintain genomic stability, cell growth, differentiation and molecular genetics. These faculty groupings are listed below; detailed descriptions of individual research programs were included in the original application.

***Breast Cancer Research Training Faculty***

W.-H. Lee, Ph.D. (Director)
C. Kent Osborne, M.D. (Co-Director)
Powell H. Brown, M.D., Ph.D.
Peter O'Connell, Ph.D.
Gary M. Clark, Ph.D.
Suzanne Fuqua, Ph.D.
Alan Tomkinson, Ph.D.
E. Lee, Ph.D.
W.-H. Lee, Ph.D.
Z. Dave Sharp, Ph.D.
Patrick Sung, Ph.D.
Greg R. Mundy
Bettie Sue Masters, Ph.D.
Bandana Chatterjee, Ph.D.
Arun K. Roy, Ph.D.
Judy M. Teale, Ph.D.
**Peter M. Ravdin, M.D. Ph.D.
**Phang-Lang Chen, Ph.D
**Renee Yew, Ph.D.
**Tom Boyer, Ph.D.
**Maria Gazynska, Ph.D.
**Paul Hasty, DVM
**Jan Vijg, Ph.D.

** New faculty additions (see Changes to the Program Faculty below except for Drs. Ravdin and Vijg who were discussed in our letter of July 8, 1999 regarding faculty issues)

*Relationship between the Breast Cancer Training Program and the Molecular Medicine Graduate Ph.D. Program*

The Breast Cancer Training Program was implemented within the context of the Molecular Medicine Graduate Ph.D. Program. The Molecular Medicine Ph.D. Program is an interdisciplinary Ph.D. training program in the Graduate School of Biomedical Sciences at the UTHSCSA. For the academic year 1999-00, there was a total of 53 students enrolled in the Molecular Medicine Program -- 45 Ph.D. and 8 M.S.

The Breast Cancer Training program takes advantage of the internationally recognized breast cancer research programs existing in the institution for many years, and offers a unique opportunity for students interested in starting careers in breast cancer research. The participating scientists in this breast cancer program represent diverse departments including the Divisions of Medical Oncology, Endocrinology in the Department of Medicine, and the Departments of Cellular and Structural Biology, and Biochemistry. In addition, the University of Texas Institute of Biotechnology and the San Antonio Cancer Institute (SACI), an NIH-designated Cancer Center, represent outstanding resources for training opportunities in clinical and basic science research. The national and international reputation of the participating faculty serve to attract a large number of excellent applicants to the breast cancer research track in the Molecular Medicine program.

The rationale for administering the breast cancer training program in the Molecular Medicine Ph.D. program was based on several important criteria: (1) The Molecular Medicine curriculum is specifically designed to provide basic science training while integrating fundamental principles of molecular biology with modern medicine. A Molecular Medicine Core course provides students with the mechanisms underlying human disease and provides intensive review of specific diseases (including breast cancer) that may serve as models for how human diseases can be studied at the molecular genetic level. (2) The Molecular Medicine program requires the participation of both clinical and basic scientists in the training process. The inclusion of MDs on all student advisory committees insures that every graduate of the program has a clear perspective on the clinical relevance of the basic research in their program that, in most instances, will serve as a guide for the project. (3) The Molecular Medicine program is an interdepartmental, interdisciplinary program that offers flexibility to students in terms of research laboratories, advisors and committee members. This arrangement offers a real potential for synergism in breast cancer research not possible in traditional department-bound programs. In summary, the Ph.D. program in Molecular Medicine offers a near perfect environment for Ph.D. training in breast cancer and has attracted many well-qualified applicants.

*Research Support for Program Faculty*

An essential component of maintaining a successful and aggressive training program in Breast Cancer Research is the continued research funding of the individual Program Faculty laboratories. Current funding for each member of the Program faculty is detailed in Table 1.The faculty have been extremely successful in obtaining research funding, including over $17 million in direct costs for the 1999-2000 reporting period.

**Key Research Accomplishments:**

- *DOD BCRP Idea Awards to New Faculty (See new additions to faculty below for a description of these training opportunities)*

**Phang-Lang Chen, Ph.D.**
Project Title: Small Chemical Molecules that Disrupt BRCA2 and Rad51 Interaction for Adjuvant Breast Cancer Therapy
Project Period: 03/01/01-02/28/04
Total Award: $300,000

**Tom Boyer, Ph.D.**
Project Title: Regulation of BRCA1 Function by DNA Damage-Induced Site-Specific Phosphorylation
Project Period: 03/01/01-02/28/04
Total Award: $286,920

**Renee Yew, Ph.D.**
Project Title: The Role of BRCA1-Dependent Ubiquitination in Breast Cancer
Project Period: 03/01/01 – 02/28/04
Total Award: $284,433

**Maria Gazcynska, Ph.D.**
Project Title: Molecular Characteristics of Multicorn, a New Large Proteolytic Assembly and
Potential Anti-Cancer Drug Target, in Human Breast Cancer Cells
Project Period: 03/31/01-02/28/04
Total Award: $224,260

- *Predoctoral Breast Cancer Research Awards to Supported Trainees*

Predoctoral training grants awarded to current trainees by the Defense Department's Breast Cancer Research Program (BCRP):

*Stefan Sigurdsson* training in Dr. Patrick Sung's laboratory.

Project Title: Functions of Human Rad51 and Other Recombination Factors In DNA Double-Strand Break Repair.
Project Period: 03/01/01 – 02/28/04
Total Award: $61,110

Homologous recombination and recombinational repair of DNA double-strand breaks are mediated by proteins of the *RAD52* epistasis group. Rad51 is a key factor in these processes and the protein can assemble on ssDNA substrates to form a nucleoprotein filament. With the help from other factors, the Rad51-ssDNA nucleoprotein filament searches for a DNA homlog and catalyzes formation of a heteroduplex DNA joint with the homolog. The biochemical reaction that forms heteroduplex DNA joints is called "homologous DNA pairing and strand exchange." A number of Rad51-like proteins are known in human cells, but their function in recombination and DNA repair is currently unknown. I have shown that two of these Rad51-like proteins, Rad51B and Rad51C, are associated in a stable heterodimer. I will further define the

homologous DNA pairing and strand exchange activity of human Rad51. In addition, a variety of experiments will be conducted to test the hypothesis that the Rad51B-Rad51C complex promotes the assembly of the Rad51-ssDNA nucleoprotein filament and enhances the efficiency of Rad51-mediated homologous DNA pairing and strand exchange. The information garnered from this study should contribute significantly to our understanding of how DNA double-strand breaks are repaired in human cells.

*Stephen Van Komen*, training in Dr. Patrick Sung's laboratory.

Project Title: Functional Interactions of Human Rad54 with the Rad51 Recombinase
Project Period: 03/01/01 – 02/28/03
Total Award: $60,345

Homologous recombination is essential for the accurate repair of DNA double-strand breaks. Products of the BRCA1 and BRCA2 breast and ovarian susceptibility genes have recently been shown to associate with key members of the recombinational machinery including the Rad51 recombinase. Rad51 is homologous to the bacterial homologous DNA pairing and strand exchange enzyme, RecA. Unlike RecA, yeast Rad51 has little ability to promote pairing between homologous linear ssDNA and covalently closed duplex to form an important recombination intermediate known as a D-loop. Importantly, yeast Rad54, another recombination factor, promotes robust D-loop formation by Rad51. Recently, I have shown that yeast Rad54 uses the free energy from ATP hydrolysis to remodel DNA structure in a fashion that generates both positively and negatively supercoiled domains in the DNA template, and that DNA supercoiling by Rad54 is important for the D-loop reaction. Given the conservation between yeast and human recombination factors, I hypothesize that human Rad54 supercoils DNA and promotes D-loop formation with human Rad51 in a similar manner. Using highly purified human Rad51 and Rad54 proteins, I will study the functional interactions between these two factors in D-loop formation and in supercoiling DNA. The results from these studies will be important for understanding the human recombinational machinery and may provide a system for dissecting the role of BRCA1, BRCA2, and other tumor suppressors in recombination and DNA double-strand break repair.

*Graduates During the Reporting Period (A description of their research and future plans is below)*

- Shyng-Shiou (Frank) Yuan, M.D., Ph.D. graduated from the program.
- Sun-Chin (Jackie) Lin, Ph.D., graduated from the program.
- Shang Li, Ph.D., graduated from the program.
- Qing Zhong, M.D., Ph.D., graduated from the program.
- Lei Zheng, M.D., Ph.D., graduated from the program.

**Reportable Outcomes**

*Supported Trainees, Research Description and Publications*

The following outstanding group of trainees were supported on the Breast Cancer Training Program during the final reporting period.

***Reporting Period 9/23/99 – 9/22/00***

Deanna Jansen – 2rd year
Shang Li – 5th year
Horng-Ru Lin – 2nd year
Teresa Motycka – 3th year
Sean Post – 3th year
***Stefan Sigurdsson – 3rd year
Qing Zhong – 4th year
Song Zhao – 4th year
***Stephen Van Komen – 2nd year
Qing Zhong – 3rd year
Guikai Wu – 2nd year

*** Mr. Sigurdsson and Mr Van Komen recently were approved for funding of DOD BRCP predoctoral fellowships. Accordingly, when those fellowships are awarded, they will be replaced with similarly qualified students training in breast Cancer Research. This is a good example of how small investments in student training can be amplified in training programs grounded in excellence.

The 1999-2000 academic year marks the seventh full year of operation for the Molecular Medicine Ph.D. Program, and is the fifth one for the Training Program in the Molecular Basis of Breast Cancer Research. The availability of highly qualified applicants to the Molecular Medicine Program was excellent. Overall, 87 applications were received for admission to the Fall 1999 entering class. Fifteen new students began classes in August of 1999. The total number of students at the start of the Fall semester 2000 in the Molecular Medicine Ph.D. Program at all levels was 51, which includes 19 women, and 4 minorities (3 Hispanic students).

*Project Summaries and Publications of Ph.D. Trainees*

- ***David Levin*** ***Mentor -- Dr. Alan Tomkinson***

DNA joining events are required to maintain the integrity of the genome. Three human genes encoding DNA ligases have been identified. David is identifying the cellular functions involving the product of the LIG1 gene. Previous studies have implicated DNA ligase I in DNA replication and some pathways of DNA repair. During DNA replication, DNA ligase I presumably functions to join Okazaki fragments. However, under physiological salt conditions, DNA ligase I does not interact with DNA. It is Mr. Levin's working hypothesis that DNA ligase I involvement in different DNA metabolic pathways is mediated by specific protein-protein interactions which serve to recruit DNA ligase I to the DNA substrate. To detect proteins that bind to DNA ligase I, David has fractionated a HeLa nuclear extract by DNA ligase I affinity chromatography. PCNA was specifically retained by the DNA ligase I matrix. To confirm that DNA ligase I and PCNA interact directly, Mr. Levin found that in vitro translated and purified recombinant PCNA bind to the DNA ligase I matrix. In similar experiments, he has shown that DNA ligase I interacts with a GST (glutathione S transferase)-PCNA fusion protein but not with GST. Using in vitro translated deleted versions of DNA ligase I, Mr. Levin determined that the amino terminal 120 residues of

this polypeptide are required for the interaction with PCNA. During DNA replication PCNA acts as a homotrimer that encircles DNA and tethers the DNA polymerase to its template. He showed that DNA ligase I forms a stable complex with PCNA that is topologically linked to a DNA duplex. Thus, it appears that PCNA can also tether DNA ligase I to its DNA substrate. A manuscript describing these studies has been published
in the Proc. Natl. Acad. Sci. U.S.A.

In addition to interacting with PCNA, the amino terminal domain of DNA ligase I also mediates the localization of this enzyme to replication foci. To determine whether these are separable functions David fine mapped the region that interacts with PCNA and, in collaboration with Dr. Montecucco's group, the region required for recruitment to replication foci. Since the same 19 amino acids are necessary and sufficient for both functions and the same changes in amino acid sequence inactivate both functions, we conclude that DNA ligase I is recruited to replication foci by its interaction with PCNA. A manuscript describing these studies has been published in the EMBO Journal.

In recent studies, Mr. Levin has a constructed a mutant version of DNA ligase I that does not interact with PCNA. Importantly the amino acid substitutions do not affect the catalytic activity of DNA ligase I. By transfecting cDNAs encoding the mutant and wild type DNA ligase I into a DNA ligase I-mutant cell line, he has demonstrated the biological significance of the DNA ligase I/PCNA interaction in DNA replication and long patch base excision repair.

This project is relevant to breast cancer since problems with DNA replication and repair undoubtedly underlie the genomic instability associated with tumor formation.

Publications:

Matsumoto, Y., Gary, R., Levin, D.S., Tomkinson, A.E. and Park, M. Reconstitution of long patch base excision repair with purified human proteins. J. Biol. Chem (1999) 274(47):33703-8

Levin DS, McKenna AE, Motycka TA, Matsumoto Y, Tomkinson AE. Interaction between PCNA and DNA ligase I is critical for joining of Okazaki fragments and long-patch base-excision repair. Curr Biol. (2000) 10(15):919-22.

- ***Zachary Mackey*** ***Mentor -- Alan Tomkinson***

DNA joining events are required to maintain the integrity of the genome. Three human genes encoding DNA ligases have been identified. In this project we are intending to identify the cellular functions involving the product of the *LIG3* gene. Mammalian cell lines with reduced DNA ligase III activity exhibit spontaneous genetic instability and increased sensitivity to DNA damaging agents. We have cloned human and mouse cDNAs encoding DNA ligase III. In both mouse and humans, we have identified two forms of DNA ligase III cDNA that differ at their 3' end and encode polypeptides with different C-termini. At the site where the cDNA sequences diverge, the nucleotide sequence resembles consensus splice donor/acceptor sequences. We have confirmed that these cDNAs represent alternatively spliced products from the same gene by cloning and analysis of the 3' end of the mouse *LIG3* gene. Analysis of DNA ligase III expression by northern blotting demonstrated that this gene is highly expressed in the testes. Using RT-PCR, we have examined the expression of the two forms of DNA ligase III cDNA in mouse tissues and cells. One form of DNA ligase III mRNA, DNA ligase III□ is ubiquitously expressed. In contrast, expression of DNA ligase III□ mRNA is restricted to the testis. During

spermatogenesis, DNA ligase III□ mRNA expression occurs during the latter stages of meiotic prophase. This restricted expression pattern suggests that DNA ligase III□ mRNA may have a specific role in the completion of meiotic recombination. In support of this idea we have shown that DNA ligase III-a interacts with the DNA strand break repair protein Xrcc1 whereas DNA ligase III□ does not. We suggest that the DNA ligase III□/Xrcc1 complex functions in DNA repair in both somatic and germ cells whereas DNA ligase III□ functions in meiotic recombination. A manuscript describing these studies has been published in Molecular and Cellular Biology.

A unique feature of the DNA ligases encoded by the *LIG3* gene is an amino terminal zinc finger that binds to DNA single-strand breaks. This motif is not required for DNA joining vitro or for the functional complementation of an *E. coli* DNA ligase mutant. However, the presence of this motif allows DNA ligase III to interact with and join nicked DNA molecules at physiological salt concentrations. Using site-directed mutagenesis, we have identified amino acid residues within the catalytic C-terminal domain that are required for interaction with nicked DNA. A manuscript describing these studies has been published in the Journal of Biological Chemistry.

This project is relevant to breast cancer since genomic instability is likely to be involved at several stages during the progression to malignant breast cancer. Methods to intervene and stabilize the genome could prevent progression and spread of the disease. In addition, information about DNA repair processes in normal and cancer cells may lead to the development of treatment regimes that more effectively kill cancer cells and minimize damage to normal tissues and cells.

**Dr. Mackey received his Ph.D. in Molecular Medicine in the summer of 2000, and has taken a post-doctoral position in C.C. Wang's laboratory at the University of California, San Francisco.**

Publications:

Zachary B. Mackey, Niedergang, C., Menissier-de Murcia, J., Leppard, J., Au, K., Chen, J., de Murcia, G. and Tomkinson, A.E. DNA ligase III is recruited to DNA strand breaks by a zinc finger motif homologous to that of Poly (ADP-ribose) polymerase. (1999) J. Biol. Chem. 274, 21679-21687

- ***John Leppard*** ***Mentor -- Alan Tomkinson***

Three genes, *LIG1*, *LIG3* and *LIG4*, encoding DNA ligases have been identified in the mammalian genome. Unlike the *LIG1* and *LIG4* genes, there are no homologues of the *LIG3* gene in lower eukaryotes such as yeast. Biochemical and genetic studies suggest that DNA ligase III participates in base excision repair and the repair of DNA single-strand break. A feature of DNA ligase III that distinguishes it from other eukaryotic DNA ligases is a zinc finger. In published studies we have shown that this zinc finger binds preferentially to nicks in duplex DNA and allows DNA ligase III to efficiently ligate nicks at physiological salt concentrations. These studies will be extended by determining how the zinc finger of DNA ligase III binds to DNA single-strand breaks but does not hinder access of the catalytic domain of DNA ligase III to ligatable nicks. Furthermore, we will reconstitute the base excision and single-strand break repair pathways mediated by DNA ligase III and elucidate the functional consequences of

interactions between DNA ligase III and other DNA repair proteins such as Xrcc1, DNA polymerase beta and poly (ADP-ribose) polymerase that participate in these repair pathways.

Mr. Leppard's research is relevant to breast cancer since genomic instability is likely to be involved at several stages during the progression to malignant breast cancer. Methods to intervene and stabilize the genome could prevent progression and spread of the disease. In addition, information about DNA repair processes in normal and cancer cells may lead to the development of treatment regimes that more effectively kill cancer cells and minimize damage to normal tissues and cells.

Publications:

Mackey, Z.B., Niedergang, C., Menissier-de Murcia, J., Leppard, J., Au, K., Chen, J., de Murcia, G. and Tomkinson, A.E. DNA ligase III is recruited to DNA strand breaks by a zinc finger motif homologous to that of Poly (ADP-ribose) polymerase. J. Biol. Chem. 274, 21679-21687 (1999).

- ***Teresa Motycka*** ***Mentor -- Alan Tomkinson***

The repair of DNA double strand breaks (DSBs) is critical for maintaining genomic stability. These cytotoxic lesions can be repaired by two different processes, one of which occurs by end-to end joining whereas the other process involves a homologous duplex. Genetic studies in Saccharomyces cerevisiae have identified a group of genes known as the *RAD52* epistasis group that are involved in the repair of DSBs by homologous recombination. The identification of mammalian homologs of these genes indicates that this type of repair is conserved among eukaryotes. The severe phenotype of yeast rad52 strains suggests that the RAD52 gene product plays key role in recombinational repair of DSBs. To understand how human Rad52 protein functions, we have fractionated a HeLa cell nuclear extract by hRad52-affinity chromatography and identified proteins that were specifically retained by the resin. A protein implicated in Rad52-dependent recombination pathway by yeast genetic studies was identified by immunoblotting. Two other proteins were identified by amino acid sequencing. One of these proteins is conserved in yeast but encoded by an ORF of unknown function. Inactivation of the gene encoding that protein results in hypersensitivity to DNA damaging agents suggesting that we have identified a novel DNA repair gene.

An understanding of the mechanisms of DSB in mammalian cells is relevant to breast cancer because the accumulating evidence linking the products of the breast cancer susceptibility genes, BRCA1 and BRCA2, with DSB repair.

Publications:

Levin DS, McKenna AE, Motycka TA, Matsumoto Y, Tomkinson AE. Interaction between PCNA and DNA ligase I is critical for joining of Okazaki fragments and long-patch base-excision repair. Curr Biol. (2000) 10(15):919-22.

- ***Ashby Morrison*** ***Mentor -- Dr. Kent Osborne***

Ms. Morrison worked in three labs, breast cancer research being the primary area of research in each lab. Her first lab rotation, which was in the lab of Peter O'Connell, Ph.D., She was involved in the preliminary work of locating a gene that when mutated may be involved in process of metastasis. The second lab rotation was done in the lab of Jolene Windle, Ph.D. There she was exposed to the technique of using mouse models to study breast cancer. Specifically, her project involved transgenic and knockout mice to research the effects of oncogenes and tumor suppressors on breast cancer development. During her third lab rotation, in the lab of Kent Osborne, MD., Ms. Morrison was involved in a more clinical area of breast cancer research. Her project was to study the effects of varying levels of estrogen receptor coactivators and corepressors during tamoxifen treatment. Ms. Morrison was accepted into Dr. Osborne's laboratory where she continues to make good progress on the identification of estrogen receptor-associated proteins that are hypothesized to be co-activator/repressor proteins.

- ***Jennifer L. Gooch*** ***Mentor -- Dr. Doug Yee***

Dr. Yee's laboratory is interested in the growth regulation of breast cancer cells by insulin-like growth factors (IGFs). Data from several laboratories had suggested that interleukin-4 (IL-4) and IGFs share common signaling pathways. Since it was known that IL-4 could directly inhibit breast cancer cell proliferation, Jennifer began examining the potential overlap of growth stimulatory and growth inhibitory signaling pathways in breast cancer cells.

Ms. Gooch first confirmed that IL-4 was inhibitory for breast cancer cells. This inhibition was dependent on expression of the IL-4 receptor and blocking antibodies to the receptor neutralized the effects of IL-4. She discovered that IL-4's growth inhibitory effects were dependent on cell proliferation. Quiescent cells were not affected by IL-4. Moreover, IL-4 induced apoptosis in estradiol-stimulated cells. She documented apoptosis by morphologic change, TUNEL assay, PARP cleavage, DNA laddering and generation of a sub-G1 peak by flow cytometry. Thus, she has shown that IL-4 inhibits breast cancer cell growth by inducing apoptosis to some, but not all, growth stimuli.

Because IL-4 and IGF-I share a common signaling pathway through insulin receptor substrate protein-1 (IRS-1), it is possible that this molecule coordinates both growth promoting and cell death signals. It is also possible that additional signals generated by IL-4 are responsible for its growth inhibitory effects. To date, she has documented Stat-6 activation by IL-4. She has shown that IL-4 treatment induces Stat-6 binding to a synthetic oligonucleotide in gel mobility shift assays. She has also shown that IRS-1 is activated by IL-4 in responsive cell lines. However, IL-4 differs from IGF-I in its kinetics of IRS-1 activation. While IGF-I rapidly phosphorylates IRS-1 to high levels followed by rapid dephosphorylation, IL-4 causes tonic levels of IRS-1 to appear in the cell. Furthermore, it appears that IRS-1 is rapidly degraded after IGF-I treatment, while such degradation does not occur after IL-4. Preliminary evidence suggests that IRS-1 may be ubiquitinated after IGF-I treatment, but not IL-4. Her future projects involve the detailed characterization of these pathways and determination of their contribution to IL-4's growth inhibitory effects.

Finally, she has shown that interferon-gamma (IFNg) stimulates Jak/Stat activation in human breast cancer cells. As in other epithelial tumors, activation of Stat-1 and Stat-3 appear to be growth inhibitory compared to their function in lymphocytes.

These projects are relevant to breast cancer since intracellular signaling pathways are almost certainly involved in the growth stimulation at some stage of mammary cell tumor development

or progression. Since growth inhibitory (IL-4) and growth stimulatory (IGF-I) pathways may be coordinated through a single molecule, the precise definition of the mechanism of IL-4 action, as compared to IGF-I action, could define molecular targets to inhibit breast cancer cell growth.

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**Jennifer L. Gooch**, Yee D. Strain-specific differences in the formation of apoptotic DNA ladders in MCF-7 breast cancer cells. (1999) *Cancer Letters*, 144:31-7.

**Jennifer L. Gooch,** Herrera R, Yee D. The role of p21 in IFN-gamma-mediated growth inhibition in human breast cancer cells. *Cell Growth and Differentiation*, 11:335-42.

Lee AV, **Gooch JL**, Osterreich S, Guler B, Yee D. IGF-I-induced degradation of IRS-1 is mediated by the 26S proteosome and requires PI-3 kinase. (2000) *Molecular Cell Biology*, 20:1489-96

- ***Linda DeGraffenried*** ***Mentor -- Dr. Suzanne Fuqua***

Ms. deGraffenried's current project is to determine the cis-acting sequences responsible for the regulation of the human estrogen receptor gene. Deletion and site-directed mutagenesis of the ER promoter combined with transient transfection assays have revealed elements located proximal as well as distal to the primary transcriptional start site to be responsible. Mobility gel shift analysis suggests that a number of factors in whole cell extracts from ER-positive MCF-7 cells bind to the ER promoter between nucleotides -245 and -192, as indicated by the formation of four specific protein/DNA complexes. This region of the promoter contains a GC box between -223 bp and -211 bp as well as a non-consensus binding site for Sp1 between -203 bp and -192 bp. Antibodies to the transcription factors Sp1 and Sp3 supershift two of the specific complexes. Co-transfection of expression plasmids for Sp1 and Sp3 with an ER promoter-driven luciferase reporter plasmid into Sp1-void *Drosophila* SL2 cells induces a one-hundred- and a thirty-fold activation of the ER promoter, respectively. Transient transfection assays using linker-scanner mutants of the ER promoter spanning -245 bp to -182 bp also suggest an important role for elements flanking the Sp binding sites in the regulation of ER gene transcription. A detailed elucidation of these elements as well as the DNA-binding proteins that mediate transcriptional response will be characterized.

This project is directly relevant to breast cancer. Elucidating the basis for regulation of ER expression is an important issue in breast cancer research.

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- ***Jill Gilroy*** ***Mentor – Dr. Hanna Abboud***

Signal transduction pathways are a vital part of development, proliferation, and tumorigenesis. In my work, I am interested in the involvement of growth factors, primarily Platelet Derived Growth Factor (PDGF) and its receptor (PDGFR), in signaling pathways. PDGFRs are tyrosine kinase receptors and upon stimulation dimerize and autophosphorylate, which in turn induces many downstream signaling molecules including, Mitogen Activated Protien Kinase (MAPK), and Phosphatidylinositol 3-kinase (PI3K). One of my goals was to determine the role of PI3K and MAPK in mediating biological processes such as cell migration and proliferation by PDGFR activation. Activation of PI3K was assayed using thin layer chromtography of anti-phosphotyrosine immunoprecipitates. MAPK activation was measured by immune complex assay of MAPK immunoprecipitates and SDS-PAGE using anti-phospho-MAPK antibodies. Functional assays, chemotaxis and $^{3}$H-thymidine assays, were also preformed to test for cell migration and proliferation respectively. Inhibitors of MAPK and PI3K were also used in these studies to further show the involvement of these pathways in the aforementioned biological processes.

This project is relevant to breast cancer since signal transduction pathways are a vital part of tumorigenesis.

Ghosh Choudhury G, **Ricono JM** Increased effect of interferon gamma on PDGF-induced c-fos gene transcription in glomerular mesangial cells: differential effect of the transcriptional coactivator CBP on STAT1alpha activation. *Biochem Biophys Res Commun* 2000 273:1069-77

***Also, please note that Ms. JM Gilroy is publishing under her married name (JM Ricono)***

- ***Shyng-Shiou (Frank) Yuan, M.D.*** ***Mentor -- Dr. Eva Lee***

The response of mammalian cells to DNA damage is complex, involving cell cycle arrest, DNA repair and, under certain conditions, apoptosis. Cells from individuals with the recessive disorder ataxia telangiectasia (AT) are hypersensitive to ionizing radiation. ATM (mutated in AT) protein contains a PI-3 kinase domain and is predominantly localized in the nucleus. c-Abl, a non-receptor tyrosine kinase, interacts with ATM and is a substrate of ATM kinase. Dr. Yuan demonstrated that ATM, c-Abl, and Rad51, a homologue of bacterial RecA protein required for DNA recombination and repair, can be co-immunoprecipitated from cell extracts. c-Abl interacts with and phosphorylates Rad51 *in vitro*. This phosphorylation enhances complex formation between Rad51 and Rad52, which functions with Rad51 in recombination and repair. After g-irradiation, an increase in both tyrosine phosphorylation of Rad51 and association between Rad51 and Rad52 occurs in wild-type cells but not in ATM-/- or c-Abl-/- cells. These findings implicate the ATM/c-Abl signaling pathway in promoting the assembly of the recombinational repair machinary.

Nijmegen breakage syndrome (NBS) is a rare autosomal recessive disease characterized by microcephaly, immunodeficiency, chromosomal instability and high cancer risk. There are many common features shared by AT and NBS, including loss of cell cycle checkpoint and sensitivity to IR. It has been shown recently that the gene product of NBS, Nibrin, is a 95 kDa protein. We

demonstrated that nibrin forms a stable complex with repair proteins Rad50 and Mre11. The Rad50/Mre11/nibrin complexes possess nuclease activities which are likely to be important for recombination, repair,and genomic stability.

Whether there is a biochemical link between ATM and p95 is being studied. This information will provide a biochemical basis for the A-T and NBS cellular phenotypes as well as the mechanism of IR sensitivity in these cells.

The recombinase, Rad51, plays a key role in homologous recombination. Multiple Rad51-interacting proteins including Rad52, Rad54 and RPA are also required for homologous recombination. Several labs have reported that the protein product of breast cancer susceptibility gene, BRCA2, interacts with Rad51 directly through its BRC domains. In normal cells, a redistribution of Rad51 protein, manifested as formation of Rad51 nuclear foci, is seen upon ionizing radiation (IR). We show that in cells harboring BRCA2 mutation, there is little IR-induced Rad51 foci formation. In addition, introduction of GFP-BRC/BRCA2 fusion protein but not GFP compromised IR-induced Rad51 foci formation. This study suggests a specific dependence of IR-induced nuclear distribution on BRCA2.

These projects are highly relevant to breast cancer since recent studies indicate that the protein product of breast cancer susceptibility gene BRCA1 interacts with Rad50 (Dr. Wen-Hwa Lee, personal communication). Furthermore, it has been reported that ATM carriers may have a higher risk of breast cancer.

Dr. Yuan received his Ph.D. in Molecular Medicine in the Spring of 2000 and has accepted a faculty position as an Assistant Professor in the Department of Obstetrics and Gynecology at the Kaohsiung Medical University, Kaohsiung, Taiwan.

**Shyng-Shiou (Frank) Yuan**, Cox, L. A., Dasika, G.K. and Lee, E.Y.-H.P. Cloning and functional studies of a novel gene aberrantly expressed in *Rb-/-* mouse embryos. Dev. Biol. 207:62-75 (1999).

Chen, G., **Yuan, S.-S. F.**, Liu, W., Xu, Y., Trujillo, K., Song, B.-W., Cong, F., Goff, S.P., Arlinghaus, R., Baltimore, D., Park, M.S., Sung, P. and Lee, E.Y.-H. P. Radiation-induced Assembly of Rad51 and Rad52 recombination complex requires ATM and c-Abl. J. Biol. Chem. 274:12748- 12752 (1999).

**Shyng-Shiou (Frank) Yuan.**, Lee, S.-Y., Chen, G., Song, M., Tomlinson, G. E., and Lee, E.Y.-H.P. BRCA2 is Required for Ionizing Radiation-induced Assembly of Rad51 Complex *in Vivo*. Cancer Res. 59: 3547-3551 (1999).

Zhao S, Weng YC, **Yuan SS**, Lin YT, Hsu HC, Lin SC, Gerbino E, Song MH, Zdzienicka MZ, Gatti RA, Shay JW, Ziv Y, Shiloh Y, Lee EY. Functional link between ataxia-telangiectasia and Nijmegen breakage syndrome gene products. (2000) *Nature* 405:473-7

- ***Suh-Chin(Jackie) Lin*** ***Mentor -- Dr. Eva Lee***

The tumor suppressor gene, p53, is frequently mutated in human tumors, including breast carcinoma. P53 null mice develop multiple spontaneous tumors, predominantly lymphoma and sarcoma, within the first 6 months of age. To establish a mouse model of p53-mediated mammary tumor development, Ms. Lin initiated a bigenetic approach employing the cre-loxp system. Through gene targeting in embryonic stem (ES) cells, mice carrying floxed p53 genes in which exons 5 and 6 are flanked by the loxp sequence were generated. A second mouse line carrying a cre transgene under the control of mouse mammary tumor virus LTR (MMTV-

cre) has also been generated. Floxed p53 mice were mated with MMTV-cre transgenic mice to produce mice with p53 inactivation in mammary tissue. Indeed, we observed p53 excision in the tissues of double transgenic mice. In addition, adenoviral vectors carrying cre recombinase are being used to inactivate p53. These approaches should provide a mouse mammary tumor model for studies of mammary tumor progression resulting from p53 mutation and for testing therapeutic interventions of mammary tumorigenesis. The resulting mice have demonstrated interesting patterns of tumor development including those of the mammary gland. These animals will be valuable models for testing new approaches to breast cancer treatment and understanding its etiology.

Upon DNA damage, p53 protein becomes phosphorylated and stabilized, leading to subsequent activation of cell cycle checkpoints. It has been shown that ATM is required for IR induced phosphorylation on Ser15 residue of p53. Based on the involvement of p53 in mammary tumorigenesis and on the higher risk of ATM carriers for breast cancer, we have carried out studies to address the cancer susceptibility of ATM heterozygous and ATM null mammary epithelial cells by transplanting mammary gland to wild-type sibling mice. Initial studies have indicated differential checkpoint and apoptotic responses in cells harboring ATM mutation. These studies will establish whether ATM plays important roles in mammary tumorigenesis.

Both of these projects are highly relevant to breast cancer, especially the Ms. Lin's animal models which hold promise in terms of new therapies for breast cancer and its metastases.

Ms. Lin sucessfully defended her dissertation on December 18, 2000. She will begin interviewing for postdoctoral positions.

Zhao S, Weng YC, Yuan SS, **Lin YT**, Hsu HC, Lin SC, Gerbino E, Song MH, Zdzienicka MZ, Gatti RA, Shay JW, Ziv Y, Shiloh Y, Lee EY. Functional link between ataxia-telangiectasia and Nijmegen breakage syndrome gene products. (2000) *Nature* 405:473-7

- ***Sean Post*** ***Mentor -- Dr. Eva Lee***

Recent studies indicate that breast cancer susceptibility genes,BRCA1 and BRCA2, are involved in DNA repair. Cells harboring mutations in either gene are hypersensitive to ionizing radiation (IR). Extensive genetic evidence in yeast indicates that DNA double-stranded breaks are processed by Rad50/Mre11 nuclease complex. It has also been shown that in response to IR, Rad50 assembles into nuclear foci. In mammalian cells, such IR-induced Rad50 foci are not observed in cells established from Nijmegen breakage syndrome (NBS). We and others have shown that the protein product of gene mutated in NBS, Nibrin, forms a stable complex with Rad50/Mre11 and the complex possesses nuclear activity. The E. Lee laboratory demonstrated that IR-induced Rad50 redistribution requires ATM kinase activity. Rad50 is phosphorylated upon IR. Their preliminary studies indicate that such IR-induced Rad50 foci formation and phosphorylation are defective in A-T cells. In addition, IR-induced Rad50 foci formation is aberrant in some sporadiac cancers that express normal ATM, Rad50, Mre11, nibrin, BRCA1 and BRCA2 suggesting involvement of additional protein in this DNA damage response.

Mr. Post is a fourth year graduate student who is characterizing IR-induced Rad50 phosphorylation. How phosphorylation affects Rad50 function will be studied. In addition, cross-linking experiments will be carried out to investigate whether there is defective Rad50 protein complex formation in breast cancer cells. These studies will provide insights into the role of ATM kinase cascade in the assembly of double-stranded breakage repair protein. Furthermore,

characterization of components in the repair protein complex may lead to the identification of additional players involved in breast carcinoma.

These projects are highly relevant to breast cancer since genomic instability is a hallmark of cancer and is thought to be a major contributor to the tumorigenic process. Mr. Post's research will contribute toward a greater understanding of the mechanisms responsible for maintaining genomic integrity that is undoubtedly involved in breast cancer development and progression.

- ***Song Zhao*** ***Mentor – Dr. Eva Lee***

Mr. Zhao is working on the functional interactions between ATM and DNA repair proteins with a focus on NBS1. Ataxia-telangiectasia (A-T) and Nijmegen breakage syndrome (NBS) are recessive genetic disorders with susceptibility to cancer and similar cellular phenotypes. The protein product of the gene responsible for A-T, designated ATM, is a member of a family of kinases characterized by a carboxy-terminal phosphatidylinositol 3-kinase-like domain. The NBS1 protein is specifically mutated in patients with Nijmegen breakage syndrome and forms a complex with the DNA repair proteins Rad50 and Mrel1. Mr. Song has shown that phosphorylation of NBS1, induced by ionizing radiation, requires catalytically active ATM. Complexes containing ATM and NBS1 exist in vivo in both untreated cells and cells treated with ionizing radiation. He, along with others in the lab, have identified two residues of NBS1, Ser 278 and Ser 343 that are phosphorylated in vitro by ATM and whose modification in vivo is essential for the cellular response to DNA damage. This response includes S-phase checkpoint activation, formation of the NBS1/Mrel1/Rad50 nuclear foci and rescue of hypersensitivity to ionizing radiation. Together, these results demonstrate a biochemical link between cell-cycle checkpoints activated by DNA damage and DNA repair in two genetic diseases with overlapping phenotypes

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Dasika GK, Lin S-C J, **Zhao S**, Sung P, Tomkinson A and Lee E Y-H P. DNA Damage-induced cell cycle checkpoints and DNA strand break repair in development and tumorigenesis. Oncogene (1999) 18, 7883-7899.

**Song Zhao**, Weng YC, Yuan SS, Lin YT, Hsu HC, Lin SC, Gerbino E, Song MH, Zdzienicka MZ, Gatti RA, Shay JW, Ziv Y, Shiloh Y, Lee EY. Functional link between ataxia-telangiectasia and Nijmegen breakage syndrome gene products. (2000) *Nature* 405:473-7

- ***Shang Li*** ***Mentor -- Dr. Wen-Hwa Lee***

Mutations of the *BRCA1* gene predispose women to the development of breast cancer. The *BRCA1* gene product [BRCA1] is a nuclear phosphoprotein whose cellular function is poorly understood. The C-terminal region of the BRCA1 protein contains an activation domain and two repeats termed BRCT (for BRCA1 C-terminal). In his recent work, Mr. Li identified a BRCT-interacting protein previously identified as CtIP, a protein that interacts with the C-terminal-binding protein (CtBP) of E1A. Together, CtIP and CtBP are postulated to form a transcription corepressor complex. The ability of BRCA1 to transactivate the p21 promoter can be inactivated by mutation of the C-terminal conserved BRCT domains. To explore the mechanisms of this BRCA1 function, the BRCT domains were used as bait in a yeast two-hybrid screen. A known protein, CtIP, a co-repressor with CtBP, was found. CtIP interacts specifically with the BRCT domains of BRCA1, both *in vitro* and *in vivo*, and tumor-derived mutations abolished these interactions. The association of BRCA1 with CtIP was also

abrogated in cells treated with DNA-damaging agents including UV, γ-irradiation and adriamycin, a response correlated with BRCA1 phosphorylation. The transactivation of the p21 promoter by BRCA1 was diminished by expression of exogenous CtIP and CtBP. These results suggest that the binding of the BRCT domains of BRCA1 to CtIP/CtBP is critical in mediating transcriptional regulation of p21 in response to DNA damage.

This project is directly relevant to breast cancer since it involves the study of a protein whose function appears to central to the mobilizing the response of cells to DNA damage. Perturbations in the systems that maintain genomic integrity underlie initiation and progression of most cancers, including those of the breast.

Publications:

**Shang Li**, Phang-Lang Chen, Thirugnana Subramanian, G. Chinnadurai, Gail Tomlinson, C. Kent Osborne, Z. Dave Sharp, and Wen-Hwa Lee. 1999 Dissociation of BRCA1 Binding to CtIP upon DNA Damage Mediates p21 Expression. J. Biol. Chem. 274:11334-11338

Zheng L, Pan H, **Li S.**, Flesken-Nikitin A, Chen P, Boyer TG, Lee W. Sequence-Specific Transcriptional Corepressor Function for BRCA1 through a Novel Zinc Finger Protein, ZBRK1. (2000) *Mol Cell* 6:757-768

**Shang Li**, Ting NS, Zheng L, Chen PL, Ziv Y, Shiloh Y, Lee EY, Lee WH Functional link of BRCA1 and ataxia telangiectasia gene product in DNA damage response. (2000) *Nature*;406:210-5

Dr. Li defended his dissertation in Fall of 2000. He is going to the University of California, San Francisco for postdoctoral training.

- ***Qing Zhong*** ***Mentor -- Dr. Wen-Hwa Lee***

One of Mr. Zhong's project in Dr. Lee's laboratory is a study of the tumor suppressor protein, TSG101. *tsg101* was identified as a tumor susceptibility gene by homozygous function inactivation of allelic loci in mouse 3T3 fibroblasts. To confirm its relevance to breast cancer that was originally reported, antibodies specific for the putative gene product were prepared and used to identify cellular 46 kDa TSG101 protein. A full size 46 kDa TSG101 protein was detected in a panel of 10 breast cancer cell lines and 2 normal breast epithelial cell lines with the same antibodies. A full-length *TSG101* mRNA was also detected using rtPCR. These results indicate that homozygous intragenic deletion of *TSG101* is rare in breast cancer cells. In more recent work, Mr. Zhong demonstrated that TSG101 is a cytoplasmic protein that translocates to the nucleus during S phase of the cell cycle. Interestingly, TSG101 is distributed mainly around the chromosomes during M phase. Microinjection of antibodies selective for TSG101 during G1 or S results in cell cycle arrest and overexpression leads to cell death. These data indicate that neoplastic transformation due to lack of TSG101 could be due to a bypass of cell cycle checkpoints.

Another more recent interest of Mr. Zhong is the role of the breast tumor suppressor BRCA1 in cancer formation. *BRCA1*, encodes a tumor suppressor that is mutated in familial breast and ovarian cancers. Mr. Zhong's work showed that BRCA1 interacts *in vitro* and *in vivo* with human Rad50, which forms a complex with hMre11 and p95/nibrin. BRCA1 was detected in discrete foci in the nucleus that colocalize with hRad50 after irradiation. Formation of irradiation-induced foci positive for BRCA1, hRad50, hMre11 or p95 were dramatically reduced in HCC1937 breast cancer cells carrying a homozygous mutation in *BRCA1*, but was restored

by transfection of wild-type *BRCA1*. Ectopic expression of wild-type, but not mutated *BRCA1* in these cells rendered them less sensitive to the DNA damage agent, methyl methanesulfonate. These data suggest that BRCA1 is important for the cellular responses to DNA damage that are mediated by the hRad50-hMre11-p95 complex.

Mr. Zhong's work on TSG101 and, especially, BRCA1 are is highly relevant for breast cancer research. By understanding the interaction and functional role of BRCA1 in the DNA repair process could lead to a greater understanding of its role in tumorigenesis and to new forms of cancer thearpy aimed at interactions with the repair proteins.

Publications:

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Dr. Zhong successfully defended his dissertation in November, 2000. He is currently completing additional work as a postdoctoral fellow in Dr. Wen-Hwa Lee's laboratory toward additional work for publications related to his doctoral research.

- ***Lei Zheng*** ***Mentor -- Dr.Wen-Hwa Lee***

In his work, Mr. Zheng explored the role of the retinoblastoma tumor suppressor (Rb) in the process of chromosome segregation. A yeast homologue (scHec1p) of the Rb-associated protein hHEC was shown to be essential for survival of yeast. The human HEC protein rescues the lethal phenotype of the null-mutation of sc*hec1* by complementing a critical role in modulation of chromosome segregation. A temperature-sensitive (ts) mutation of hs*HEC1* leads to a high frequency of errors in chromosome segregation. hsHec1p binds to Rb at an IxCxE motif specifically during M phase. In yeast carrying a ts allele of hs*hec1,* the expression of wild-type Rb reduced chromosome segregation errors by approximately 5-fold, suggesting that Rb enhances the fidelity of chromosome segregation. These results may aloso help explain why $Rb^{+/-}$ cells convert to $Rb^{-/-}$ at high frequency by loss of the wild-type *Rb* allele. How Rb and HEC facilitate chromosome segregation is the continuing pursuit of Mr. Zheng.

This is research that is highly relevant to breast cancer. Aneuploidy is the hallmark of cancer cells, including those of the mammary glands. Also, RB mutations are implicated in breast cancer. Accordingly, understanding this new role for the RB is relevant for a full understanding of breast cancer development and may provide new avenues for the development of novel therapies.

In another project, Mr. Zheng has identified and characterized a BRCA1-interacting protein, ZBRK1, that is a zinc finger-containing protein capable of repressing transcription of BRCA1-targeted genes such as those induced upon DNA damage. This is highly significant for breast cancer as this possibly explains the mechanism for the biological activity of BRCA1 through sequence-specific transcriptional regulation and the cellular response to DNA damage.

**Lei Zheng**, Chen, Y., and Lee, W. H. (1999). Hec1p, an evolutionarily conserved coiled-coil protein, modulates chromosome segregation through interaction with SMC proteins. Mol Cell Biol *19*, 5417-5428.

**Lei Zheng**, Chen, Y., Riley, DJ, Chen, PL., and Lee, WH. Retinoblastoma protein enhances the fidelity of chromosome segregation mediated by a novel coiled-coil protein, HsHec1p (2000) Molecular Cell Biology. 20:3529-37.

**Lei Zheng**, Pan H, Li S., Flesken-Nikitin A, Chen P, Boyer TG, Lee W. Sequence-Specific Transcriptional Corepressor Function for BRCA1 through a Novel Zinc Finger Protein, ZBRK1. (2000) *Mol Cell* 6:757-768

Li, S., Ting NS, **Zheng L**, Chen PL, Ziv Y, Shiloh Y, Lee EY, Lee WH. Functional link of BRCA1 and ataxia telangiectasia gene product in DNA damage response. (2000) *Nature*;406:210-5

**Lei Zheng**, Li S, Boyer TG, and and Lee, W-H. Lessons learned from BRCA1 and BRCA2 (2000) Oncogene, 19.

Dr. Zheng successfully defended his Ph.D. dissertation in Novermber, 2000. He is currently exploring other laboratories for postdoctoral training.

- ***Horng-Ru Lin*** ***Mentor -- Dr.Wen-Hwa Lee***

Mutations of the *BRCA2* gene predispose women to the development of breast cancer. The *BRCA2* gene product [BRCA2] is a nuclear phosphoprotein whose cellular function is poorly understood. BRCA2 is known to interact with other members of the DNA repair machinery such Rad51 and Rad50. The functional consequences of these interactions are unknown except for the fact that replacement of functional BRCA2 in BRCA2-deficient cells restores cell survival in response to ionizing radiation. Mr. Lin is focusing his studies on the phosphorylation of BRCA2 and its relationship to signaling induced by DNA damage. Upon DNA damage and BRCA2 phosphorylation, the proteins associated with BRCA2 are changed. The relationship of these changes is being pursued at the molecular and cellular levels. These studies have a direct bearing on breast cancer research since the findings could lead to new strategies for the treatment of breast cancer.

- ***Stephen Van Komen*** ***Mentor – Dr. Patrick Sung***

In yeast homologous recombination, Rad54, a member of Swi2/Snf2 family of proteins, functionally cooperates with the Rad51 recombinase in making D-loop, the first DNA joint formed between recombining chromosomes. Our biochemical studies have indicated that yeast Rad54 modulates DNA topology at the expense of ATP hydrolysis, producing extensive unconstrained supercoils in DNA. This supercoiling ability is likely to be indispensable for D-loop formation. Given the high degree of structural and functional conservation among yeast and human recombination factors, we hypothesize that human Rad51 and Rad54 also function together to make D-loop. This hypothesis is being tested with human Rad51 and Rad54 proteins purified from insect cells infected with recombinant baculoviruses. In addition, whether human Rad54 has ATP hydrolysis-driven DNA supercoiling ability is also being examined. Our work is directly relevant to breast cancer, since recent studies have implicated the breast tumor suppressor BRCA2 in modulating the activities of the recombination machinery via Rad51.

Mr. Van Komen's research is directly relevant to breast cancer since double strand breaks in DNA and their repair is an issue pertinent to breast cancer. Since the tumor suppressor, BRCA2, interacts with Rad51, it is critically important to understand the biochemistry of this important enzyme in DNA repair.

Petukhova, G., S. **Van Komen, S.** Vergano, H. Klein, and P. Sung. (1999) Yeast Rad54 promotes Rad51-dependent homologous DNA pairing via ATP hydrolysis-driven change in DNA double helix conformation. (1999) J. Biol. Chem. 274:29453-62.

Sung, P. Trujillo, K., and **S. Van Komen.** Recombination factors of Saccharomyces cerevisiae. (2000) Mutation Research 451:257-75.

**Stephen Van Komen**, Petukhova G, Sigurdsson S, Stratton S, Sung P. Superhelicity-driven homologous DNA pairing by yeast recombination factors Rad51 and Rad54. (2000) *Mol Cell* 6:563-72

- **Deanna Jansen** **Mentor – Dr. Patrick Sung**

Homologous recombination is required for the repair of DNA double-strand breaks. The products of the yeast RAD50, MRE11, and XRS2 genes mediate the nucleolytic processing of DNA double-strand breaks, and as such, are indispensable for the recombinational repair of these breaks. As evidenced by co-immunoprecipitation and two-hybrid experiments, Rad50, Mre11, and Xrs2 proteins are associated in a complex, which has been reconstituted in our laboratory using purified components. Likewise, the human counterparts of these proteins - hRad50, hMre11, and NBS1 - have also been purified as a stable complex from human cells in our laboratory. To address the role of yeast Rad50 in recombination and repair, we have introduced specific mutations into this protein to inactivate its ability to bind and hydrolyze ATP. Ongoing studies are aimed at defining the effects of these mutations on the cellular capacity to carry out recombination and the repair of DNA double-strand breaks. Recombinant baculoviruses are being constructed to facilitate the purification of the mutant rad50 proteins for biochemical analyses. Our work is germane for understanding the molecular basis of certain forms of breast cancer, as in human cells, the activities of Rad50 and its associated proteins are modulated by the breast tumor suppressor BRCA1.

- **Stefan Sigurdsson** **Mentor – Dr. Patrick Sung**

Among members of the RAD52 group required for homologous recombination and DNA double-strand break repair, the RAD51 encoded product is of particular interest because of its structural and functional similarities to the Escherichia coli recombination protein RecA. RecA promotes the pairing and strand exchange between homologous DNA molecules to form heteroduplex DNA. In published studies, human Rad51 (hRad51) was found to have a weak ability to make DNA joints and little DNA strand exchange activity. We have explored a variety of reaction parameters that could influence the recombinase activity of hRad51 and demonstrated that under certain conditions, hRad51 makes DNA joints avidly and promotes highly efficient DNA strand exchange. Recently, hRad51 was shown to interact with the breast tumor suppressor BRCA2. The in vitro DNA strand exchange system developed in our laboratory should be well suited for examining the functions of various human recombination factors including BRCA2 in the homologous DNA pairing and strand exchange reaction.

Publications:

Van Komen S, Petukhova G, **Sigurdsson S**, Stratton S, Sung P. Superhelicity-driven homologous DNA pairing by yeast recombination factors Rad51 and Rad54. (2000) *Mol Cell* 6:563-72

**Stefan Sigurdsson**, Trujillo K, Song B, Stratton S, Sung P. Basis for avid homologous DNA strand exchange by human Rad51 and RPA (2000) J. Biol. Chem. In Press.

- **Guikai Wu** **Mentor – Dr. Phang-Lang Chen**

Nijmegan breakage syndrome (NBS), a chromosomal instability disorder, is characterized in part by cellular hypersensitivity to ionizing radiation. The NBS1 gene product, p95 (NBS1 or nibrin) forms a complex with Rad50 and Mre11. Cells deficient in the formation of this complex are defective in DNA double-strand break repair, cell cycle checkpoint control, and telomere length maintenance. How the NBS1 complex is involved in telomere length maintenance remains unclear. In published studies the Chen laboratory showed that NBS1 and Mre11 co-localize with TRF1 in PML nuclear bodies in telomerase-negative immortalized cells during G2 phase of the cell cycle. Significantly, the NBS1/TRF1 foci undergo active BrdU incorporation during late S/G2 transition, suggesting a novel role for NBS1 in telomere lengthening in telomerase-negative immortalized cell lines. These results suggest that NBS1 may be invovled in alternative lengthening of telomeres in telomerase-hegative immortalized cells. Given that multiple key players in homolgous recombination area localized to PML bodies in telomerase-hegative immortalized cells, it is reasonable to speculate that active DNA synthesis in these nuclear domains in late S/G2 may reflect a telomere maintenance process potentially involving a homologous recombination. Mr. Wu will investigate the mechanistic details of how these factors work in concert. An understanding of the mechanisms of telomere length maintenance is relevant to breast cancer.

Publications:

**Wu G**, Lee W-H, and Chen P-L. NBS1 and TRF1 Colocalize at Promyelocytic Leukemia Bodies during Late S/G2 Phases in Immortalized Telomerase-negative Cells. (2000) J Biol Chem 275:39, 30618-30622

*Changes to the Program Faculty:*

Removals: The following individuals have been removed from the faculty since they have moved to other institutions.

Doug Yee, M.D.
Jolene Windle, Ph.D.

Additions: The following investigators have been added to the faculty. All have expertise and funded research programs relevant to breast cancer as indicated by their descriptions below and appended NIH Biosketches.

*Jan Vijg, Ph.D.*, Professor, Department of Physiology.

The association between cancer and aging is not simply a matter of chronological time, since other animal species with shorter life spans show the same increase in cancer incidence much earlier, i.e., at about the same relative point in their life history as humans. Interestingly, at very old age, i.e., over 80 years in humans, cancer mortality levels off and starts to decline. Our laboratory focuses on the biological basis of the aging-cancer link, following two broad approaches. The first approach involves the use of transgenic and mutant mouse models to study mutation accumulation in both normal mice and mice with specific defects in one or more genome stability systems (e.g., DNA Repair). The second line of research involves the lucky few in the human population who survived the ravages of old age for at least a century (centenarians), virtually without signs of cancer or other age-related diseases. By screening populations of centenarians in comparison to groups of younger control individuals for polymorphic variation in genes involved in genome stability pathways, we attempt to test the hypothesis that such genes play a major deterministic role in keeping cancer at bay thereby promoting extreme longevity.

Trainees in Dr. Vijg's laboratory will use animal models to examine the effects of DNA repair deficiencies on genome stability and will apply state-of-the-art technologies to detect and analyze mutations and polymorphisms.

*Phang-Lang Chen, Ph.D.*, Assistant Professor, Department of Molecular Medicine.

Dr. Chen's laboratory is interested in understanding the critical role of tumor suppressor genes in the genesis and progression of cancer. Her early work centered on understanding the suppressor of retinoblastoma, RB and p53, and directly led to the identification of the functions of RB in cell growth and cell proliferation. More recently, she has turned her attention to the breast carcinoma gene product, BRCA2. We have demonstrated that BRCA2 binds to the DNA double-strand break repair protein Rad51 through the BRC repeats, and have shown that the BRC repeats are necessary for normal resistance of cells to the effects of DNA-damaging agents. Our recent studies have identified a physical interaction between BRCA2 and another double-strand break repair factor Rad50. Other ongoing studies focus on the product of the gene mutated in Nijmegen breakage syndrome (NBS). NBS cells are also deficient for radiation-induced nuclear foci containing the repair factor Mre11. An analysis of the kinetic relationship between Mre11 phosphorylation and the appearance of its radiation-induced foci indicates that the former precedes the latter. Together, these data suggest that specific phosphorylation of Mre11 is induced by DNA damage and NBS1 is essential in this process, perhaps by recruiting specific kinases. We are currently focusing on identifying and deciphering the signaling systems and kinases responsible for critical regulatory events in DNA double-strand break repair.

Trainees in Dr. Chen's laboratory will study signal tranduction pathways involved in the cellular response to DNA damage and will gain experience in mammalian genetics, DNA repair protein biochemistry, and cell biology.

*Tom Boyer, Ph.D.*, Assistant Professor, Department of Molecular Medicine.

Dr. Boyer's research is focused on understanding the molecular mechanisms through which the encoded protein product of the **BR**east **CA**ncer susceptibility gene, ***BRCA1***, effects its tumor suppressive properties. Toward this end, we have undertaken the biochemical fractionation of human cell extracts in an effort to identify, isolate, and functionally characterize BRCA1-containing protein complexes. Thus far, his laboratory has succeeded in the biochemical

resolution of two distinct BRCA1-containing multiprotein complexes involved in DNA double-strand break repair and transcription. Efforts are currently underway to purify these complexes to homogeneity and to identify and clone their respective constituent subunits. Ultimately, our goal will be to reconstitute these activities in vitro from entirely recombinant proteins in an effort to facilitate future mechanistic studies. Fundamental knowledge of how BRCA1 functionally interacts with the cellular DNA double-strand break repair and transcription machinery will expedite the rational design of small molecule drugs capable of specifically interrupting this interaction. Such compounds could theoretically render targeted breast tumor cells hypersensitive to the DNA-damaging effects of radiation or other genotoxic agents, thereby radically improving their therapeutic efficacy.

Trainees in Dr. Boyer's laboratory will apply biochemical methodologies to study the cellular response to DNA damage and will gain experience in DNA repair protein biochemistry, and macromolecular complex identification and function.

*Paul Hasty, DVM*, Associate Professor, Department of Molecular Medicine.

Dr. Hasty's laboratory is interested in DNA repair and cell cycle responses to DNA damage. He emphasizes biology by studying mice with specific mutations in DNA repair genes using embryonic stem cell/gene targeting technology. Currently, they are studying mice mutated for proteins important for the repair of DNA double–strand breaks by two different pathways. The first pathway is called recombinational repair by virtue of the fact that it utilizes a homologous template usually provided by the sister chromatid. To disrupt recombinational repair, Dr. Hasty's group mutated Rad51 and found it to be essential for repairing gamma radiation-induced damage and cellular proliferation. Mutant embryos died shortly after implantation. We showed that a p53-mediated cell cycle response contributed to embryonic lethality. p53 is a tumor suppressor that is essential for stopping cellular proliferation after DNA damage. We also discovered that Rad51 functions by binding to a breast cancer susceptibility gene called Brca2 and a subtle brca2 mutation causes genomic instability and increased sensitivity to genotoxic agents. Thus, we established that the Rad51 pathway is important for suppressing tumors. The second pathway is called nonhomologus end joining (NHEJ) because it joins chromosomal ends without the use of a homologous template. To disrupt NHEJ, Dr. Hasty mutated Ku80 (a.k.a. Ku86) and found that ku80 mutant mice are relatively normal at birth; however, exhibit an early onset of characteristics associated with aging that include osteopenia, skin and follicular atrophy, liver degeneration and shortened life span. Early onset of sepsis and cancer shortened life span. In addition, cells derived from ku80 mutant mice undergo premature cellular senescence that is dependent on p53. Ongoing research focuses on the biological role of Ku80 in both aging and oncogenesis.

Trainees will have a variety of opportunities to develop animal models for studying DNA repair and cell-cycle checkpoint gene function. Using these animal models, trainees will learn about molecular basis for the pathological changes caused by genetic instability.

*Renee P. Yew, Ph.D.*, Assistant Professor, Department of Molecular Medicine.

Mutational inactivation of the *BRCA1* gene accounts for a large percentage of hereditary breast cancer. Although the *BRCA1* gene product has been implicated to function in a number of different cellular processes including DNA repair and transcription, it is still unclear how BRCA1 biochemically mediates its cellular function as a tumor suppressor protein. Recently, the highly conserved ring finger domain of BRCA1 has been implicated in the ubiquitination of proteins. This ubiquitinating activity of BRCA1 has only recently been discovered and how this

biochemical activity of BRCA1 may mediate the functions of BRCA1 in breast cancer prevention has yet to be addressed. It has been suggested that the BRCA1 gene product may function as an ubiquitin protein ligase or E3 enzyme in a manner similar to a growing number of proteins that comprise a family of ring finger proteins. If this putative E3 activity of BRCA1 can be shown to be a physiological function of BRCA1 in the cell, this could greatly aid in determining the molecular mechanism by which BRCA1 mediates its cellular function. Based on results showing that the ring finger domain of BRCA1 possesses a putative ubiquitin protein ligase activity, our hypothesis is that BRCA1 mediates its biological function by targeting proteins for ubiquitination. The Yew laboratory is testing the idea that BRCA1 effects the ubiquitination of proteins that are either negative regulators of DNA repair or are positive regulators of growth proliferation, leading either to their degradation or to an alteration of their activity. The long-term goal of their study is to characterize the role of BRCA1-dependent E3 ubiquitination in the prevention of breast cancer.

Students will have the opportunity to train in the biochemistry and cell biology of important cancer-related molecules by studying the ubiquitinating activity of BRCA1 *in vivo* and *in vitro*. In addition, the role of these activities in the cellular responses to DNA damage will also be significant training prospects.

*Maria Gazcynska, Ph.D.*, Assistant Professor, Department of Molecular Medicine.

Dr. Gazcynska's laboratory is interested in the regulation of cell division and cell differentiation, which are major factors preventing neoplastic growth. Proteolysis is one of the widely accepted controlling mechanism of these processes. To a large extent, proteolytic control is executed by the proteolytic multicatalytic assembly called the proteasome. For example, the proteasome regulates cell cycle progression, destroys or activates numerous oncogenic proteins and transcription factors, receptor proteins and antigenic proteins, and multiple evidence links proteasome-mediated proteolysis with the breast cancer. One of specific inhibitors of the proteasome is already under clinical trials as anti-cancer drug. Dr. Gazcynska's laboratory has discovered a new giant proteolytic complex distinct from the proteasome and ubiquitous among eukaryotes. The enzyme, named multicorn, apparently takes part in cell cycle regulation and is involved in partial overcoming the physiological effects of proteasome inhibitors. Moreover, there is significant differences in the amount and composition of the multicorn in human breast cancer MCF-7 cells, as compared with non-cancerous MCF-10A cells. Data indicate that the multicorn enzyme is involved in progression of mitosis. Since mitosis is the period of greatest vulnerability of cells to anti-cancer drugs, it can be considered an attractive potential drug target. Dr. Gazcynska's laboratory focuses on the role of multicorn in cell cycle regulation and on mechanisms underlying the regulation of the multicorn activity.

Trainees will have opportunities to apply macromolecular biochemistry and imaging to the understanding vital proteolytic complexes and their role in the cell cycle, as well as cancer development and progression.

*Course Changes:* None during the last funding period.

**SUMMARY**: The Breast Cancer Training Program made excellent progress toward attracting and retaining excellently qualified students in breast cancer research. The students received a high level of training in the modern research methods and theory. A total of 30 publications on breast cancer was achieved by students supported by the program. Two of our students obtained predoctoral training awards from the Defense Department's Breast Cancer Research Program. There were new faculty additions, four of which obtain DOD BCRP Idea awards, that

will greatly expanded the training opportunities in Breast Cancer Research. Combined with the basic instruction they receive in the Molecular Medicine Ph.D. Program, students will graduate as highly skilled researchers who will competitive effectively for post doctoral positions in the premiere breast cancer laboratories in the world.

**Appendix: NIH Biosketches and Reprints of Trainee Publications.**

## BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Photocopy this page or follow this format for each person.

| NAME | POSITION TITLE |
|---|---|
| Thomas G. Boyer, Ph.D. | Assistant Professor |

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

| INSTITUTION AND LOCATION | DEGREE *(if applicable)* | YEAR(s) | FIELD OF STUDY |
|---|---|---|---|
| Frostburg State College, Frostburg, MD | B.S. | 1983 | Biology |
| Frostburg State College, Frostburg, MD | B.S. | 1983 | Wildlife/Fisheries Mgt |
| State University of New York at Buffalo | Ph.D. | 1990 | Biochemistry |
| University of California, Los Angeles | PostDoc | 1998 | Molecular Genetics |

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. If the list of publications in the last three years exceeds two pages, select the most pertinent publications. **DO NOT EXCEED TWO PAGES.**

## PROFESSIONAL EXPERIENCE

| | |
|---|---|
| 1983-1985 | Research Assistant, Biotech Research Laboratories, Rockville, MD |
| 1985-1990 | Graduate Research Assistant, State University of New York at Buffalo |
| 1990-1991 | Fellow, California Institute for Cancer Research, UCLA |
| 1991-1997 | Fellow, American Cancer Society, UCLA |
| 1999 – Present | Assistant Professor, Dept. of Molecular Medicine/Institute of Biotechnology<br>The University of Texas Health Science Center at San Antonio |

## AWARDS

| | |
|---|---|
| 1983 | Frostburg State College Biology Departmental Honors |
| 1990 | California Institute for Cancer Research Fellowship |
| 1991 | American Cancer Society (National Division) Junior Postdoctoral Research Fellowship |
| 1995 | American Cancer Society (California Division) Senior Postdoctoral Research Fellowship |
| 1999 | Parvin Postdoctoral Recognition Award |

## PUBLICATIONS

**Boyer, T.G.**, Krug, J.R., and Maquat, L.E. (1989). Transcriptional regulatory sequences of the housekeeping gene for human triosephosphate isomerase. J. Biol. Chem. **264**: 5177-5187.

**Boyer, T.G.,** and Maquat, L.E. (1990). Minimal sequence and factor requirements for the initiation of transcription from an atypical, TATATAA box-containing housekeeping promoter. J. Biol. Chem. **265**: 20524-20532.

**Boyer, T.G.**, and Maquat L.E. (1991). Modulation of human triosephosphate isomerase gene transcription by serum. J. Biol. Chem. **266**: 13350-13354.

Zhou, Q., Lieberman, P.M., **Boyer, T.G.**, and Berk, A.J. (1992). Holo-TFIID supports transcriptional stimulation by diverse activators and from a TATA-less promoter. Genes & Dev. **6**: 1964-1974.

Zhou, Q., **Boyer, T.G.**, and Berk, A.J. (1993). Factors (TAFs) required for activated transcription interact with the TATA box-binding protein conserved core domain. Genes & Dev. **7**: 180-187.

**Boyer, T.G.** and Berk, A.J. (1993). Functional interaction of adenovirus E1A with holo-TFIID. Genes & Dev. **7**: 1810-1823.

Kerr, L.D., Ransone, L.J., Wamsley, P., Schmitt, M.J., **Boyer, T.G.**, Zhou, Q., Berk, A.J., and Verma, I.M. (1993). Association between proto-oncoprotein Rel and TATA-binding protein mediates transcriptional activation by NF-kappa B. Nature **365**: 412-419.

Kobayashi, N., **Boyer, T.G.**, and Berk, A.J. (1995). A class of activation domains interacts directly with TFIIA and stimulates TFIIA-TFIID-promoter complex assembly. Mol. Cell. Biol. **15**: 6465-6473.

Kobayashi, N., Horn, P., Sullivan, S., Triezenberg, S.J., **Boyer, T.G.**, and Berk, A.J. (1998). DA-Complex assembly activity required for VP16C transcriptional activation. Mol. Cell. Biol. **18**: 4023-4031.

Berk, A.J., **Boyer, T.G.**, Kapanidis, A.N., Ebright, R.H., Kobayashi, N., Horn, P.J., Sullivan, S.M., Koop, R., Surby, M.A., and Triezenberg,, S.J. (1998). Mechanisms of viral activators. Cold Spring Harbor Symp. Quant. Biol. Volume **LXIII**. 243-252.

**Boyer, T.G.**, Martin, M.E.D., Lees, E., Ricciardi, R.P., and Berk, A.J. (1999). Mammalian Srb/Mediator complex targeted by adenovirus E1A protein. Nature **399**: 276-279.

Zheng, L., Pan, H., Li, S., Fleskin-Nitikin, A., Chen, P.-L., **Boyer, T.G.**, and Lee, W.-H. (2000). Sequence-specific transcriptional corepressor function for BRCA1 through a novel zinc finger protein, ZBRK1. Mol. Cell **6**: 757-768.

Zheng, L., Li, S., **Boyer, T.**, and Lee, W.-H. (2000). Lessons learned from BRCA1 and BRCA2. Oncogene, *In press*.

Zhong, Q., **Boyer, T.G.**, Chen, C.-F., Chen, P.-L., and Lee, W.-H. (2000). BRCA1 promotes non-homologous end-joining of DNA double-strand breaks. Submitted.

## BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Photocopy this page or follow this format for each person.

| NAME | POSITION TITLE |
|---|---|
| Phang-Lang Chen | Assistant Professor |

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

| INSTITUTION AND LOCATION | DEGREE *(if applicable)* | YEAR(s) | FIELD OF STUDY |
|---|---|---|---|
| National Chung-Hsing University | B.S. | 1984 | Food Science |
| National Taiwan University | M.S. | 1986 | Biochemistry |
| University of California, San Diego | Ph.D. | 1991 | Molec. Pathology |

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. If the list of publications in the last three years exceeds two pages, select the most pertinent publications. **DO NOT EXCEED THREE PAGES.**

### Professional Positions:

| | |
|---|---|
| 1986-1987 | Research Assistant, Department of Biochemistry,<br>National Taiwan University |
| 1990 - 1991 | Research Assistant, Department of Pathology, School of Medicine,<br>University of California, San Diego |
| 1992 - 1996 | Instructor |
| 1996 - Present | Assistant Professor<br>Department of Molecular Medicine/Institute of Biotechnology<br>University of Texas Health Science Center at San Antonio |

### Honors:

| | |
|---|---|
| 1984-1986 | Graduate Scholarship of the Ministry of Education, Taiwan |
| 1987-1990 | Scholarship of the Ministry of Education, Taiwan |
| April, 1995 | Texas Triangle Award for outstanding accomplishments at Texas Triangle Meeting in Molecular Medicine |
| 1999 | V Foundation Scholar |

### Research Projects Ongoing or Completed During the Last 3 Years:

SPORE in Breast Cancer – Development Grant – "Identification and characterization of BRCA2-associated proteins in mammary epithelial cells"
Program Director: C. Kent Osborne, M.D. Project Leader: Phang-Lang Chen, Ph.D.
Agency: National Cancer Institute (NCI)
5 P50 CA58183-06 Period: August 1, 1997 – July 31, 1998
Test the hypothesis that the interactions between BRCA2 and target proteins have an important role in the biological function of BRCA2. To test the hypothesis, accomplish following specific aims: 1) to identify BRCA2-associated proteins (referred to as B2APs), and prioritize them for further analysis; 2) to acquire full-length cDNAs and immunological reagents for the B2AP clones; and 3) to determine whether the interactions of BRCA2 and B2APs is altered in breast cancer cells.

"BRCA2 Regulates DNA Double-Strand Break Repair Through Interactions with Rad51"
Principal Investigator: Phang-Lang Chen, Ph.D.
Agency: Susan G. Komen Breast Cancer Foundation
Grant No. 9733 Period: January 1, 1998 – December 31, 1999
The overall goal of this proposal is to understand the cellular function of BRCA2 and to elucidate its role in the development and progression of breast cancer. The goals are to use cellular, biochemical and molecular methods to purify and characterize the BRCA2 complexes, determine the mechanism of macromolecular assembly, determine the signaling pathway involved in regulating the interaction between BRCA2 and Rad51, and to determine if BRCA2 has a modulatory role in the DNA pairing strand exchange reaction. These studies will, for the first time, begin to elucidate the role of the breast cancer suppressor genes BRCA2, which could lead to the development of new diagnostic or therapeutic regimens.

"Breast Tumorigenesis in Mice Heterozygous for Brca2 Mutations"
Principal Investigator: Phang-Lang Chen, Ph.D.

Agency: San Antonio Cancer Institute
5 P30 CA54174-07 Period: June 23, 1998 – June 22, 1999
(1) To test the hypothesis that cooperation between the protooncogene *neu* and *Brca2* will result in a reduced latency and an increase in multiplicity in tumor formation; and (2) To test the postulate that heterozygous *Brca2* knockout mice will demonstrate an increased sensitivity to _-irradiation in the mammary gland as evidenced by an increase in tumor formation and multiplicity.

"The BRCA Genes, the DNA Repair Machinery and Cancer"
Principal Investigator: Phang-Lang Chen, Ph.D.
Agency: The V Foundation
Period: August 1, 1999 – July 31, 2001

"BRCA2 and the DNA Double-Strand Break Repair Machinery"
Principal Investigator: Phang-Lang Chen, Ph.D.
Agency: U.S.A.M.R.M.C.
Period of Grant: November 1, 1999-September 30, 2002
To investigate the role of the breast cancer suppressor, BRCA2, with the strand scission reaction in DNA double-strand break repair through its interactions with a novel protein termed Rad50 linked to BRCA (RLB1). This is a novel interaction, and understanding BRCA2's role in this aspect of DNA repair will have important implications for the genesis of breast cancer and the development of future treatments.

"Molecular Basis of the NBS1 protein in genomic stability"
Principal Investigator: Phang-Lang Chen, Ph.D.
1R01CA85605-01 Period: April 1, 2000- March 31, 2004
Agency: National Institute Health (NIH)
To test the hypothesis that Mre11 and CtIP represent downstream effectors through which NBS1 coordinately regulates two distinct processes involved in the maintenance of genome integrity: DNA double-strand break repair and cell cycle checkpoint control. This postulate will be tested by addressing the following objectives. 1. To test whether phosphorylation of Mre11 induced by DNA-damaging agents is important for changes in the Mre11 complex formation or its enzymatic activity. 2.To test whether NBS1 mediates the DNA damage-induced phosphorylation of the CtIP/CtBP co-repressor complex of BRCA1, and to determine if this results in its dissociation and activation of p21 expression. 3. To identify the potential kinase(s) which phosphorylates Mre11 and CtIP mediated by NBS1.

"Small chemical molecules that disrupt BRCA2 and Rad51 interaction for adjuvant breast cancer therapy"
Principal Investigator: Phang-Lang Chen, Ph.D.
Period: September 1, 2001- August 31, 2004
Agency: U.S.A.M.R.M.C.
The interaction between BRCA2 and Rad51 mediated by BRC repeats is critical for cellular response to DNA damage. To exploit the importance of this interaction toward the development of new adjuvant anti-breast cancer chemotherapeutic agents, we planned to isolate small chemical molecules that disrupt interaction between BRCA2 and Rad51. The efficacy of these molecules will be evaluated by the following aims: 1). To test the cytotoxicity of the identified molecules in breast cancer cells. 2). To examine the effect of small chemical molecules on radiation response in an experimental mammary carcinoma *in vivo*.

**RELEVANT PUBLICATIONS (partial listing from total of 45):**

1. Huang H-JS, Yee J-K, Shew J-Y, **Chen P-L**, Bookstein R, Friedmann T, Lee EY-HP and Lee W-H: Suppression of the Neoplastic Phenotype by Replacement of the Rb Gene in Human Cancer Cells. Science, 242: 1563-1566 (1988).
2. **Chen P-L**, Scully P, Shew J-Y, Wang J-YJ, and Lee W-H: Phosphorylation of the Retinoblastoma Gene Product is Modulated during the Cell Cycle and Cellular Differentiation. Cell, 58: 1193-1198 (1989).
3. Bookstein R, Shew J-Y, **Chen P-L**, Scully P and Lee W-H: Suppression of Tumorigenicity of Human Prostate Carcinoma Cells by Replacing a Mutated *RB* Gene. Science, 247: 712-715 (1990).
4. **Chen P-L,** Chen Y, Bookstein R and Lee W-H: Genetic Mechanisms of Tumor Suppression by the Human p53 Gene. Science, 250: 1576-1580 (1990).
5. **Chen P-L**, Chen Y, Shan B, Bookstein R, and Lee W-H: Stability of Retinoblastoma Gene Expression Determines the Tumorigenicity of Reconstituted Retinoblastoma Cells. Cell Growth Differ., 3: 119-125 (1992).
6. Lee W-H, Hollingsworth RE, Qian Y-W, **Chen P-L,** Hong F and Lee EY-HP: RB Protein as a Cellular "Corral" for Growth-promoting Proteins. Cold Spring Harbor Symp. on Quantitative Biol. Cell Cycle, 56: 211-217 (1992).

7. Shan B, Zhu X, **Chen P-L**, Durfee T, Yang Y, Sharp ZD and Lee W-H: Molecular Cloning of Cellular Genes Encoding Retinoblastoma-Associated Proteins: Identification of a Gene with Properties of the Transcription Factor E2F. Mol. & Cell. Biol., 12: 5620-5631 (1992).
8. Durfee T, Becherer K, **Chen P-L**, Yeh S-H, Yang Y, Kilburn A, Lee W-H and Elledge S: The retinoblastoma protein associates with the protein phosphatas type 1 catalytic subunit. Genes & Development, 7: 555-569 (1993).
9. Chen Y, **Chen P-L**, and Lee W-H: Hot Spot p53 Mutants Interact Specifically with Two Cellular Proteins during Progression of the Cell Cycle. Mol. & Cell. Biol., 14: 6764-6772 (1994).
10. **Chen P-L**, Ueng Y-C, Durfee T, Chen K-C, Yang-Feng T, and Lee W-H: Identification of a Human Homologue of Yeast nuc2 Which Interacts with the Retinoblastoma Protein in a Specific Manner. Cell Growth Differ., 6: 199-210 (1995).
11. **Chen P-L**, Riley D, and Lee W-H: The Retinoblastoma Protein as a Fundamental Mediator of Growth and Differentiation Signals -- Critical Reviews in Eukaryotic Gene Expression, 5(1): 79-95 (1995).
12. Chen Y, Chen C-F, Riley DJ, Allred DC, **Chen P-L**, Von Hoff D, Osborne CK, and Lee W-H: Aberrant Subcellular Localization of BRCA1 in Breast Cancer. Science, 270: 789-791 (1995).
13. **Chen P-L**, Riley D, Chen-Kiang S, and Lee W-H: Retinoblastoma protein directly interacts with and activates the transcription factor NF-IL6. PNAS, 93: 465-469 (1996).
14. Chen Y, Farmer A, Chen C-F, Jones D, **Chen P-L**, and Lee W-H: BRCA1 is a 220 kDa nuclear phosphoprotein that is expressed and phosphorylated in a cell cycle dependent manner. Cancer Research, 56: 3168-3172 (1996).
15. Chen C-F, Chen Y, Dai K, **Chen P-L**, Riley DJ, and Lee W-H: A New Member of the hsp90 Family of Molecular Chaperones Interacts with Retinoblastoma Protein during Mitosis and after Heat Shock. Mol. & Cell. Biol., 16: 4691-4699 (1996).
16. **Chen P-L**, Riley DJ, Chen Y, and Lee W-H: Retinoblastoma protein positively regulates terminal adipocyte differentiation through direct interaction with C/EBPs. Genes & Dev., 10: 2794-2804 (1996).
17. Chen C-F, Li S, Chen Y, **Chen P-L**, Sharp ZD, and Lee W-H: The Nuclear Localization Sequences of the *BRCA1* Protein Interact with the Importin-α Subunit of the Nuclear Transport Signal Receptor. J. Biol. Chem., 271: 32863-32868 (1996).
18. Chang K-H, Chen Y, Chen T-T, Chou W-H, **Chen P-L**, Ma Y-T, Yang-Feng TL, Leng X, Tsai M-J, O'Malley BW, and Lee W-H: A thyroid hormone receptor coactivator negatively regulated by the retinoblastoma protein. PNAS, 94: 9040-9045 (1997).
19. Chen Y, Riley DJ, **Chen P-L**, and Lee W-H: HEC, a Novel Nuclear Protein Rich in Leucine Heptad Repeats Specifically Involved in Mitosis. Mol. & Cell. Biol., 17: 6049-6056 (1997).
20. Lee W-H, Chew H, Farmer A, and **Chen P-L**: "Biological Functions of the BRCA1 Protein." Breast Disease, an International Journal, Edited by Edison Liu, IOS Press, 10(1,2): 11-22 (1998).
21. **Chen P-L**, Chen C-F, Chen Y, Xiao J, Sharp ZD, and Lee W-H: The BRC repeats in BRCA2 are critical for RAD51 binding and resistance to methyl methanesulfonate treatment. PNAS, 95: 5287-5292 (1998).
22. Chen Y, **Chen P-L**, Chen C-F, Sharp ZD, and Lee W-H: Thyroid hormone, T3-dependent phosphorylation and translocation of Trip230 from the Golgi complex to the nucleus. PNAS, 96: 4443-4448 (1999).
23. Li S, **Chen P-L**, Subramanian T, Chinnadurai G, Tomlinson G, Osborne CK, Sharp ZD, and Lee W-H: Binding of CtIP to the BRCT Repeats of BRCA1 Involved in the Transcription Regulation of p21 is Disrupted Upon DNA Damage. JBC, 274: 11334-11338 (1999).
24. Dong Z, Zhong Q, and **Chen P-L**: The NBS protein is essential for MRE11 phosphorylation upon DNA damage. JBC, 274: 19513-19516 (1999).
25. Zhong Q, Chen C-F, Li S, Chen Y, Wang C-C, Xiao J, **Chen P-L**, Sharp ZD, and Lee W-H: Association of BRCA1 with the hRad50-hMre11-p95 Complex and the DNA Damage Response. Science, 285: 747-750, (1999).
26. Chen C-F, **Chen P-L**, Zhong Q, Sharp ZD, and Lee W-H; Expression of BRC repeats in breast cancer cells disrupts the BRCA2-Rad51 complex and leads to radiation hypersensitivity and loss of G2/M checkpoint control. JBC, 274:32931-32935, (1999).
27. Zheng L, Chen Y, Riley DJ, **Chen P-L**, and Lee W-H: Retinoblastoma Protein Enhances the Fidelity of Chromosome Segregation Mediated by hsHec1p. Mol. & Cell. Biol., 20: 3529-3537, (2000).
28. Li S, Ting N S.Y., Zheng L, **Chen P-L**, Ziv Y, Shiloh Y, Lee E Y-H, and Lee W-H: Functional link of BRCA1 and ataxia-telangiectasia gene product in DNA damage response. Nature, 406: 210-215, (2000).
29. Zheng L, Pan H, Li S, Flesken-Nikitin A, **Chen P-L**, Boyer T, and Lee W-H: A novel zinc-finger protein, ZBRK1, represses transcription of the GADD45 gene mediated by BRCA1. Mole. Cell, 6: 757-768, (2000)
30. Wu G, Lee W-H, and **Chen P-L**: NBS1 and TRF1 colocalize at PML bodies during late S/G2 phases in immortalized telomerase-negative cells: Implication of NBS1 in alternative lengthening of telomeres. J. Biol. Chem., 275: 30618-30622, (2000).

## BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed on Form Page 2.
Photocopy this page or follow this format for each person.

| NAME | POSITION TITLE |
|---|---|
| Maria E. Gaczynska, Ph.D. | Assistant Professor |

EDUCATION (*Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.*)

| INSTITUTION AND LOCATION | DEGREE | YEAR CONFERRED | FIELD OF STUDY |
|---|---|---|---|
| University of Lodz, Poland | M.Sc | 1985 | Molecular Biology |
| University of Lodz, Poland | Ph.D. | 1989 | Biophysics |
| Nicolaus Copernicus University, Poland | B.S. | 1990 | Physics |

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. If the list of publications in the last three years exceeds two pages, select the most pertinent publications. DO NOT EXCEED TWO PAGES.

**PROFESSIONAL EXPERIENCE**

1984 - 1989 **Research Associate**, Laboratory of Dr. Grzegorz Bartosz, Department of Biophysics, University of Lodz, Poland.

1989 -1990 **Senior Researcher**, Laboratory of Dr. Grzegorz Bartosz, Department of Biophysics, University of Lodz, Poland.

1990 - 1991 **Postdoctoral Fellow**, Laboratory of Dr. Marguerite M.B. Kay, Department of Immunology and Microbiology, College of Medicine, University of Arizona

1991 - 1995 **Postdoctoral Fellow**, Laboratory of Dr. Alfred L. Goldberg, Department of Cell Biology, Harvard Medical School

1996 - 1997 **Postdoctoral Fellow**, Massachusetts Institute of Technology, Center for Cancer Research, Laboratory of Dr. Hidde Ploegh.

1997-Present **Assistant Professor**, Department of Molecular Medicine, University of Texas Health Science Center at San Antonio

**RESEARCH PROJECTS ONGOING OR COMPLETED DURING THE LAST 3 YEARS**

*"Immunosenescence and the interplay of cytosolic proteases in antigen presentation"* Aging Research and Education Center, San Antonio. Principal Investigator: Maria Gaczynska; Period: 07/01/00-06/30/01. The goal is to establish how the age-related changes in the equilibrium between cytosolic proteases influence the MHC class I antigen processing and presentation.

*"Nanoenzymology of the proteasome as a tool for drug design"*. San Antonio Area Foundation Research Award. Principal Investigator: Maria Gaczynska; Period: 06/01/00-05/31/01. The goal is to develop the automated system of image analysis to study the dynamic structural changes of the proteasome using the AFM technique.

*"Multicorn as an Example of Regulation of Proteolytic Activities of Large Complexes on a Molecular Level"*. MCB-9906434; National Science Foundation; Principal Investigator: Maria Gaczynska, Period: o7/01/99 through 06/30/02 The major goal of this project is to broaden our knowledge about activities of large enzymatic complexes.

*"Molecular Cloning and Expression of the Gene Encoding a Subunit of the Multicorn: A New Large Proteolytic Complex from Fission Yeast"*, Competitive Research Enhancement Fund (CREF) Agency: UTHSCSA. Principal Investigator: Maria Gaczynska, Period: 04/01/99 – 03/31/00
The goal of this project is to clone a gene encoding a subunit of the new proteolytic complex from fission yeast. The gene will be subsequently expressed in homologous and heterologous systems and an active recombinant multicorn will be obtained.

*"Interactions of Gly-Ala Domain with Proteasome,* Institutional Research Grant, Agency: UTHSCSA, Principal Investigator: Maria Gaczynska, Period: 03/01/98 – 02/28/99
The objective of this project was to understand the mechanism of action of Gly-Ala repeat domains. The domains act as a highly specific inhibitor of the proteasome, protect the viral protein EBNA1 from degradation by the proteasome and prevent immune presentation.

## PUBLICATIONS (selected)


**Gaczynska, M.** (1989) Changes in proteolytic activity of human erythrocyte membrane during red cell aging. *Biochem. Biophys. Acta;* 981: 173-177.

Klinger, MHF, Halbhuber, KJ, **Gaczynska, M**, KJ, Feuerstein, H, Linss, W. (1989) Cytochemical detection of band 3 antibodies on in vitro aged red blood cells. *Studia Biophysica*; 134: 27-32.

Klinger, MHF, Halbhuber, KJ, Linss, W, Feuerstein, H, **Gaczynska, M.** (1990) The binding of polyclonal antibodies against human band 3 to in vitro aged erythro-cytes. *Acta Histochem.*; 88: 71-76.

Retelewska, W, **Gaczynska, M**, Bartosz, G, Judkiewicz, L. (1991) Consequences of the presence of elongated variant of the major transmembrane protein (Band 3 protein) in the human erythrocyte membranes. *Clin. Chim. Acta*; 198:255-260.

**Gaczynska, M**, Bartosz, G, Judkiewicz, L. (1991) Proteolytic susceptibility of erythrocyte membrane proteins in hereditary spherocytosis. *Clin. Chim. Acta*; 198: 267-270.

**Gaczynska, M**, Chwialkowski, M, Olejniczak, W, Wojczuk, S, Bartosz, G. (1991) Scanning tunneling microscopy of human erythrocyte membranes. *Biochem. Biophys. Res. Comm.*; 181: 600-603.

**Gaczynska, M**, Bartosz, G. (1993) Neutral serine proteinase and metalloproteinase from human erythrocyte membranes. *Cytobios*; 74: 29-33.

**Gaczynska, M**, Rock, KL, Goldberg, AL. (1993) γ-Interferon and expression of MHC genes regulate peptide hydrolysis of proteasomes. *Nature* (London); 365: 264-266.

**Gaczynska, M**, Rock, KL, Goldberg, AL. (1993) Role of proteasomes in antigen presentation. *Enzyme & Protein*; 47: 354-369 (review).

Van Kaer, L, Ashton-Rickardt, PG, Eichelberger, M, **Gaczynska, M**, Nagashima, K, Rock, KL, Goldberg, AL, Doherty, PC, Tonegawa, S. (1994) Altered peptidase and viral-specific T cell response in LMP2 mutant mice. *Immunity*; 1: 533 541.

**Gaczynska, M**, Rock, KL, Spies, T, Goldberg, AL. (1994) Peptidase activities of proteasomes are differently regulated by the LMP2 and LMP7 genes. *Proc. Natl. Acad. Sci. U.S.A.*; 91: 9213-9217.

Goldberg, AL, **Gaczynska, M**, Grant, E, Michalek, M, Rock, KL (1995) The fuctions of the proteasome in antigen presentation. In *Cold Spring Harbor* Symposia on Quantitative Biology, vol. LX: 479-490 ; Cold Spring Harbor Laboratory Press (review).

**Gaczynska, M**, Goldberg, AL, Tanaka, K, Hendil, KB, Rock, KL (1996) Proteasome subunits X and Y alter peptidase activities in opposite ways to the interferon-gamma - induced subunits LMP2 and LMP7. *J. Biol. Chem.* ; 271: 17275-17280.

Craiu, A, **Gaczynska, M,** Akopian, T, Gramm, CF, Fenteany, G, Goldberg, AL, Rock, KL. (1997) Lactacystin and *clasto*-lactacystin ß-lactone modify multiple proteasome ß subunits and inhibit intracellular protein degradation and MHC class I antigen presentation. *J. Biol. Chem.* 272: 13437-13445.

Bogyo, M, McMaster, J, **Gaczynska, M**, Tortorella, D, Ploegh H. (1997) Covalent modification of the active site threonine of proteasomal β subunits and the Escherichia coli homolog HsIV by a new class of inhibitors. *Proc. Natl. Acad. Sci. U.S.A.;* 94: 6629-6634.

Bogyo, M, **Gaczynska, M,** Ploegh H. (1997) Proteasome Inhibitors and Antigen Presentation. *Biopolymers-Peptide Science* ; 43: 269-280

Glas, R., Bogyo, M., McMaster, J.S., **Gaczynska, M**, Ploegh, H. (1998) A proteolytic system that compensates for loss of proteasome function. *Nature* (London), 392: 618-622.

Osmulski, P, **Gaczynska, M.** (1998) A new large proteolytic complex distinct from the proteasome I present in the cytosol of fission yeast. *Current Biol*, 8:1023-1026.

Osmulski, P, **Gaczynska, M.** (2000) Atomic Force Microscopy Reveals Two Conformations of the 20S Proteasome from Fission Yeast. *J. Biol. Chem.*,275: 13171-13174.

**Gaczynska, M.**, Osmulski, P.A., Ward W.F (2000) Caretaker or undertaker? The role of proteasome in aging. *Mech Age. Dev.,* in press (review).


## AWARDS AND OTHER PROFESSIONAL ACTIVITIES

1996 Anna Fuller Fund Fellowship in Molecular Oncology
1989 Award (Third Degree) of Polish Ministry of Public Education for Ph.D. Thesis
1989 Collective Award (First Degree) of the President of the University of Lodz for Academic Achievements in Year
1989 Award of Polish Biophysical Society for Young Biophysicists
1988 Collective Award (First Degree) of the President of the University of Lodz for Academic Achievements in Year
1988 Award of Polish Biophysical Society for Young Biophysicists
1988 The Wl. Mozolowski Award of Polish Biochemical Society for Outstanding Biochemists
1987 Collective Award (First Degree) of the President of the University of Lodz for Academic Achievements in Year
1987 The Wl. Mozolowski Award of Polish Biochemical Society for Outstanding Biochemists
1986 The Wl. Mozolowski Award of Polish Biochemical Society for Outstanding Biochemists
1985 Medal for Outstanding Study, University of Lodz

## BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Photocopy this page or follow this format for each person.

| NAME | POSITION TITLE |
|---|---|
| E. Paul Hasty, D.V.M. | Associate Professor |

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

| INSTITUTION AND LOCATION | DEGREE *(if applicable)* | YEAR(s) | FIELD OF STUDY |
|---|---|---|---|
| Texas A&M University, College Station, TX | B.S. | 1985 | Veterinary Medicine |
| Texas A&M University, College Station, TX | D.V.M. | 1987 | Veterinary Medicine |
| Baylor College of Medicine, Houston, Tx | Postdoc | 1992 | Molecular Medicine |

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. If the list of publications in the last three years exceeds two pages, select the most pertinent publications. **DO NOT EXCEED TWO PAGES.**

Professional Positions:

1987 – 1992 Postdoctoral Fellow, Institute for Molecular Genetics, Baylor College of Medicine, Houston, Tx.
1992 – 1996 Assistant Professor, Department of Biochemistry and Molecular Biology, University of Texas M.D. Anderson Cancer Center, Houston, Tx.
1996 – 2000 Director of DNA Repair, Lexicon Genetics.
2000 – present Associate Professor, Department of Molecular Medicine, University of Texas Health Science Center at San Antonio, Tx.

Awards and Other Professional Activities:

1983 The National Dean's List
1988 – 1990 Postdoctoral Research Fellow of Granada Corporation
1990 – 1992 Postdoctoral Research Fellow of the Cystic Fibrosis Foundation
1993 March of Dimes Basil O'Connor Scholar

Research Projects Ongoing or Completed During the Last 3 Years:

*"Investigation of xrcc5 Mutant Mice and Cell Lines"* ( Investigates the function of Ku80 in tissue culture cells and in mice so that we may better understand those pathways that repair DNA double-strand breaks (DSBs) and monitor DNA damage.)
Principal Investigator: E. Paul Hasty, D.V.M.
Agency: National Institutes of Health/National Cancer Institute
Type: R01 CA76317-01, Years 1-4, July 7, 1997 to September 30, 2001
The long-term objective of this project is to investigate the function of Ku80 in tissue culture cells and in mice so that we may better understand those pathways that repair DNA double-strand breaks (DSBs) and monitor DNA damage.

Relevant Publications:

Hasty, P., Ramirez-Solis, R., Krumlauf, R., and Bradley, A. (1991). Introduction of a subtle mutation into the *Hox-2.6* locus in embryonic stem cells. Nature 350:243-246.

Hasty, P., Rivera-Perez, J., Chang, C., and Bradley, A. (1991). Target frequency and integration pattern for insertion and replacement vectors in embryonic stem cells. Mol. Cell. Biol. 11:4509-4517.

Hasty, P., Rivera-Perez, J., and Bradley, A. (1991). The length of homology required for gene targeting in embryonic stem cells. Mol. Cell. Biol. 11:5586-5591.

Zheng, H., Hasty, P., Brennenman, M., Grompe, M., Gibbs, R., Wilson, J., and Bradley, A. (1991). Fidelity of targeted recombination in human fibroblasts and murine embryonic stem cells. Proc. Natl. Acad. Sci. USA 88:8067-8071.

Hasty, P., Rivera-Perez, J., and Bradley, A. (1992). The role and fate of DNA ends for homologous recombination in embryonic stem cells. Mol. Cell. Biol. 12:2464-2474.

Bradley, A., Hasty, P., Davis, A., and Ramirez-Solis, R. (1992). Modifying the mouse: design and desire. Bio/Technology 10:534-539

Hasty, P., Bradley, A., Morris, J., H., Edmondson, D., G., Venuti, J., M., Olson, E., N., and Klein, W., H. (1993). Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. Nature 364:501-506.

Hasty, P., Crist, M., Grompe, M., and Bradley, A. (1993). The efficiency of insertion versus replacement vector targeting varies at different chromosomal loci. Mol. Cell. Biol. 14:8385 - 8390.

Rudolph, U., Brabet, P., Kaplan, J., Hasty, P., Bradley, A., and Birnbaumer, L. (1993). Gene targeting of the $G_{i2a}$ gene in ES cells with replacement and insertion vectors. J. Receptor Research 13:619-637.

Rudolph, U., Brabet, P., Hasty, P., Bradley, A., and Birnbaumer, L. (1993). Targeting and intrachromosomal recombination at the $G_{i2a}$ locus in embryonic stem cells. Transgen. Research 2:345-355.\

O'Neil, W.K., Hasty, P., McCray, P.B. Jr., Casey, B., Rivera-Perez, J., Welsh, M.J., Beaudet, A.L., and Bradley, A. (1993). Targeted disruption of the murine cystic fibrosis locus. Human Mol. Genetics 2:1561-1569.

Zhang, H., Hasty, P., and Bradley, A. (1994). Targeting Frequency for Deletion Vectors in Embryonic Stem Cells. Mol. Cell. Biol. 14:2404 - 2410.

Hasty, P., O'Neil, W.K., Liu, Q., Morris, A.P. Bebok, Z., Shumyatsky, G.P. Jilling, T., Sorcher, E.J., Bradley, A., and Beaudet, A.L. (1995). Severe phenotype in mice with a termination mutation in exon of cystic fibrosis gene. Som. Cell. Molec. Gen. 21:177-187.

Mitani, K., Wakamiya, M., Hasty, P., Graham, F.L., Bradley, A., and Caskey, T. (1995). Gene targeting in mouse embryonic stem cells with an adenoviral vector. Som. Cell. Molec. Gen. 21:221-231.

Hasty, P., Rivera-Perez, J., and Bradley, A. (1995). Gene conversion during vector insertion in embryonic stem cells. Neuclic Acids Res. 23:2058-2064.

Zhu, B., Bogue, M. A., Lim, D.-S., Hasty, P., and Roth, D. B. (1996). Ku86-deficient mice exhibit severe combined immunodeficiency and defective processing of V(D(J recombination intermediates. Cell 86:379-389.

Lim D.-L., and Hasty, P. (1996). A mutation in mouse *rad51* results in an early embryonic lethal that is suppressed by a mutation in p53. Mol. Cell. Biol. 16:7133-7143.

Lu, Q., Hasty, P., and Shur, B.D. (1997) Targeted mutation in _1,4-Galactosylt transferase leads to pituitary insufficiency and neonatal lethality. Dev. Biol. 181: 257-267.

Sharan, S. K., Morimatsu, M., Albrecht, U., Lim, D.-S., Regel, E., Dinh, C., Sands, A., Eichele, G., Hasty, P., and Bradley, A. (1997) Embryonic lethality and radiation hypersensitivity mediated by Rad51 in mice lacking Brca2.Nature 386:804-810.

Morimatsu, M., Donoho, G., and Hasty, P. (1998) Cells deleted for *Brca2* COOH terminus exhibit hypersensitivity to radiation and premature senescence. Can. Res. 58:3441-3447.

Vivian, J. L., Klein, W. H., and Hasty, P. (1999) Temporal, Spacial and tissue-specific expression of a myogenin-lacZ transgene targeted to the *Hprt* locus in mice. Biotechniques 27:154-163.

Osipovich, O., Duhe, R., Hasty, P., Durum, S. K. and Muegge, K., (1999) Defining functional domains of Ku80: DNA end binding and survival after radiation. Biochem. and Biophys. Res. Commun. 261:802-807.

Vogel, H., Lim, D-L., Karsenty, G., Finegold, M., and Hasty, P. (1999) Deletion of Ku86 causes early onset of senescence in mice. Proc. Natl. Acad. Sci. 96:10770-10775.

Gu, Y., Sekiguchi, J., Gao, Y., Dikkes, P., Frank, K., Ferguson, D., Hasty, P., Chun, J., and Alt, F. W. (2000) Defective embryonic neurogenesis in Ku-deficient but not DNA-dependent protein kinase catalytic subunit-deficient mice. Proc. Natl. Acad. Sci. 97:2668-2673.

Lim, D-L., Vogel, H., Willerford, D. M., Sands, A., Platt, K. A., and Hasty, P. (2000) Analysis of ku80-mutant mice and cells with deficient levels of p53. Mol. Cell. Biol. 20:3773-3780.

## BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed on Form Page 2
Photocopy this page or follow this format for each person.

| NAME | POSITION TITLE |
|---|---|
| Jan Vijg, Ph.D. | Professor of Physiology |

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

| INSTITUTION AND LOCATION | DEGREE *(if applicable)* | YEAR(s) | FIELD OF STUDY |
|---|---|---|---|
| State University of Leiden, The Netherlands | B.A. | 1980 | Biology |
| State University of Leiden, The Netherlands | M.Sc. | 1982 | Molecular Biology |
| State University of Leiden, The Netherlands | Ph.D. | 1987 | Molecular Biology |

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. If the list of publications in the last three years exceeds two pages, select the most pertinent publications. **PAGE LIMITATIONS APPLY. DO NOT EXCEED 2 PAGES FOR THE ENTIRE BIOGRAPHICAL SKETCH PER INVESTIGATOR.**

**Professional Appointments:**

| | |
|---|---|
| 1987-90 | Head, Department of Molecular Biology, Institute for Experimental Gerontology, Rijswijk, The Netherlands |
| 1990-94 | Lecturer on Medicine, Division on Aging, Harvard Medical School, Boston, MA |
| 1990-93 | Scientific Director, Ingeny B.V., Leiden, The Netherlands |
| 1993- | Director, Molecular Genetics Section, Gerontology Division, Beth Israel Hospital, Boston, MA |
| 1996-98 | Associate Professor of Medicine, Harvard Medical School, Boston, MA |
| 1998- | Professor of Physiology, University of Texas Health Science Center, San Antonio, TX |
| 1998- | Director of Basic Research, Institute for Drug Development, San Antonio, TX |

**Postdoctoral Training:**

| | |
|---|---|
| 1982-87 | Research Associate, TNO Institute for Experimental Gerontology, Rijswijk, The Netherlands |

**Honors:**

| | |
|---|---|
| 1985-90 | Founder and Chairman of the Board, Biomedical Study Group on the Etiology of Aging, the Dutch Organization MEDIGON (Foundation of Medical and Health Research) |
| 1987-90 | Member, MEDIGON Committee on Dementia and Alzheimer's Disease Research |
| 1987 | Founder, "EURAGE Molecular Biology Research Group" |
| 1987 | Schreuder Award, Netherlands Society of Gerontology |
| 1988- | Member, Editorial Board of *Mutation Research* and *Aging: Clinical and Experimental Research* |
| 1994 | Nathan Shock New Investigator Award, Gerontological Society of America |
| 1999 | Editor for the Americas, *Mechanisms of Ageing and Development* |

**Selected publications since 1995:**

Ziao S, Li D, Vijg J, Sugarbaker DJ, Corson JM, Fletcher JA. Codeletion of p15 and p16 in primary malignant mesothelioma. Oncogene 1995;11:511-515.

Ziao S, Li D, Vijg J, Fletcher JA. Codeletion of p15 and p16 genes in primary non-small cell lung carcinoma. Cancer Res 1995;55:2968-2971.

Mullaart E, Borglum AD, te Meerman GJ, Kruse TA, Vijg J. Two-dimensional DNA typing of human pedigrees: spot pattern characterization and segregation. Genomics 1995;29:641-646.

Boerrigter METI, Dollé MET, Martus H-J, Gossen JA, Vijg J. Plasmid-based transgenic mouse model for studying in vivo mutations. Nature 1995;377:657-659.

Borglum AD, Mullaart E, Kvistgaard AB, Uitterlinden AG, Vijg J, Kruse TA. Two-dimensional DNA typing as a genetic marker system in humans. Cytogenet Cell Genet 1995;71:260-265.

Dollé M, Martus HJ, Gossen JA, Boerrigter METI, Vijg J. Evaluation of a plasmid-based transgenic mouse model for detecting in vivo mutations. Mutagenesis 1996;11:111-118.

Li D, Vijg J. Multiplex co-amplification of 24 retinoblastoma gene exons after pre-amplification by long-distance PCR. Nucleic Acids Res 1996;24:538-539.

van Orsouw N, Li D, van der Vlies P, Scheffer H, Eng C, Buys CH, Li FP, Vijg J. Mutational screening of large genes by extensive PCR multiplexing and 2-D electrophoresis: application to the RB1 gene. Hum Mol Genet 1996;5:755-761.

Wu Y, Scheffer H, Uitterlinden AG, Mullaart E, Buys CHCM, Vijg J. Comprehensive and accurate mutation scanning of the CFTR-gene by two-dimensional DNA electrophoresis. Human Mutation 1996;8:160-167.

van Orsouw N, Li D, Vijg J. Denaturing gradient gel electrophoresis (DGGE) increases resolution and informativity of Alu directed inter-repeat PCR. Molecular and Cellular Probes 1997;11:95-101.

Eng C., Vijg J. Genetic testing: The problems and the promise. Nature Biotechnology 1997;15:422-426.

Marsh DJ, Roth S, Lunetta KL, Sistonen P, Dahia PLM, Hemminki A, Zheng Z, Caron S, van Orsouw NJ, Bodmer W, Cottrel SE, Dunlop MG, Eccles D, Hodgson SV, Jarvinen H, Kellokumpu I, Markie D, Neale K, Phillips R, Rosen P, Syngal S, Vijg J, Tomlinson IPM, Aaltonen L, Eng C. Exclusion of PTEN.MMAC1/TEP1 and 10q22-24 as the susceptibility locus for juvenile polyposis syndrome (JPS). Cancer Res 1997; 57:5017-5021.

Dollé MET, Giese H, Hopkins CL, Martus H-J, Hausdorff JM, Vijg J. Rapid accumulation of genome rearrangements in liver but not in brain of old mice. Nat Genet 1997;17:431-434.

De Vries A, Dollé MET, Broekhof JLM, Muller JJA, Kroese ED, van Kreijl CF, Capel JA, Vijg J, van Steeg H. Induction of DNA adducts and mutations in spleen, liver and lung of XPA-deficient/lacZ transgenic mice after oral treatment with benzo[a]pyrene: correlation with tumour development. Carcinogenesis 1997;12:2327-2332.

Borglum AD, Nyegaard M, Kvistgaard AB, Mullaart E, Uitterlinden AG, Vijg J, Kruse TA Mapping of 34 minisatellite loci resolved by two-dimensional DNA typing. Cytogenet Cell Genet 1997;79:248-256.

Rines DR, van Orsouw NJ, Sigalas I, Li FP, Eng C, Vijg J. Comprehensive mutational scanning of the p53 coding region by two-dimensional gene scanning. Carcinogenesis 1998;19:979-984.

Van Orsouw NJ, Dhanda RK, Rines RD, Smith WM, Sigalas I, Eng C, Vijg J. Rapid design of denaturing gradient-based two-dimensional electrophoretic gene mutational scanning tests. Nucleic Acids Res. 1998;26:2398-2406.

Perls TT, Bubrick E, Wager CG, Vijg J, Kruglyak L. Siblings of centenarians live longer. Lancet 1998;351:1560.

Smith WM, Van Orsouw NJ, Fox EA, Kolodner RD, Vijg J, Eng C. Accurate, high throughput "snapshot" detection of hMLH1 mutations by two-dimensional DNA electrophoresis. Genet Testing 1998;2(1):43-53.

Dhanda RK, Van Orsouw NJ, Sigalas I, Eng C, Vijg J. Critical factors in the performance and costs of two-dimensional gene scanning: RB1 as a model. BioTechniques 1998;25:664-675.

Dhanda RK, Smith W, Scott CB, Eng C, Vijg J. A simple system for automated two-dimensional electrophoresis: applications to genetic testing. Genet Testing 1998;2(1):67-70.

Van Orsouw NJ, Zhang X, Wei JY, Johns DR, Vijg J. Mutational scanning of mitochondrial DNA by two-dimensional electrophoresis. Genomics 1998;52:27-36.

Giese H, Dollé MET, Hezel A, van Steeg H, Vijg J. Accelerated accumulation of somatic mutations in mice deficient in the nucleotide excision repair gene XPA. Oncogene 1999;18(5):1257-1260.

Van Orsouw, NJ and Vijg J. Design and application of 2-D DGGE-based gene mutational scanning tests. Genetic Analysis (Biomolecular Engineering), 1999;14:205-213.

Dollé MET, Novak M, Martus H-J, Vijg J. Characterization of color mutants in lacZ plasmid-based transgenic mice, as detected by positive selection. 1999, Mutagenesis 1999;14:287-293.

Khrapko K, Bodyak N, Thilly WG, van Orsouw NJ, Zhang X, Coller HA, Perls TT, Upton M, Vijg J, Wei JY. Cell-by-Cell scanning of whole mitochondrial genomes in aged human heart reveals a significant fraction of myocytes with clonally expanded deletions. Nucleic Acids Res 1999;27:2434-2441.

van Orsouw NJ, Dhanda RK, Xu S-H, Elhaji Y, Narod SA, Li FP, Eng C, Vijg J. A highly accurate, low-cost test for mutations in BRCA1. J. Med. Genet. 1999;36:747-753.

Dollé MET, Snyder WK, Van Orsouw NJ, Vijg J. Background mutations and polymorphisms in *lacZ*-plasmid transgenic mice. Env Molec Mutagen., 1999;34:112-120.

van Oostrom CT, Boeve M, van Den Berg J, de Vries A, Dollé MET, Beems RB,van Kreijl CF, Vijg J, van Steeg H. Effect of heterozygous loss of p53 on benzo[a]pyrene-induced mutations and tumors in DNA repair-deficient XPA mice. Env Mol Mutagen., 1999;34(2-3):124-130.

Yeh JJ, Lunetta KL, van Orsouw NJ, Moore FD Jr, Mutter GL, Vijg J, Dahia PL, Eng C. Somatic mitochondrial DNA (mtDNA) mutations in papillary thyroid carcinomas and differential mtDNA sequence variants in cases with thyroid tumours. Oncogene. 2000;19;2060-2066.

Vijg J. Somatic mutations and aging: a re-evaluation. Mutation Res, 2000; 447:117-135.

Dollé MET, Snyder WK, Gossen JA, Lohman PHM, Vijg J. Distinct spectra of somatic mutations accumulated with age in mouse heart and small intestine. Proc Natl Acad Sci USA, 2000; 97(15):8403-8408.

## BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed on Form Page 2.
Photocopy this page or follow this format for each person.

| NAME | POSITION TITLE |
|---|---|
| P. Renee Yew, Ph.D. | Assistant Professor |

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

| INSTITUTION AND LOCATION | DEGREE *(if applicable)* | YEAR(s) | FIELD OF STUDY |
|---|---|---|---|
| University of California, Berkeley, CA | B.A. | 1983 | Genetics |
| University of California, Santa Barbara, CA | M.A. | 1985 | Biochem and Mole Biol |
| University of California, Los Angeles, CA | Ph.D. | 1993 | Microbiol and Mole Gen |
| Harvard Medical School, Boston, MA | Post-doc | 1993-1998 | Biochem and Mole Biol |

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. If the list of publications in the last three years exceeds two pages, select the most pertinent publications. **DO NOT EXCEED TWO PAGES.**

### Professional Positions

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|---|---|
| 1984 - 1985 | Teaching Assistant and Tutor, Department of Biochemistry and Molecular Biology, University of California, Santa Barbara, CA |
| 1985 – 1987 | Research Associate, Amgen Inc., Thousand Oaks, CA |
| 1987 - 1988 | Teaching Assistant, Department of Microbiology and Molecular Genetics, University of California, Los Angeles, CA |
| 1993 - 1998 | Postdoctoral Research Fellow, Department of Cell Biology, Harvard Medical School, Boston, MA |
| 11/1/98 - Present | Assistant Professor, Department of Molecular Medicine, Institute of Biotechnology, University of Texas Health Science Center at San Antonio, San Antonio, TX |

### Honors and Awards

NIH Pre-doctoral Trainee, Training Grant in Genetics, University of California, Los Angeles, 1989-1992
Sidney C. Rittenberg Award, University of California, Los Angeles, CA, 1993
Jane Coffin Childs Memorial Fund, Postdoctoral Fellowship, New Haven, CT, 1994-1997
Leukemia Society of America Special Fellowship, New York, NY, 1997-2000

### Research Projects Ongoing or Completed During the Last 3 Years

Principal Investigator: P. Renee Yew, Ph.D.
Agency: Howard Hughes Medical Institute
Type: HHMI sub-grant "New Faculty Start-Up" Award, UTHSCSA
Period: 2/01/1999 to 1/31/2000
The goal of this project was to determine how Cdc34-mediated protein degradation regulated the complicated process of DNA replication onset in higher eukaryotes. The responsibility of the PI was to supervise the students in the laboratory and guide their experimental approach toward the problem.

Principal Investigator: P. Renee Yew, Ph.D.
Agency: San Antonio Cancer Institute
Type: Pilot Project
Period: 5/01/1999 to 4/30/2000

The goal of this project was to identify the proteins that associate with Cdc34 and function in DNA replication. The responsibility of the PI was to supervise the students in the laboratory and guide their experimental approach toward the problem.

Principal Investigator: P. Renee Yew, Ph.D.
Agency: Institutional Research Grant, UTHSCSA
Type: New Faculty Start Up, Subgrant Award
Period: 9/01/1999 to 1/14/2000
The goal of this project was to understand how Cdc34 mediated the degradation of the Xenopus substrate, p27-Xic1. The responsibilities of the PI were to supervise the students in the laboratory and guide their experimental approach toward the problem.

Principal Investigator: P. Renee Yew, Ph.D.
Agency: Competitive Research Enhancement Fund, UTHSCSA
Type: New Faculty Start Up Award
Period: 1/01/2000 to 12/31/2000
The goal of this project is to understand how Cdc34 mediates the degradation of the Xenopus substrate, p27-Xic1. The responsibilities of the PI are to supervise the students in the laboratory and guide their experimental approach toward the problem.

Principal Investigator: P. Renee Yew, Ph.D.
Agency: National Science Foundation
Period: 3/01/2000 to 2/28/2003
The goal of this project is to identify and study the proteins that associate with Cdc34 during the onset of DNA replication. The responsibility of the PI is to participate in the biochemical purification of Cdc34-associated proteins and supervise the students working on this project.

Principal Investigator: P. Renee Yew, Ph.D.
Agency: Department of Defense, Breast Cancer Research Program, Idea Award
Period: 3/01/2001 to 2/28/2004
The goal of this project is to test the hypothesis that BRCA1 mediates its biological functions by targeting proteins for ubiquitination. The responsibility of the PI is to participate in the generation of required cDNA libraries and to supervise the students working on this project.

**Publications**

1. Yew, P.R., Tripathi, B., Rajavashisth, J.F., Barath, P., and Lusis, A.J. (1989) NIH 3T3 transforming gene not a general feature of atherosclerotic plaque DNA. *Biochem.Biophy., Res. Comm.* 165: 1067-1071.
2. Yew, P.R., Kao, C.C., and Berk, A.J. (1990) Dissection of functional domains in the adenovirus 2 early 1B 55K polypeptide by suppressor-linker insertional mutagenesis. *Virology* 179: 795-805.
3. Kao, C.C., Yew, P.R., and Berk, A.J. (1990) Domains required for in vitro association between the cellular p53 and the adenovirus 2 E1B 55K proteins. *Virology* 179: 806-814.
4. Yew, P.R. and Berk, A.J. (1992) Inhibition of p53 transactivation required for transformation by adenovirus early 1B protein. *Nature* 357: 82-85.
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6. Pagano, M., Tam, S.W., Theodoras, A.M., Beer-Romano, P., Del Sal,. G., Chau, V., Yew, P.R., Draetta, G.F., and Rolfe, M. (1995) Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27. *Science* 269: 682-685.
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8. Chuang, L.-C. and Yew, P.R.. (2000). Regulation of nuclear transport and degradation of the Xenopus cyclin-dependent kinase inhibitor, $p27^{Xic1}$. *J. Biol. Chem. In press.* (Epub ahead of print 10/23/2000).
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# Interaction between PCNA and DNA ligase I is critical for joining of Okazaki fragments and long-patch base-excision repair

David S. Levin*, Allison E. McKenna†, Teresa A. Motycka*, Yoshihiro Matsumoto† and Alan E. Tomkinson*

**DNA ligase I belongs to a family of proteins that bind to proliferating cell nuclear antigen (PCNA) via a conserved 8-amino-acid motif [1]. Here we examine the biological significance of this interaction. Inactivation of the PCNA-binding site of DNA ligase I had no effect on its catalytic activity or its interaction with DNA polymerase β. In contrast, the loss of PCNA binding severely compromised the ability of DNA ligase I to join Okazaki fragments. Thus, the interaction between PCNA and DNA ligase I is not only critical for the subnuclear targeting of the ligase, but also for coordination of the molecular transactions that occur during lagging-strand synthesis. A functional PCNA-binding site was also required for the ligase to complement hypersensitivity of the DNA ligase I mutant cell line 46BR.1G1 to monofunctional alkylating agents, indicating that a cytotoxic lesion is repaired by a PCNA-dependent DNA repair pathway. Extracts from 46BR.1G1 cells were defective in long-patch, but not short-patch, base-excision repair (BER). Our results show that the interaction between PCNA and DNA ligase I has a key role in long-patch BER and provide the first evidence for the biological significance of this repair mechanism.**

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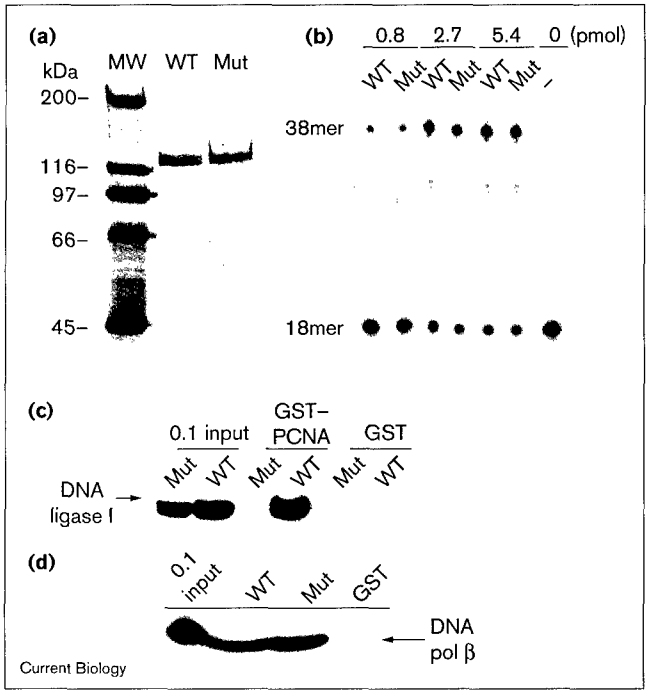
**Figure 1**




Effect of amino-acid substitutions that inactivate the PCNA-binding site of DNA ligase I on its catalytic and Pol β-binding activities. **(a)** After separation by SDS–PAGE, the wild-type (WT) and mutant (Mut) DNA ligase I purified from baculovirus-infected insect cells were detected by Coomassie blue. Molecular mass standards (in kDa) are on the left. **(b)** DNA joining by recombinant DNA ligase I. WT and mutant DNA ligase I were incubated with a labeled nicked oligonucleotide substrate (see Supplementary material). After separation by denaturing gel electrophoresis, labeled oligonucleotides were detected by autoradiography. The positions of the substrate (18mer) and ligated product (38mer) are indicated on the left. **(c)** Binding of DNA ligase I to PCNA. WT and mutant DNA ligase were incubated with glutathione beads bound by either GST–PCNA or GST (see Supplementary material). Ligase bound to the beads was detected by immunoblotting. **(d)** Binding of DNA ligase I to Pol β. Glutathione beads with the amino-terminal 118 amino acids of DNA ligase I (WT or mutant) fused to GST, or GST alone as the ligand, were incubated with Pol β (see Supplementary material). Pol β bound to the beads was detected by immunoblotting. The lane labeled 0.1 input contained one-tenth of the protein in the binding reactions.

## Results and discussion

We have suggested that interaction of DNA ligase I with PCNA provides a molecular explanation for the unique involvement of this ligase in DNA replication [2]. As the PCNA-binding motif of DNA ligase I also mediates recruitment of the ligase to replication foci [3], PCNA binding could be required for subnuclear targeting to sites of DNA replication but not for the catalytic reactions at the replication fork. We first investigated whether inactivation of PCNA binding had an effect on other biochemical properties of DNA ligase I. Using site-directed mutagenesis, the adjacent phenylalanine residues in the conserved PCNA-binding motif of human DNA ligase I (amino acids 8 and 9) were replaced by alanines. After subcloning into baculovirus expression vectors, wild-type and mutant DNA

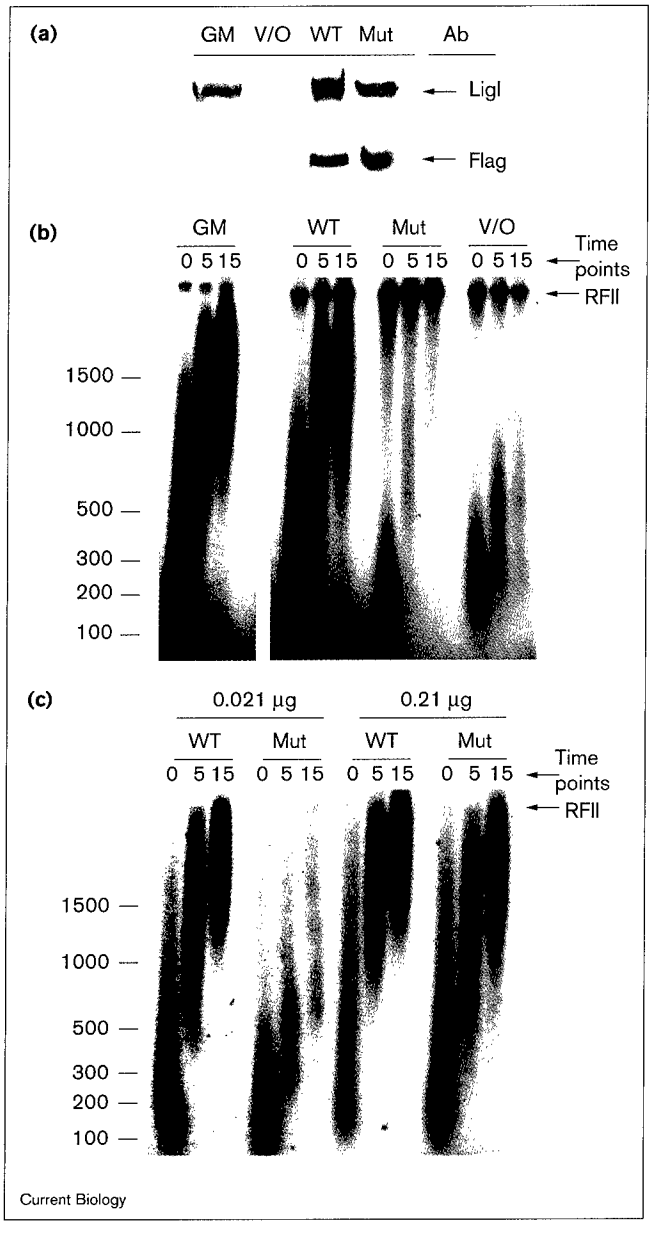



**Figure 2**

Expression of endogenous and tagged DNA ligase I in 46BR.1G1 cells and the effect of amino-acid substitutions that inactivate the PCNA-binding site of DNA ligase I on Okazaki fragment processing. Whole-cell extracts were prepared from the control cell line GM00847 (GM) and from derivatives of 46BR.1G1 stably transfected with empty expression vector (V/O), plasmid expressing Flag-tagged WT DNA ligase I, or plasmid expressing Flag-tagged mutant DNA ligase I (Mut) (see Supplementary material). **(a)** Endogenous and Flag-tagged DNA ligase I were detected in extracts (60 µg) by immunoblotting with anti-DNA ligase I and anti-Flag antibodies. **(b)** Analysis of DNA replication intermediates by pulse-chase labeling. Aliquots from DNA replication assays with the indicated extracts (360 µg) (see Supplementary material) were collected at the times indicated. **(c)** Aliquots from pulse-chase DNA replication assays supplemented with purified DNA ligase I (WT or Mut) were collected at the times indicated. The position of replicative form II DNA (RFII) is indicated. Positions of molecular mass standards (in nucleotides) are shown. After separation by alkaline agarose gel electrophoresis, labeled DNA replication intermediates were detected by autoradiography.

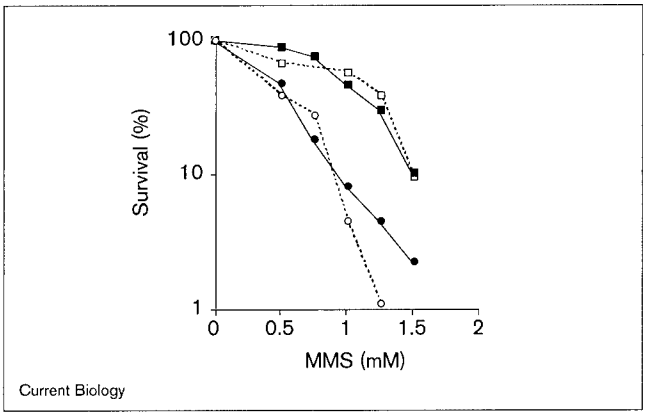
ligase I were purified to near homogeneity from infected insect cells (Figure 1a). The alanine substitutions abolished PCNA binding ([3] and Figure 1c), but did not alter DNA joining activity (Figure 1b) or inactivate binding to DNA polymerase β (Pol β) (Figure 1d). Nor did the amino-acid changes abolish the ability of DNA ligase I to complement the temperature-sensitive phenotype of the *Escherichia coli lig* strain (see Supplementary material).

To elucidate the role of PCNA binding in the various DNA transactions involving DNA ligase I, we transfected DNA ligase I-deficient 46BR.1G1 cells with cDNAs encoding Flag-tagged versions of either wild-type DNA ligase I or the mutant enzyme that does not interact with PCNA. Figure 2a shows immunoblots of extracts from two stable transfected derivatives of 46BR.1G1 that express comparable amounts of the wild-type and mutant ligase. The level of endogenous ligase in 46BR.1G1 cells is about half that in control GM00847 cells, whereas the levels of tagged DNA ligase I in transfected 46BR.1G1 cells are about five times those of the endogenous protein.

A unique characteristic of 46BR.1G1 cells is the abnormal processing of Okazaki fragments [4,5], which can be revealed by pulse-labeling DNA replication intermediates in the SV40 *ori*-dependent DNA replication assay (Figure 2b) [5]. As expected, the replication intermediates produced by extracts from the 46BR.1G1 derivative expressing wild-type DNA ligase I were essentially identical to those produced by a control cell extract (Figure 2b). In contrast, extracts from 46BR.1G1 expressing the mutant DNA ligase I still exhibited the defect in lagging-strand DNA synthesis (Figure 2b). Addition of 21 nanograms of wild-type DNA ligase I to 46BR.1G1 extracts restored the pattern of replication intermediates to that produced by a control extract, whereas addition of an equivalent amount of mutant DNA ligase I had no effect (Figure 2c). When a 10-fold larger amount of purified ligase protein was added to the 46BR.1G1 extract, both wild-type and mutant DNA ligase I corrected the replication defect, indicating that high levels of DNA ligase protein can suppress the requirement for PCNA binding (Figure 2c). This is the first direct evidence that the interaction between DNA ligase I and PCNA coordinates the synthesis and ligation of Okazaki fragments. The possibility remains, however, that recruitment of DNA ligase I to replication foci via PCNA binding [3] is also important for cellular DNA replication.

As 46BR.1G1 cells are hypersensitive to killing by methyl methanesulfonate (MMS) [6], we compared the ability of wild-type and mutant DNA ligase I to correct this phenotype. Expression of wild-type DNA ligase I complemented the sensitivity to DNA damage (Figure 3),

**Figure 3**

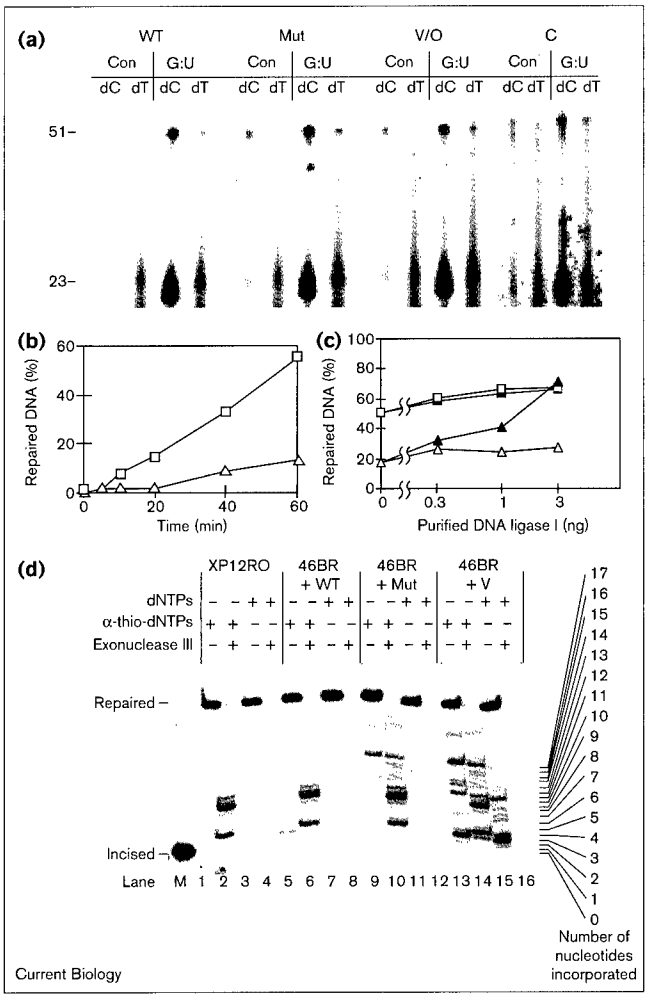



Effect of inactivation of the PCNA-binding site of DNA ligase I on the complementation of the MMS sensitivity of 46BR.1G1 cells. The control cell line GM00847 (open squares) and 46BR.1G1 stably transfected with the empty expression vector (open circles), or WT DNA ligase I cDNA (filled squares), or mutant DNA ligase I cDNA (filled circles) were incubated with MMS (see Supplementary material).

whereas the mutant DNA ligase I had no significant effect (Figure 3). These results show that participation of DNA ligase I in repair of cytotoxic DNA damage induced by MMS is mediated through its interaction with PCNA. It is generally accepted that the major cytotoxic DNA lesion introduced by MMS, 3-methyl adenine, is repaired by BER. Two subpathways of BER can be distinguished on the basis of the length of repair DNA synthesis and the requirement for PCNA [7]; the relative importance of these subpathways for the repair of different base lesions *in vivo* has not been established. As DNA ligase I has been linked with both short-patch BER (one-nucleotide repair) [8] and long-patch BER (repair tract of 2–11 nucleotides) [9], we compared the abilities of 46BR.1G1 extracts to catalyze different BER subpathways. To measure short-patch BER, we used a linear DNA duplex with a single uracil:guanine pair. The linear nature of this substrate makes it refractory to repair by PCNA-dependent BER [10]. In these assays, the 46BR.1G1 extract had essentially the same activity as a control extract both in terms of the amount of ligated product and the patch size, which was predominantly one nucleotide (Figure 4a). Similar results were obtained in assays with extracts from cells expressing either form of DNA ligase I (Figure 4a).

To measure long-patch BER, we used a circular DNA substrate with a single synthetic abasic (AP) site that cannot be repaired by short-patch BER (see Supplementary material). In these assays, the 46BR.1G1 extract generated significantly less repaired product than the control extract (Figure 4b). The addition of purified wild-type DNA ligase I protein to the 46BR.1G1 extract corrected the defect in AP site repair but had no effect on the repair reaction catalyzed by the control cell extract (Figure 4c).

**Figure 4**




Effect of inactivation of the PCNA-binding site of DNA ligase I on short-patch and long-patch BER. **(a)** Extracts (10 μg) from the control cell line XP12RO (C) and from 46BR.1G1 stably transfected with the empty expression vector (V/O), or expressing WT DNA ligase I (WT), or mutant DNA ligase I (Mut), were assayed for short-patch BER activity using a linear oligonucleotide duplex with (G:U) or without (Con) a single uracil residue (see Supplementary material). Repair reactions contained either [α-$^{32}$P]dCTP or [α-$^{32}$P]TTP to detect single or multiple nucleotide incorporation events, respectively. The positions of labeled 23mer reaction intermediates and 51mer ligated products are indicated. **(b)** Extracts (2 μg) from either XP12RO (open squares) or 46BR.1G1 cells (open triangles) were assayed for long-patch BER activity using a labeled circular substrate containing a single synthetic AP site (see Supplementary material). **(c)** Long-patch BER reactions catalyzed by extracts from 46BR.1G1 cells were supplemented with either WT (closed triangles) or mutant (open triangles) purified DNA ligase I. In similar assays, control XP12RO extracts were supplemented with either WT (closed squares) or mutant (open squares) purified DNA ligase I. **(d)** Assays to determine the length of repair DNA synthesis in long-patch BER reactions catalyzed by extracts (5 μg) from control cells (XP12RO, lanes 1–4) and 46BR.1G1 expressing WT DNA ligase I (46BR + WT, lanes 5–8); mutant DNA ligase I (46BR + Mut, lanes 9–12) or containing the empty expression vector (46BR + V, lanes 13–16) (see Supplementary material). The positions of the incised product (lane M) and the repaired product are indicated on the left. DNA repair synthesis events of 0–17 nucleotides are indicated in the right.

In contrast, addition of equal amounts of the mutant form of DNA ligase I had no effect on the efficiency of AP site repair in either 46BR.1G1 or control extract (Figure 4c).

To characterize the defect in long-patch BER, we examined the effect of DNA ligase I deficiency on DNA repair synthesis. Under these reaction conditions (see Supplementary material), the repair reaction catalyzed by the control extract was essentially complete (Figure 4d, lanes 1,2), with most of the repair events having repair synthesis tracts of either two or seven nucleotides (Figure 4d, lane 2). In contrast, incomplete DNA repair events with abnormally long repair synthesis tracts, up to 17 nucleotides, were detected in assays with 46BR.1G1 extracts (Figure 4d, lane 15). This effect was more pronounced in reactions with α-thio-dNTPs (Figure 4d, lanes 13,14). As expected, extracts from 46BR.1G1 cells expressing tagged wild-type DNA ligase I produced the same pattern of repair tracts as the control extract (Figure 4d, lanes 5–8). Extracts from 46BR.1G1 cells expressing mutant DNA ligase I did not show a marked defect in the overall repair reaction (Figure 4d, lane 11). The presence of repair tracts greater than seven nucleotides, however, indicated that the abnormality in DNA repair synthesis was only partially corrected (Figure 4d, lanes 9,10). We conclude that the interaction between PCNA and DNA ligase I has a key role in the coordination of the DNA synthesis and ligation steps that complete long-patch BER. Taken together with the cell survival studies, our observations provide the first strong evidence that long-patch BER is an important DNA repair mechanism *in vivo* and is not functionally redundant with short-patch BER.

Extracts from the cell line EM9, which is deficient in DNA ligase III [11] and also hypersensitive to MMS [12], are defective in short-patch but not long-patch BER [13]. Thus, it appears that short-patch BER is completed by the DNA ligase IIIα–Xrcc1 complex, whereas long-patch BER is completed by DNA ligase I in a PCNA-dependent reaction. The simplest explanation of the alkylation-sensitive phenotypes shown by cell lines deficient in DNA ligase III or I is that these enzymes are involved in functionally distinct BER subpathways that repair different MMS-induced cytotoxic lesions. Alternatively, either DNA ligases I or III might function in DNA repair pathways, other than BER, that repair cytotoxic DNA lesions induced by alkylating agents.

*Supplementary material*

Supplementary material including additional methodological detail and a figure showing the complementation of the temperature-sensitive phenotype of the *E. coli lig* strain is available at http://current-biology.com/supmat/supmatin.htm.

## Acknowledgements

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# Functional link of BRCA1 and ataxia telangiectasia gene product in DNA damage response

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***BRCA1*** **encodes a familial breast cancer suppressor that has a critical role in cellular responses to DNA damage[1,2]. Mouse cells deficient for *Brca1* show genetic instability, defective G2–M checkpoint control and reduced homologous recombination[3,4]. BRCA1 also directly interacts with proteins of the DNA repair**

**machinery[5] and regulates expression of both the *p21* and *GADD45* genes[6–8]. However, it remains unclear how DNA damage signals are transmitted to modulate the repair function of BRCA1. Here we show that the BRCA1-associated protein CtIP[9–12] becomes hyperphosphorylated and dissociated from BRCA1 upon ionizing radiation. This phosphorylation event requires the protein kinase (ATM) that is mutated in the disease ataxia telangiectasia[13]. ATM phosphorylates CtIP at serine residues 664 and 745, and mutation of these sites to alanine abrogates the dissociation of BRCA1 from CtIP, resulting in persistent repression of BRCA1-dependent induction of GADD45 upon ionizing radiation. We conclude that ATM, by phosphorylating CtIP upon ionizing radiation, may modulate BRCA1-mediated regulation of the DNA damage-response *GADD45* gene, thus providing a potential link between ATM deficiency and breast cancer.**

BRCA1 interacts with the transcriptional co-repressor complex of CtIP and CtBP through its BRCT domains, and DNA damage-induced dissociation of BRCA1 from this complex may be important for BRCA1 function[9]. To explore the mechanism regulating this interaction, we examined the phosphorylation of CtIP in T24 cells treated with ionizing radiation (IR), ultraviolet or methylmethane sulphonate (MMS). CtIP immunoprecipitated from extracts of treated cells showed slower migrating forms (Fig. 1a, compare lanes 3–5 with lane 2), which were especially prominent in cells γ-irradiated at the dose of 20–40 Gy (Fig. 1a, lane 3; and Fig. 1b). The alteration in electrophoretic mobility was attributed to phosphorylation, as it was sensitive to λ-phosphatase but inhibited by NaF and $Na_3VO_4$ (Fig. 1c). λ-Phosphatase-treated CtIP migrated faster than the CtIP immunoprecipitated from control cells (Fig. 1c, compare lanes 4 and 2), suggesting that CtIP is a phosphoprotein that becomes hyperphosphorylated after exposure of cells to DNA-damaging agents.

The phosphatidylinositol3-OH kinase [PI(3)K] related family of serine/threonine protein kinases, which includes ATM and DNA-PK (DNA-dependent protein kinase), have been implicated in cell signalling events in response to DNA double-strand breaks[13,14]. It appears that a PI(3)related kinase is involved in the phosphorylation of CtIP, as the IR-induced hyperphosphorylation of CtIP was inhibited in cells treated with the PI(3)K inhibitor, wortmannin (20 μM) (Fig. 1d, lane 4). In two isogenic cell lines, M059J (with deficient DNA-PK activity) and M059K (with normal levels of DNA-PK activity)[15], the IR-induced hyperphosphorylation of CtIP remains unchanged (data not shown); however, the hyperphosphorylated forms of CtIP appeared only in ATM wild-type GM00637G cells, but not in ATM-deficient GM09607A cells after IR (Fig. 1e). Two other ATM-deficient cell lines, GM05849B and GM02052C, showed identical results (Fig. 1f). Similarly, in two isogenic stable clones derived from ATM-deficient fibroblasts (AT22IJE-T) carrying either vector alone (plasmid pEBS7) or Flag-tagged wild-type ATM (plasmid pEBS7-YZ5)[16], CtIP hyperphosphorylation was restored only in cells expressing wild-type ATM (Fig. 1g, bottom panel, compare lanes 2 and 4). The presence of wild-type ATM in these cells was confirmed by western blot analysis (Fig. 1g, top panel). These observations suggest that ATM is responsible for the phosphorylation of CtIP following IR.

ATM has been shown to phosphorylate Ser 15 in the highly conserved amino terminus of p53 with the sequence $_{10}$VEPPLSQE$_{17}$ in response to IR[17,18]. The amino-acid sequence of CtIP has six SQ sites bearing similar sequences surrounding Ser 15 of p53. Two of these serines, 664 and 745, are conserved between human and

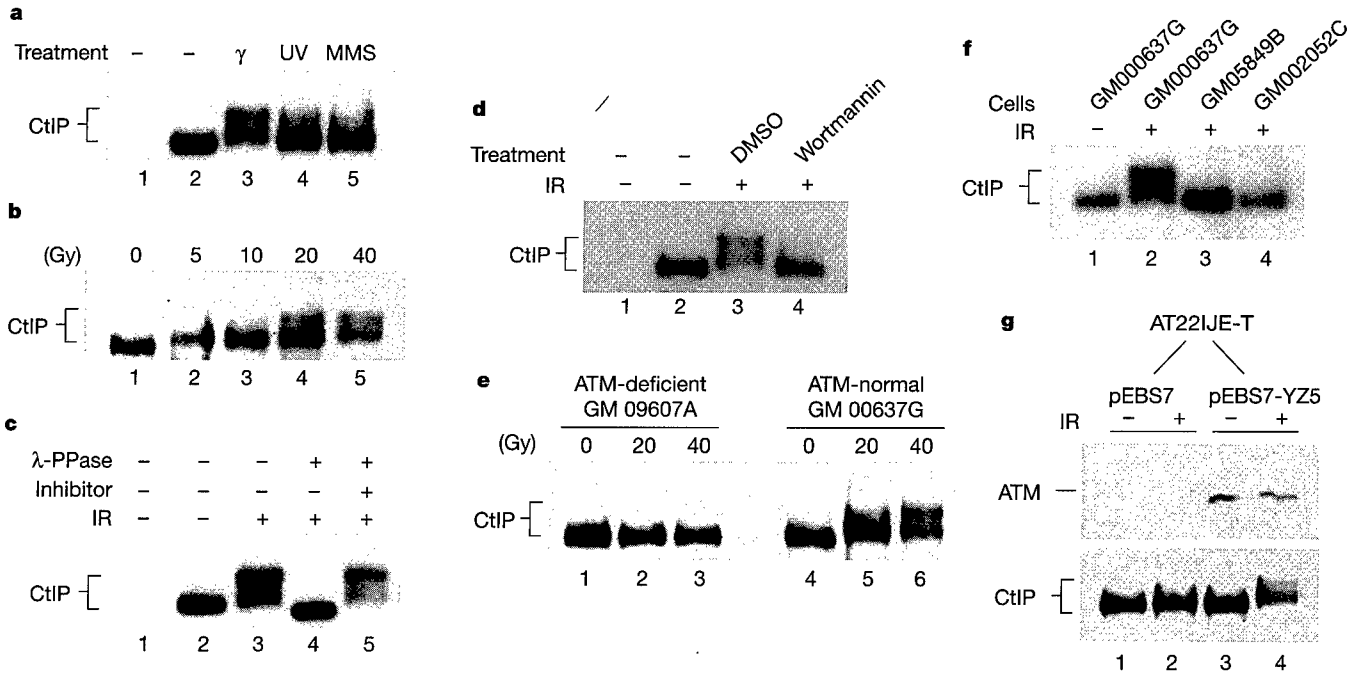


**Figure 1** IR-induced phosphorylation of CtIP is ATM dependent. **a**, Change in CtIP gel mobility upon DNA damage. T24 cells were untreated (–) or treated with IR (γ), ultraviolet (UV) or MMS; extracts were immunoprecipitated using anti-CtIP antibody (lanes 2–5) and immunoblotted for CtIP. **b**, Dose-dependent IR phosphorylation of CtIP. T24 cells treated with IR (5–40 Gy) were immunoblotted for CtIP. **c**, Changes in mobility are due to the phosphorylation of CtIP. CtIP immune complexes from untreated (–) or γ-irradiated (+) T24 cells were untreated or treated with λ-phosphatase ± phosphatase inhibitors, as indicated (lanes 2–5), and immunoblotted with anti-CtIP antibodies. **d**, Inhibition of CtIP phosphorylation by wortmannin. T24 cells were untreated (lanes 1 and 2), or pre-incubated with either dimethyl-sulphoxide (DMSO; lane 3) or 20 μM wortmannin (lane 4) before IR (40 Gy); the extracts were then immunoprecipitated and blotted for CtIP (lanes 2–4). Extracts were immunoprecipitated using pre-immune serum in lane 1 (**a**,**c**,**d**). **e**, Phosphorylation of CtIP upon IR is compromised in ATM-deficient cells. Cell extracts from ATM-deficient (GM09607A, lanes 1–3) or normal human fibroblasts (GM00637G, lanes 4–6) treated with IR for the indicated dosages were immunoblotted for CtIP. **f**, Absence of CtIP hyperphosphorylation in additional ATM-deficient cell lines (GM05849B, lane 3; GM02052C, lane 4) upon IR (40 Gy). **g**, Ectopic expression of wild-type ATM in A-T cells restored the phosphorylation of CtIP upon IR. ATM-deficient AT22IJE-T cells were stably transfected with pEBS7 (vector) or pEBS7-YZ5 (carrying Flag-tagged wild-type ATM cDNA). Extracts from IR-treated cells (40 Gy) were immunoblotted with monoclonal antibodies: 2C1, for ATM (top); C11 for CtIP (bottom).

mouse (Fig. 2a), and we mutated these to alanines. Recombinant glutathione *S*-transferase (GST)–CtIP proteins carrying either the double mutations [GST–CtIP(C-S664/745A)] or no mutation [GST–CtIP(C)] were expressed and isolated for *in vitro* ATM kinase assays[17,18]. Immunoprecipitated Flag-tagged wild-type ATM was capable of phosphorylating recombinant GST–CtIP(C), but not GST–CtIP(C-S664/745A) (Fig. 2b, bottom panel, compare lanes 2 and 5), whereas inactive mutant ATM(D2870A) did not phosphorylate either GST–CtIP(C) or GST–CtIP(C-S664/745A) (Fig. 2b, bottom panel, lanes 3 and 6,). The presence of Flag-tagged ATM and ATM(D2870A) was confirmed by immunoblot analysis (Fig. 2b, top panel). These data indicate that ATM may directly phosphorylate CtIP on Ser 664 and Ser 745 *in vitro*.

To determine whether these residues on CtIP are phosphorylated *in vivo*, we transfected Flag-tagged CtIP(WT), CtIP(S664A) or CtIP(S664/745A) into human osteosarcorma U2OS cells and metabolically labelled these cells with inorganic $^{32}$P-phosphate. After IR, CtIP(WT), CtIP(S664A) or CtIP(S664/745A) were immunopreci-

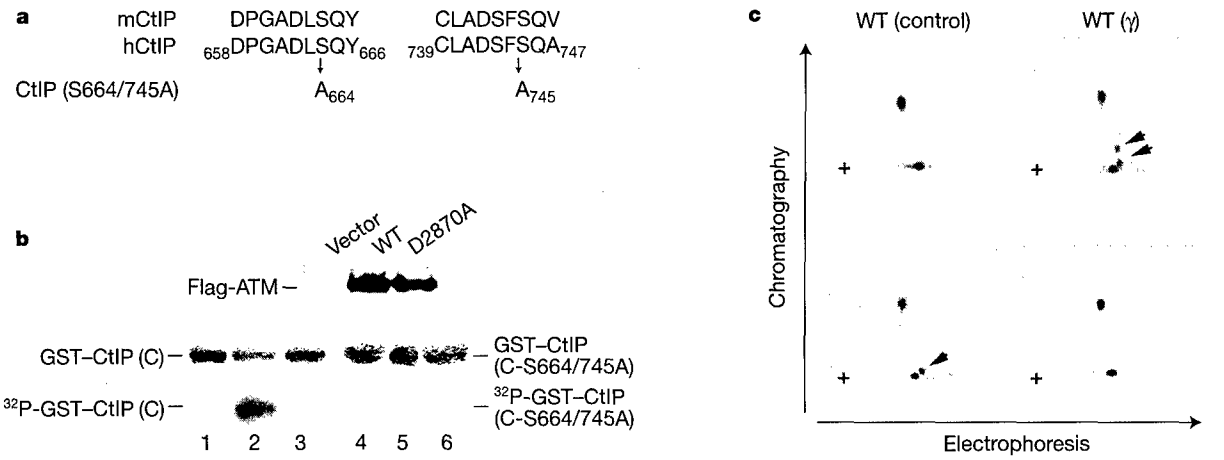


**Figure 2** Identification of ATM phosphorylation sites on CtIP. **a**, Sequences of two potential ATM-phosphorylation sites in CtIP and the alignment between human (hCtIP) and mouse (mCtIP) CtIP. For the CtIP phosphorylation site mutant, CtIP(S664/745A), Ser 664 and Ser 745 were mutated to alanine. **b**, Phosphorylation of CtIP by ATM-associated kinase *in vitro*. 293 cells were transiently transfected with vector alone (lanes 1 and 4), or vectors expressing Flag-tagged wild-type ATM (WT) (lanes 2 and 5) or mutated ATM(D2870A) (lanes 3 and 6). Transfected cell extracts were immunoprecipitated with anti-Flag mAb for *in vitro* kinase assay using GST–CtIP(C) (lanes 1–3) and GST–CtIP(C-S664/745A) (lanes 4–6) as substrates. Top, western blot of ectopically expressed ATM using anti-Flag mAb. Middle, Coomassie blue gel of GST–CtIP(C) and GST–CtIP(C-S664/745A). Bottom, autoradiogram of phosphorylated GST–CtIP(C) and CtIP(C-S664/745A). **c**, Phosphorylation of Ser 664 and Ser 745 of CtIP *in vivo* upon IR. U2OS cells transiently transfected with Flag-tagged CtIP(WT), CtIP(S664A) or CtIP(S664/745A) expression vectors, were metabolically labelled with $^{32}$P, then either untreated or treated with IR (20 Gy). CtIP was isolated for tryptic phosphopeptide mapping analysis[19]. Two new spots appeared for CtIP(WT) upon IR (arrowheads). Mutation of Ser 664 to alanine resulted in the disappearance of one IR-induced spot, whereas mutation of both Ser 664 and Ser 745 to alanine resulted in the loss of both spots on the tryptic phosphopeptide map.

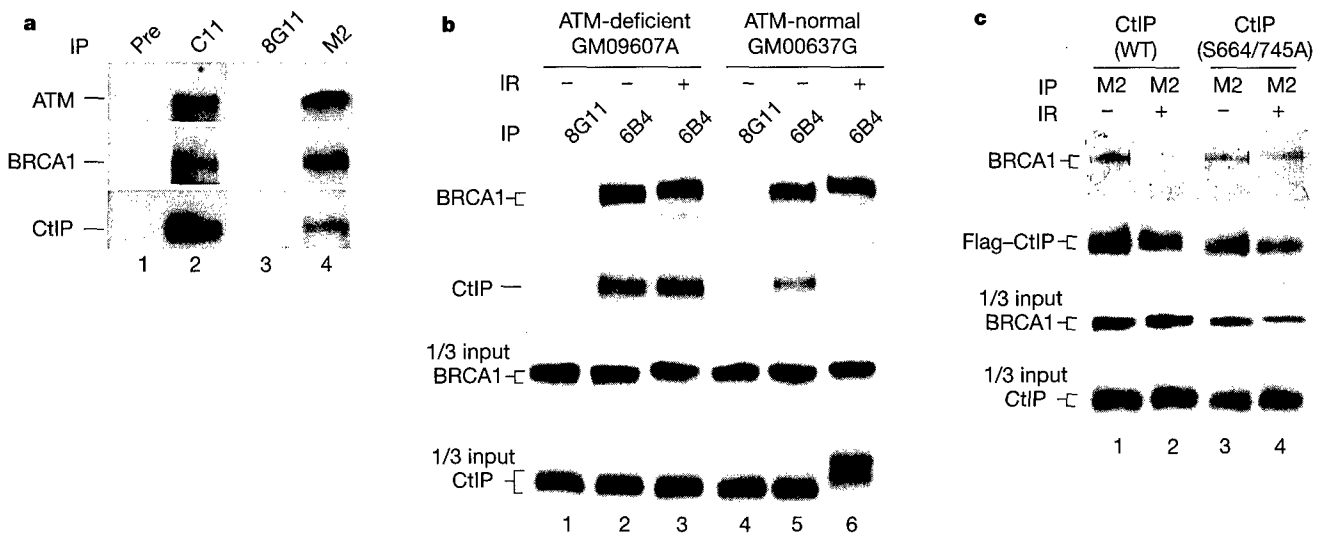


**Figure 3** Phosphorylation of CtIP by ATM is essential for dissociation of CtIP from BRCA1 upon IR. **a**, Formation of ATM–BRCA1–CtIP complex *in vivo*. HCT116 cell lysates were immunoprecipitated using pre-immune serum (lane 1) or anti-CtIP polyclonal antibody, C11 (lane 2), and immunoblotted for ATM, BRCA1 or CtIP. In lanes 3 and 4, HCT116 cells were transiently transfected with Flag-tagged ATM expression construct. The lysates were immunoprecipitated using anti-GST (8G11) monoclonal antibody (mAb) as a control or anti-Flag mAb, and immunoblotted for ATM, BRCA1 or CtIP. **b**, Dissociation of BRCA1 and CtIP is abrogated in ATM-deficient cells upon IR. ATM deficient (GM09607A, lanes 1–3) or normal human fibroblasts (GM00637G, lanes 4–6) were untreated (–) or treated with IR (+, 40 Gy). Extracts were immunoprecipitated using control (8G11) mAb or anti-BRCA1 (6B4) mAb, and immunoblotted for BRCA1 (top) or CtIP (bottom). Total input of BRCA1 and CtIP is also shown. **c**, CtIP phosphorylation mutant fails to dissociate from BRCA1 upon IR. Flag-tagged wild-type (lanes 1 and 2) or mutated (lanes 3 and 4) CtIP were transiently transfected into U2OS cells, and the cells were either untreated (–) or treated with IR (40 Gy). The extracts were immunoprecipitated using anti-Flag mAb (M2). Top, immunoblot for BRCA1; bottom, immunoblot for Flag-tagged CtIP. Total input of BRCA1 and Flag-tagged CtIP is also shown.

pitated with anti-Flag antibody (M2), and subjected to two-dimensional tryptic peptide analysis[19]. Two additional tryptic $^{32}$P-phosphopeptide spots arose from CtIP(WT) immunoprecipitated from γ-irradiated cells compared with non-irradiated cells (Fig. 2c, top panel, arrows); however, both of these two $^{32}$P-phosphopeptide spots were absent in CtIP(S664/745A), and only one of these $^{32}$P-phosphopeptides spots was present in CtIP(S664A) (Fig. 2c, bottom panel). Together, these data indicate that both Ser 664 and Ser 745 on CtIP may be phosphorylated *in vivo* in response to IR.

To explore the biological significance of ATM-dependent phosphorylation of CtIP upon IR, we initially tested whether ATM associates with the CtIP and BRCA1 complex *in vivo* by co-immunoprecipitation. BRCA1, CtIP and ATM were co-immunoprecipitated from the human colon carcinoma cell line HCT116 using an anti-CtIP polyclonal antibody (Fig. 3a, compare lanes 1 and 2). As the ATM-specific antibody, 2C1, was not efficient for immunoprecipitation, HCT116 cells were first transfected with a Flag-tagged wild-type ATM expression vector, and subsequently immunoprecipitated with anti-Flag M2 antibody. BRCA1 and CtIP were both co-immunoprecipitated with Flag-tagged ATM (Fig. 3a, lane 4). These data suggest that ATM is present in the previously identified BRCA1–CtIP complex *in vivo*.

We then proceeded to evaluate the consequence of IR-induced ATM-dependent phosphorylation of CtIP on CtIP–BRCA1 complex formation. In the absence of IR, CtIP associated with BRCA1 in both ATM-deficient (GM09607A) and ATM-normal (GM00637G) cells (Fig. 3b, lanes 2 and 5). Upon IR, CtIP dissociated from BRCA1 in ATM-normal cells, but not in ATM-deficient cells (Fig. 3b, compare lanes 3 and 6). Western blot analysis of the cell lysates clearly indicated that IR-induced hyperphosphorylation of CtIP is absent in ATM-deficient cells (Fig. 3b, '1/3 input' panel, compare lanes 3 and 6). Because ATM also phosphorylates BRCA1 (ref. 20), the persistent association of CtIP and BRCA1 in ATM-deficient cells after IR could be attributed to a deficiency in the phosphorylation of BRCA1, of CtIP, or of both. To discriminate between these alternatives, we performed similar experiments with U2OS cells transiently transfected with Flag-tagged CtIP(WT) or CtIP(S664/745A). BRCA1 associated with both CtIP(WT) or CtIP(S664/745A) in the absence of IR (Fig. 3c, lanes 1 and 3), whereas BRCA1 dissociated from CtIP(WT), but remained associated with CtIP(S664/745A) upon IR (Fig. 3c, compare lanes 2–4). Because U2OS cells contain wild-type ATM, IR-induced ATM-dependent phosphorylation of BRCA1 has no apparent effect on the status of the CtIP–BRCA1 complex (Fig. 3c). It should be noted that the change in electrophoretic mobility of Flag-tagged wild-type CtIP was less prominent after IR, perhaps due to the addition of the Flag epitope. These

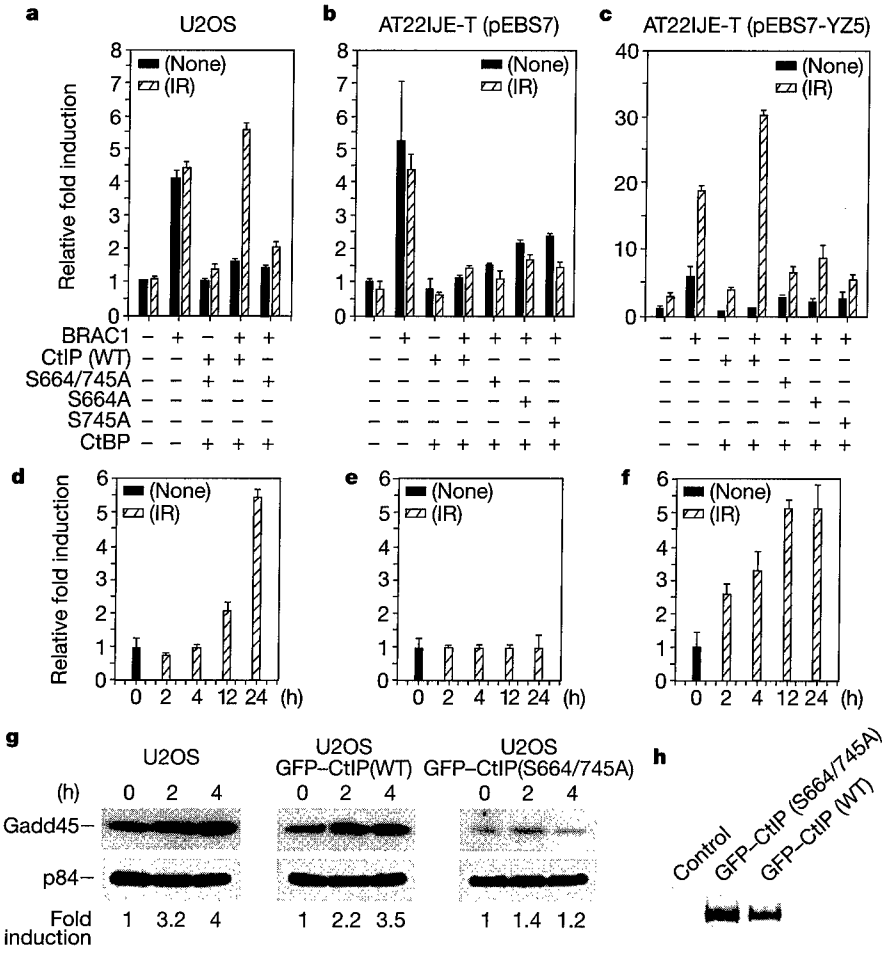


**Figure 4** Regulation of GADD45 expression by BRCA1, CtIP and CtBP. **a**–**c**, U2OS, AT22IJE-T (pEBS7) or AT22IJE-T (pEBS7-YZ5) cells were co-transfected with pI-3 (containing *GADD45* intron 3 driving expression of a luciferase reporter gene), pSV40–β-gal (control plasmid) and the plasmids indicated. The luciferase activity was measured and normalized with β-galactosidase activity. Scale in **c** is different from in **a** and **b**. Results were derived from three independent transfection experiments. **d**–**f**, pI-3 reporter activity in U2OS, AT22IJE-T (pEBS7), or AT22IJE-T (pEBS7-YZ5) cells treated with IR. Cells were co-transfected with pI-3 and pSV40–β-gal plasmid, and untreated (solid bar) or treated (hatched bar) with IR (30 Gy) at 36 h after transfection. Transfected cells were then assayed for luciferase activity at the time points indicated. **g**, IR-induced expression of cellular GADD45 protein. U2OS cells stably expressing either GFP–CtIP (WT) or GFP–CtIP(S664/745A) were collected 2 and 4 h after IR and immunblotted for GADD45 and a nuclear matrix protein p84 as an internal control[9]. Blots were developed using an ECL kit and quantified by an SI Densitometer (Molecular Dynamics). The protein ratio of GADD45:p84 is shown. **h**, Immunoblot analysis of ectopically expressed GFP–CtIP(WT) and GFP–CtIP(S664/745A) in the stable cell clones using anti-GFP antibody.

results recapitulate the behaviour of the CtIP–BRCA1 complex in γ-irradiated ATM-deficient cells (Fig. 3b), and suggest that ATM-dependent phosphorylation of CtIP on Ser 664 and Ser 745 is essential for its dissociation from BRCA1 upon IR.

Dissociation of the CtIP–CtBP co-repressor complex from BRCA1, leading to relief of transcription repression, could represent one of the mechanisms regulating the transcriptional activity of BRCA1 upon DNA damage. To test this hypothesis, we examined the effect of CtIP–CtBP on the transactivation activity of BRCA1 on the *GADD45* regulatory element (intron 3 of *GADD45*) which was induced by BRCA1 (ref. 8). Consistently, expression of BRCA1 activated transcription from the reporter plasmid (pI-3) containing intron 3 (+1,553 to +1,695) of *GADD45* by about fourfold compared with the pcDNA3.1 vector alone in U2OS cells (Fig. 4a). Co-expression of CtIP(WT) or CtIP(S664/745A) and CtBP with BRCA1 repressed this BRCA1-induced activity by about 70–80%. After IR, however, CtIP(WT) no longer repressed the activity of BRCA1, whereas CtIP(S664/745A) retained persistent repression (Fig. 4a). The same persistent repression was observed with both CtIP(WT) and CtIP(S664/745A) in ATM-deficient cells [AT22IJE-T(pEBS7)] after IR (Fig. 4b). Re-introduction of wild-type ATM into these cells [AT22IJE-T(pEBS7-YZ5)] eliminated repression mediated by the CtIP(WT)–CtBP complex after IR, whereas CtIP(S664/745A)–CtBP maintained its repression (Fig. 4c). These results suggest that a defect in the ATM-dependent phosphorylation of CtIP(S664/745A) inhibited the dissociation of CtIP(S664/745A) from BRCA1 upon DNA damage, leading to the continuous repression of transcriptional activity from the intron 3 of *GADD45*. Furthermore, it appears that phosphorylation of both Ser 664 and Ser 745 is required for dissociation, as a single mutant form of CtIP(S664A or S745A) maintained its repressive effect after IR treatment (Fig. 4b, c). It was noted that the overall fold induction of the reporter (pI-3) was higher in the AT22IJE-T (pEBS7-YZ5) cells compared with in the U2OS cells in these experiments. This is because the reporter activity 4 h after IR was induced 2–3-fold in AT22IJE-T (pEBS7-YZ5) cells but very little in U2OS cells (Fig. 4d, f). The transcriptional activity of the reporter construct alone in ATM-deficient cells [AT22IJE-T(pEBS7)] remained constant after IR, which is consistent with previous results showing that induction of *GADD45* in response to IR requires ATM (compare Fig. 4e and f)[21]. Together, these data suggest that phophorylation of CtIP by ATM is critical for releasing BRCA1 from its repressive state. Consistently, overexpression of BRCA1 could titrate the endogenous CtIP–CtBP repressor complexes and thereby liberate BRCA1 from repression (Fig. 4a–c).

The persistent repression exerted by CtIP(S664/745A) suggests that it may act as a dominant-negative inhibitor of BRCA1-mediated transcriptional activity following IR. To test this possibility, we created U2OS cells stably expressing green fluorescent protein (GFP) tagged CtIP(WT) or CtIP(S664/745A), challenged these cells with IR, and assessed endogenous expression of GADD45 by immunoblot analysis. Overexpression of CtIP(S664/745A) but not CtIP(WT) inhibited the induction of GADD45 after IR (Fig. 4g). The expression of GFP-tagged CtIP proteins was confirmed by immunoblot analysis (Fig. 4h). These data further support the notion that phosphorylation of CtIP at Ser 664 and Ser 745 is important for BRCA1-mediated induction of GADD45 in response to DNA damage.

In response to genomic insult, ATM probably transduces the DNA damage signal by phosphorylating downstream effector molecules involved in regulating cell-cycle progression or DNA damage repair. For example, IR-induced ATM-dependent phosphorylation of p53 and hMDM2 contributes to the activation of p53, leading to the induction of p21 and GADD45 (refs 17, 18, 22). BRCA1 has also been demonstrated to induce expression of p21 and GADD45 (refs 6–8). Our study suggests another DNA damage-response pathway in which the signal is transmitted through phosphorylation of CtIP by ATM, leading to dissociation of the CtIP–CtBP repressor complex from BRCA1, which in turn, activate transcription of *GADD45* (Fig. 5). This regulatory mechanism may also explain our previous results concerning repression by CtIP/CtBP on the expression of p21 mediated by BRCA1 (ref. 9). Apparently, BRCA1 and p53 are important in p21 and GADD45 expression in response to IR. It is likely that both p53 and BRCA1 mediate synergistic and parallel pathways to ensure a proper cellular response to DNA damage.

These results provide a link between ATM and BRCA1 through CtIP, which may explain the increased risk for breast cancer in certain populations of ataxia telangiectasia heterozygotes[23,24]. In the absence of functional ATM, the activity of BRCA1 may become disregulated leading, in turn, to a defect in the cellular response to DNA damage, concomitant genomic instability and, ultimately, tumorigenesis. □

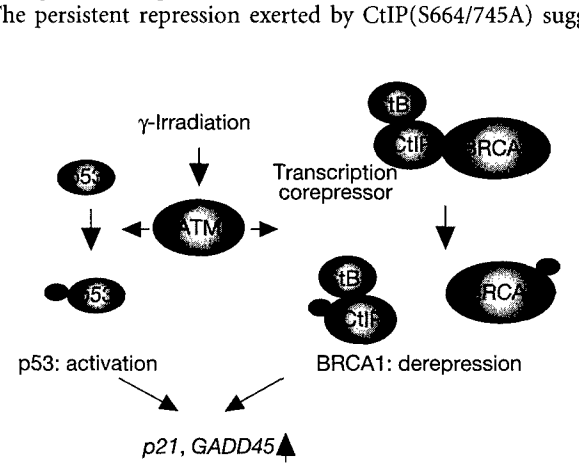


**Figure 5** Model showing how ATM modulates the BRCA1 transcriptional regulation of DNA damage-response genes following IR. In response to IR, ATM kinase becomes activated and phosphorylates CtIP to disrupt the CtIP–CtBP–BRCA1 complex. Consequently, BRCA1 is released and participates in the activation of DNA damage-response genes *p21* and *GADD45*.

## Methods

### Plasmid constructs

The pCNF–CtIP(WT) plasmid that expresses the Flag-tagged CtIP was generated by subcloning Flag-tagged CtIP cDNA into pcDNA3.1 vector (Invitrogen). The pCNF–CtIP(S664/745A) with mutations at Ser 664 and Ser 745 was engineered by site-directed mutagenesis. pCMV–ATM expresses Flag-tagged wild-type ATM, whereas pCMV–ATM(D2870A) expresses mutant ATM with Asp 2,870 changed to alanine by site-directed mutagenesis. GST–CtIP(C) was constructed by inserting the carboxy-terminal fragment of CtIP (amino acids 324–897) into the *Sma*I site of pGEPK3 vector. GFP–CtIP contains the full-length CtIP cDNA downstream of GFP-tagged expression vector[25]. pcDNA–BRCA1, pRcCMV–CtBP and pSV40–β-gal plasmids have been described[9]. The pI-3 plasmid was constructed as described[8].

### Cell treatment, immunoprecipitation and western blot analysis

Cells were incubated in fresh medium for 3 h and treated with different dosages of γ-ray (5–40 Gy), ultraviolet (1 mJ $cm^{-2}$) or 0.01% MMS. The cells were collected 1 h after treatment and lysed in Lysis 250 buffer. Immunoprecipitation and western blots were carried out as described[9]. We used the following antibodies: anti-CtIP mouse polyclonal antibody C11, anti-BRCA1 monoclonal antibody 6B4, anti-GST monoclonal antibody 8G11 (ref. 9), ATM monoclonal antibody 2C1 (GeneTex), anti-Flag monoclonal antibody M2 (Sigma), anti-GADD45 rabbit polyclonal Ab H165 (Santa Cruz Biotechnology) and anti-GFP monoclonal antibody (Clontech).

### λ-Phosphatase treatment

Immune complexes containing CtIP were washed in Lysis 250 buffer in the absence of phosphatase inhibitors. Parallel samples were resuspended in λ-phosphatase buffer (New England Biolabs) either in the presence or absence of phosphatase inhibitors, NaF (50 mM final concentration) and $Na_3VO_4$ (2 mM final concentration). λ-Phosphatase (400 U) was added to each sample followed by incubation at 30 °C for 1 h.

### Transfection and luciferase assay

Plasmid DNA including 10 μg of pCMV (vector), pCMV-ATM or pCMV-ATM(D2870A) was transfected into 293 cells ($2 \times 10^6$ in 10-cm dish) by the calcium phosphate/DNA

co-precipitation method. Transfection into HCT116 cells was performed using lipofectin. For measuring the transactivation activity by luciferase assay, U2OS cells were transfected by calcium phosphate/DNA co-precipitation with 0.5 μg of pI-3 reporter construct, along with 5 μg of pcDNA–BRCA1, 2.5 μg of pRcCMV–CtBP, 2.5 μg of pCNF–CtIP(WT), pCNF–CtIP(S664/745A), pCNF–CtIP(S664A), pCNF–CtIP(S745A), or pcDNA3.1 as the control vector to equalize the amount of the transfected DNA. One microgram of pSV40–β-gal was co-transfected for standardization of transfection efficiency by measurement of β-galactosidase activity. The cells were irradiated with 30 Gy 36 h after transfection, and assayed for luciferase and β-galactosidase activities 4 h after treatment using standard procedures[9].

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# DNA damage-induced cell cycle checkpoints and DNA strand break repair in development and tumorigenesis

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**Several newly identified tumor suppressor genes including *ATM, NBS1, BRCA1* and *BRCA2* are involved in DNA double-strand break repair (DSBR) and DNA damage-induced checkpoint activation. Many of the gene products involved in checkpoint control and DSBR have been studied in great detail in yeast. In addition to evolutionarily conserved proteins such as Chk1 and Chk2, studies in mammalian cells have identified novel proteins such as p53 in executing checkpoint control. DSBR proteins including Mre11, Rad50, Rad51, Rad54, and Ku are present in yeast and in mammals. Many of the tumor suppressor gene products interact with these repair proteins as well as checkpoint regulators, thus providing a biochemical explanation for the pleiotropic phenotypes of mutant cells. This review focuses on the proteins mediating G1/S, S, and G2/M checkpoint control in mammalian cells. In addition, mammalian DSBR proteins and their activities are discussed. An intricate network among DNA damage signal transducers, cell cycle regulators and the DSBR pathways is illustrated. Mouse knockout models for genes involved in these processes have provided valuable insights into their function, establishing genomic instability as a major contributing factor in tumorigenesis.**

**Keywords:** checkpoint; homologous recombination; non-homologous end joining; knockout; tumor suppressor genes

## Introduction

Existing evidence indicates that multiple cellular processes including checkpoint activation, DNA repair, and changes of gene transcription are initiated in response to DNA damage. Studies in budding and fission yeast have identified players involved in these processes. Some of the genes required for DNA damage-induced checkpoints are also required for cell cycle arrest upon replication block. Homologs of these genes have been identified in mammalian cells, and their roles in development and in DNA damage responses are being unraveled. In contrast to yeast, checkpoint pathways are differentially activated by UV, which mainly causes bulky adducts, and ionizing radiation (IR), which causes single-strand and double-strand breaks (DSBs) in mammals. While UV-induced mammalian cellular responses will be described briefly, this review will focus on DSB-induced cellular responses.

DSBs are the most detrimental form of DNA damage because they lead to chromosomal breakage and rearrangement, events that may result in apoptosis or tumorigenesis. Several human syndromes are characterized by chromosome instability and sensitivity to DSB-causative agents (reviewed by Khanna *et al.*, 1998). Ataxia telangiectasia (A-T) and Nijmegen breakage syndrome (NBS) (reviewed by Shiloh, 1997; Rotman and Shiloh, 1998) are among the best characterized. Clinical manifestations of A-T include progressive neurodegeneration, telangiectasia in the face, immune deficiency, gonadal dysgenesis, and cancer predisposition. A-T cells are defective in DNA damage-induced checkpoint control as well as DNA repair. The product of the *ATM* gene (**A-T** mutated) belongs to a protein kinase family whose members include Mec1 and Tel1 of budding yeast, Rad3 of fission yeast, Mei41 of fruit fly, and other mammalian kinases including ATR (ATM- and RAD3-related) and DNA-PKcs. Studies of this gene family have provided insights into the DNA damage-induced cellular responses as well as other cellular processes such as meiosis and telomere maintenance. The clinical and cellular phenotypes of A-T and NBS share significant similarities. The *NBS1* gene encodes a 95 kDa protein (Carney *et al.*, 1998; Varon *et al.*, 1998) that is a component of a stable Mre11/Rad50/NBS1 nuclease complex (Trujillo *et al.*, 1998; Paull and Gellert, 1999). This complex plays a critical role in DNA DSBR (reviewed by Haber, 1998), explaining the hypersensitivity of NBS cells to IR. However, the molecular basis of the checkpoint defect in NBS cells is less clear. The products of the tumor suppressor genes, *BRCA1* and *BRCA2*, are also involved in DSBR. BRCA1 also plays a role in checkpoint regulation.

Some checkpoint pathways, e.g., signaling from ATM to the checkpoint kinase Chk2, appear to be conserved evolutionarily (Matsuoka *et al.*, 1998), whereas others are unique to mammals (Banin *et al.*, 1998; Canman *et al.*, 1998). In this review, we focus on DNA damage-induced cell cycle checkpoints and on the biochemical and genetic studies of DSBR in mammalian cells. Phenotypes of mouse mutants harboring mutations in genes involved in these cellular processes are summarized. Network of interactions among the multifunctional products of genes involved in cellular response to DSBs is emerging.

### *Checkpoint controls in eukaryotic cells*

Checkpoints serve to monitor the order of events in the cell cycle and ensure that a cell cycle event occurs only

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after the completion of a prior event (Weinert and Hartwell, 1989). DNA damage checkpoint is one such example that is activated upon various kinds of external or internal stimuli that induce DNA damage, either programmed or accidental, and thus helps integrate DNA repair with cell cycle progression (Hartwell and Kastan, 1994). Such mechanisms are important for proper development as well as for prevention of genomic instability and cancer. Programmed breaks in DNA occur during development, e.g., meiotic recombination or during immunoglobulin gene rearrangements. Accidental DNA damage may be produced by several different ways: (i) exogenous DNA damaging agents that may be physical (UV, IR, etc.), or chemical e.g., methyl-methane-sulfonate (MMS), cisplatin, and neocarzinostatin (NCS); (ii) biological (certain viral infections) agents; (iii) endogenous DNA damaging agents such as reactive oxygen species. Such damages need to be repaired before the DNA is segregated into daughter cells to ensure faithful propagation of the genome. In keeping with the definition of checkpoints as being surveillance mechanisms acting between cell cycle phase transitions, DNA damage checkpoints may be divided into G1/S, S, and G2/M.

*The G1/S checkpoint* This ensures that damaged DNA is not replicated and is one of the better-understood DNA damage checkpoints in mammalian cells. The tumor suppressor *p53*, one of the most commonly mutated genes in cancer, plays an important role in DNA damage induced G1/S arrest and apoptosis. The p53 gene product, a transcription activator, up-regulates the expression of genes such as p21, MDM2, and GADD45 (a growth arrest and DNA damage responsive gene) through the p53-binding elements in the promoter regions. The CDK inhibitor p21 can bind several cyclin-CDK complexes *in vitro* and may mediate the p53-dependent G1/S checkpoint (Figure 1) (Dulic *et al*., 1994; Kuerbitz *et al*., 1992; Reed *et al*., 1994). Normally, the cellular level of p53 protein is low due to its relatively short half-life. The stability of p53 is enhanced in cells exposed to different DNA damage agents, including IR and UV (reviewed by Levine, 1997). Phosphorylation on serine-15 of p53 partially inhibits its interaction with MDM2, a protein that targets p53 for ubiquitin-dependent degradation (Shieh *et al*., 1997). ATM interaction with dsDNA is enhanced upon IR *in vitro* (Suzuki *et al*., 1999). ATM might be recruited to sites of DSBs *in vivo* and thus regulates the activity of downstream effectors such as p53. Both ATM and ATR proteins phosphorylate p53 on serine-15 (Banin *et al*., 1998; Canman *et al*., 1998; Tibbetts *et al*., 1999). ATM phosphorylates p53 on serine-15 when DNA strand breaks are induced by agents such as IR (Canman *et al*., 1998), while ATR may be more critical for phosphorylation of p53 upon UV damage. Expression of kinase inactive ATR ($ATR^{ki}$) in human fibroblasts interfered with late phase phosphorylation of p53 upon IR (Tibbetts *et al*., 1999) suggesting that the delayed phosphorylation of p53 observed in A-T cells (Kastan *et al*., 1992) may be mediated by ATR. IR also induces dephosphorylation of serine-376 in p53 in an ATM-dependent manner (Waterman *et al*., 1998). The identity of the ATM-dependent phosphatase that dephosphorylates serine-376 of p53 is unknown. This dephosphorylation event results in the creation of a binding site for 14-3-3 protein and in turn, an increased affinity for specific DNA sequences. Consistent with ATM-dependent activation of p53, neither dephosphorylation nor interaction with 14-3-3 protein is evident in A-T cells (Waterman *et al*., 1998). Thus both phosphorylation and dephosphorylation of specific sites in p53 may play a vital role in checkpoint activation.

Recently, a new candidate tumor suppressor, p33, which co-precipitates with p53, was identified (Garkavtsev *et al*., 1998). Overexpression of p33 causes G1 arrest or apoptosis and functional p33 is required for p53 mediated p21 induction in response to IR. Two novel members of the p53 protein family, p63 and p73, were recently cloned (reviewed by Kaelin, 1999). Although their exact role as tumor suppressors or checkpoint proteins is unknown, p73 is phosphorylated by a non-receptor tyrosine kinase c-Abl that is activated upon DNA damage (Gong *et al*., 1999; Yuan *et al*., 1999b). Both p63 and p73 share the DNA binding and transactivation domains of p53 and can activate p53 target genes in a transient transfection assay (Lee and La Thangue, 1999; Shimada *et al*., 1999). Like p53, p73 upregulates MDM2 expression, but unlike p53, it is not targeted by MDM2 for ubiquitin-dependent degradation. Instead, MDM2 negatively regulates p73 transactivator function by disrupting its interaction with p300/CBP, a component of the eukaryotic transcription complex (Zeng *et al*., 1999).

ATM when activated phosphorylates and activates c-Abl kinase which in turn can activate p73 and potentially result in the transactivation of p21 and GADD45 (Lee and La Thangue, 1999). Together, ATM and c-Abl may mediate a checkpoint function

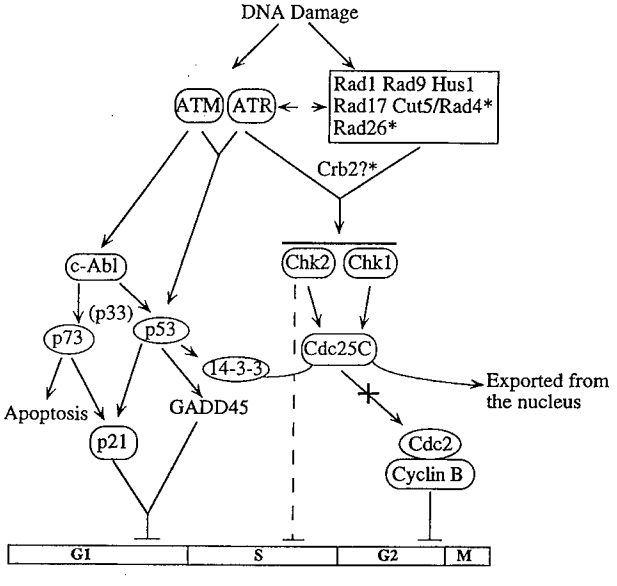


**Figure 1** Signal transduction pathways regulating UV and IR induced checkpoints in mammalian cells. A schematic representation of some of the known DNA damage checkpoint pathways in mammalian cells. Some information is based on the evidence from *S. pombe* system and where mammalian homologs have not yet been identified, they have been marked by an asterisk (*). Where precise biochemical evidence is lacking but genetic evidence is available, a broken line (- - -) has been used instead of a continuous line (-)

through p73 but it is not known if p73-negative cells have checkpoint defects. Thus ATM may mediate G1/S checkpoint through a direct phosphorylation of p53 or through phosphorylation of c-Abl which in turn can upregulate p21 through p73 activation (Figure 1). ATR may be the primary p53 kinase in response to UV damage and only a secondary kinase for p53 in response to IR. DNA-PKcs, another DNA dependent kinase related to ATM and ATR, does not appear to play an important role in the G1/S checkpoint since cells that lack DNA-PKcs have normal DNA damage checkpoints (Burma *et al.*, 1999).

*The S phase checkpoint* When DNA is damaged during early part of the DNA synthesis phase (S phase), the S phase DNA damage checkpoint (SDDC) is activated (Larner *et al.*, 1997). Although the exact mechanisms are poorly understood, several studies have demonstrated a down-regulation of DNA replication in response to radiation damage (Painter, 1986). The lack of functional ATM renders this checkpoint defective causing radio-resistant DNA synthesis (RDS), a hallmark of A-T cells (Painter and Young, 1980). Using a defined chromosomal replicon system, Larner *et al.*, (1994) demonstrated that down regulation of DNA synthesis is mediated through a block in firing of replication origins.

DNA replication in eukaryotic cells initiates from many replication origins that fire throughout the S phase in a defined manner. In A-T cells, initiation of mid or late firing of DNA origins is not inhibited upon IR (Larner *et al.*, 1999). Although most of the proteins involved are conserved through evolution, a molecular understanding of the SDDC regulation in the mammalian system is far from clear. Studies in yeast have demonstrated that Mec1 and Rad53 checkpoint proteins are required for firing of late origins suggesting that SDDC checkpoint may be mediated through a block in firing of late origins (Santocanale and Diffley, 1998). Indeed, when *S. cerevisiae* are treated with MMS, DNA replication delay is associated with a selective block to firing of late origins that can be relieved by mutation of Rad53 (Shirahige *et al.*, 1998). In *S. pombe*, Cds1 (a Rad53 ortholog) mediates the S phase checkpoint in response to DNA damage (Lindsay *et al.*, 1998).

Hydroxyurea, a ribonucleotide reductase inhibitor, which blocks the progression of replication forks from early origins also inhibits firing of late origins. A block in DNA replication activates a replication checkpoint, which may be different from SDDC. In *S. pombe*, the replication checkpoint is mediated through Cds1 and Chk1 (Boddy *et al.*, 1998; Zeng *et al.*, 1998). A human homolog of Cds1 (termed Chk2 or HsCds1) was recently cloned (Brown *et al.*, 1999; Matsuoka *et al.*, 1998). Chk2 (HsCds1) is phosphorylated upon DNA damage as well as when DNA synthesis is blocked. Only the former is dependent on ATM suggesting other kinases may activate Chk2 when replication is blocked thereby activating the replication checkpoint. In mammalian cells, whether Chk1 also plays a role in the replication checkpoint remains to be addressed.

*The G2/M checkpoint* This checkpoint is operational in late G2 phase and presumably allows for repair of DNA that was damaged in late S or in the G2 phases of cell cycle prior to mitosis. Thus, the G2 checkpoint functions to prevent damaged DNA being segregated into daughter cells. This checkpoint depends on the inhibition of Cdc2 kinase activity (Rhind *et al.*, 1997; Yu *et al.*, 1998). Chk1 and Chk2 phosphorylate Cdc25C, a dual specificity phosphatase required for removal of the inhibiting phosphorylation of tyrosine-15 of Cdc2, *in vitro* (Matsuoka *et al.*, 1998; Sanchez *et al.*, 1997). Since mammalian cells lacking Chk1 and Chk2 are unavailable at the present time, the relative contribution of each protein to the G2/M checkpoint is unclear (Figure 1). In *S. pombe* Chk1 and not Cds1 is essential for the G2/M checkpoint (Brondello *et al.*, 1999). Human Chk2 is activated in an ATM-dependent manner in the presence of DSBs (Brown *et al.*, 1999; Chaturvedi *et al.*, 1999; Matsuoka *et al.*, 1998). In response to IR, human Chk1 is also phosphorylated and activated in an NBS1- and ATM-dependent manner (Dasika *et al.*, in preparation). UV-induced activation of Chk1 does not require ATM or NBS1 but expression of dominant-negative ATR abrogates Chk1 phosphorylation suggesting ATR may be upstream of Chk1 in the UV response pathway (Dasika *et al.*, in preparation). Phosphorylation of Cdc25C on serine-216 by Chk1 or Chk2 creates a binding site for 14-3-3 proteins and results in export to and retention in the cytoplasm (Lopez-Girona *et al.*, 1999; Peng *et al.*, 1997). Nuclear Cdc2 remains phosphorylated in the absence of Cdc25C and the cells remain arrested in the G2 phase.

Recent evidence suggests that p53 may also play a role, albeit a redundant one, in the G2/M checkpoint (Passalaris *et al.*, 1999). Activation of p53 in response to DNA damage results in induction of GADD45 (Zhan *et al.*, 1994, 1996). GADD45 can destabilize Cdc2/cyclin B complexes *in vitro* suggesting that it may mediate Cdc2/cyclin inactivation *in vivo* (Zhan *et al.*, 1999). Alternatively or in addition, p53-dependent transcriptional repression of cdc2 and cyclin B promoters may contribute to the G2/M checkpoint (Passalaris *et al.*, 1999).

*Other upstream activators of checkpoint control* *S. cerevisiae* mutants incapable of arresting cell cycle progression in the S/G2 phase when subjected to radiation (rad mutants) were isolated which proved to be very useful genetic tools to study checkpoint mechanisms (Weinert and Hartwell, 1989, 1993). Similarly, in *S. Pombe* at least six 'rad' genes (Rad1, Rad3, Rad9, Rad17, Rad26 and Hus1) are required for the S and G2/M checkpoints in response to DNA damage (reviewed by Rhind and Russell, 1998). Recently, human homologs of Rad1, Rad9, Rad17 and Hus1 have been cloned (Bao *et al.*, 1999; St. Onge *et al.*, 1999; Volkmer and Karnitz, 1999). Since individual gene knockouts are not available, their exact requirement in checkpoint regulation of mammalian cells is unknown (Figure 1). However, human Rad9 is phosphorylated upon DNA damage and physically associates with Rad1 and Hus1 but not Rad17 (St. Onge *et al.*, 1999; Volkmer and Karnitz, 1999). Since ATM (a Rad3-like protein) binding to dsDNA is enhanced upon IR *in vitro* (Suzuki *et al.*, 1999), and since Rad9 phosphorylation is presumably normal in A-T cells (Volkmer and Karnitz, 1999),

whether ATM and/or Rad1/Rad9/Hus1 complex serve(s) as the damage recognition complex is still unclear.

A BRCT (BRCA1 carboxyl terminus) motif containing yeast protein designated Crb2/Rhp9 was found to be phosphorylated in response to UV damage with similar kinetics as Chk1 (Saka *et al.*, 1997). Crb2 phosphorylation was dependent on the *rad* genes but not *Chk1* suggesting that Crb2 may also be upstream of Chk1 (Saka *et al.*, 1997). Recent evidence suggests that Crb2 is required, in addition to Cds1, for the S and G2/M checkpoints (Grenon *et al.*, 1999). The identity of a human Crb2 homolog, if there is one, that may play an analogous role is currently unknown, although several BRCT motif containing candidates have been identified in mammals (Callebaut and Mornon, 1997). Fission yeast Cut5 (cell untimely torn mutant 5) is an essential component of replication checkpoint (Saka *et al.*, 1994). Like Crb2, Cut5 interacts with Chk1 in a yeast two-hybrid system (Saka *et al.*, 1997). It was recently found to be essential for the G2 checkpoint regardless of the type of DNA damage (Verkade and O'Connell, 1998).

There is much to be learnt about the upstream activators of checkpoint response in mammalian cells. Since the human homologs of Rad genes have been cloned only recently, the coming years should be very fruitful in understanding the molecular basis of the checkpoint response to DNA damage in mammalian cells. Checkpoint pathways are likely to be required not only upon DNA damage but also during normal cellular proliferation to safeguard genomic stability. Therefore, mutation of checkpoint genes is likely to result in severe phenotypes.

### *DNA double-strand break repair in eukaryotes*

Most of our knowledge on DSBR comes from studies in yeast. At least two distinct pathways have evolved for DSBR in eukaryotic cells: homologous recombinational (HR) repair and non-homologous end joining (NHEJ) (for review, see Nickoloff and Hoekstra, 1998; Paques and Haber, 1999; Petukhova *et al.*, 1999; Rathmell and Chu, 1998). HR is an error-free pathway wherein an intact template of DNA in the sister chromatid or homolog is used to repair damaged DNA. In contrast, NHEJ is an error-prone pathway that joins DNA ends with no sequence homology, although short homologous sequences ($<10$ bp) termed microhomology are frequently found at the junction. NHEJ often results in deletions or small insertions. Many subpathways have been identified in budding yeast (Paques and Haber, 1999) and the biochemical activities of many of the DSBR proteins have been characterized (reviewed by Petukhova *et al.*, 1999; Smith and Jackson, 1999). We will summarize the recent progress on DSBR in mammals and discuss similarities and differences with that of yeast.

*The Rad50/Mre11/NBS1 complex* Genetic and biochemical observations indicate that DSBs are processed by nucleases. After the introduction of a DSB, the nucleolytic processing yields a 3′-single-stranded (ss) overhang of a few hundred bases (see Figure 2; Cao *et al.*, 1990; Sun *et al.*, 1991). In *S. cerevisiae*, *mre11/rad50/xrs2* mutants are defective in the processing of DSB (Taccioli *et al.*, 1994). Deletion of each gene results in hypersensitivity to IR and MMS and defects in both NHEJ and meiotic homologous recombination. Genetic analysis has established the roles of the Mre11/Rad50/Xrs2 complex in both HR and NHEJ pathways (Boulton and Jackson, 1998). In addition to DSBR, the products of these genes are involved in other cellular processes including chromatin structure and telomere maintenance and the generation of Spo11-mediated DSB in meiosis (for review, see Haber, 1998; and references therein). Using a mutator assay, Chen and Kolodner have demonstrated that Mre11/Rad50/Xrs2 suppresses gross chromosomal rearrangements that presumably occur by non-homology-mediated re-arrangement, whereas a eukaryotic ssDNA binding protein, replication protein A (RPA) suppresses microhomology-mediated rearrangements (Chen and Kolodner, 1999). Earlier studies demonstrated that *rad50* and *xrs2* haploid cells in G2 are more sensitive to IR than *rad50* or *xrs2* diploid cells in G1 (Ivanov *et al.*, 1992), indicating these proteins play an important role in sister-chromatid interactions.

Rad50 and Mre11 proteins share sequence similarity with *E. coli* SbcC and SbcD, respectively, proteins that form a complex with nuclease activity (Connelly and Leach, 1996; Sharples and Leach, 1995). Rad50, like SbcC, is a coiled-coil protein with ATP binding motifs and shares sequence similarity with the structural maintenance of chromosome (SMC) family of proteins. This protein family is implicated in chromosome condensation and segregation, transcriptional repression and recombination (Connelly *et al.*, 1998). Mre11, like SbcD, belongs to a family of phosphoesterases (Sharples and Leach, 1995). In view of the sequence similarity with SbcD, and since DSB processing yields 3′-single strand overhang *in vivo*, it was expected that Mre11 would possess 5′-to-3′ nuclease activities. Surprisingly, purified eukaryotic Mre11 possesses $Mn^{2+}$-dependent 3′-to-5′ dsDNA exonuclease, ssDNA endonuclease and, in yeast, 3′-to-5′ ssDNA exonuclease activities as well (Haber, 1998; Moreau *et al.*, 1999). One mechanism by which Mre11 complex could potentially affect 5′- to 3′-resection of DSB ends is through endonuclease cleavage of ssDNA unwound by an associated helicase, similar to how RecBCD protein complex processes DSBs in *E. coli* (Paques and Haber, 1999; Petukhova *et al.*, 1999). The specific involvement of a helicase in DSB processing reaction remains to be addressed.

Studies of *mre11* mutants indicate that this protein (and presumably the other members of the complex) has both enzymatic and structural functions. Nuclease-deficient *mre11* mutant is not as sensitive to IR as the null mutants but has normal telomere length, suggesting that the Mre11 nuclease activities are not required for chromosome end protection and may not be as crucial in mitosis as in meiosis (Moreau *et al.*, 1999).

A protein complex consisting of Mre11/Rad50/NBS1 is found in human cells that is likely to be the functional homolog of yeast Mre11/Rad50/Xrs2 complex (Carney *et al.*, 1998; Dolganov *et al.*, 1996; Petrini *et al.*, 1995; Trujillo *et al.*, 1998). Unlike Mre11 or Rad50, there is only very limited sequence similarity between yeast Xrs2 and human NBS1. The most

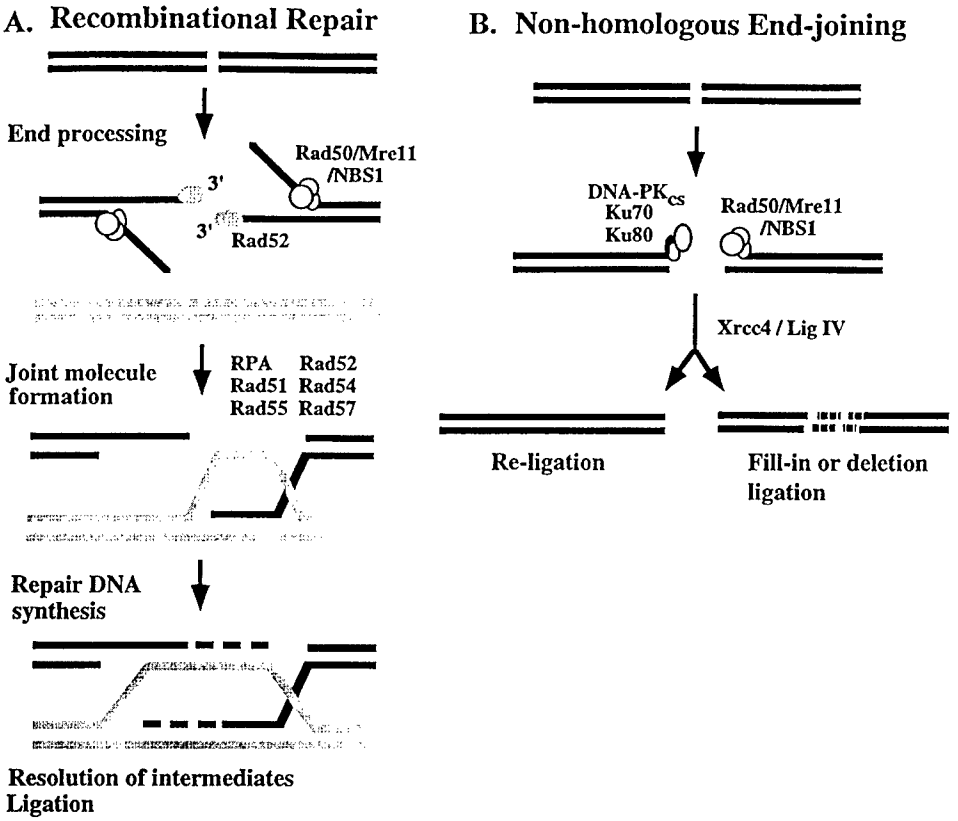


**Figure 2** Model for the double-strand break repair in mammals. (a) Homologous recombinational repair pathway. A double-strand break (DSB) is initially processed by the Mre11/Rad50/NBS1 nuclease complex yielding 3′ single-strand overhang. Rad52 protects DNA ends and also facilitate the formation of heteroduplex DNA which requires Rad51 and its associated proteins. Intact DNA from the sister chromatid or homologous chromosome (shown in grey lines) is used as a template to replace the genetic information lost during the nucleolytic process. Following the nucleolytic process, Holliday junction, branch migration, nuclease resolution of the junction and ligation of the DNA complete the recombinational repair (b) Non-homologous end-joining pathway. The Ku heterodimer binds to DNA ends and recruits DNA-PKcs. Mre11/Rad50/NBS1 have enzymatic and/or structural roles on the DNA. The XRCC4/ligase IV complex is required for the joining of DNA ends. The DNA DSB may be repaired accurately or inaccurately. In the latter case, the ends are processed resulting in the loss or addition of nucleotides and is frequently observed in mammalian cells. Other factors that may be required for this process remain to be identified

notable feature of NBS1 is a forkhead-associated (FHA) domain and a BRCT domain in its amino terminus, both of which have been implicated in protein-protein interactions (Varon *et al.*, 1998). The exonuclease activity of Mre11 is enhanced by Rad50 (Paull and Gellert, 1998). Furthermore, the Mre11/Rad50/NBS1 complex exhibits several activities not seen in the absence of NBS1 including the unwinding of DNA duplex and efficient cleavage of fully paired hairpins (Paull and Gellert, 1999). Recent studies demonstrate that many DSBR proteins are required for V(D)J recombination (see below). Thus these studies provide a molecular explanation of the DSB sensitivity of NBS cells and the defective V(D)J recombination observed in NBS patients. More studies are needed to explain how NBS1 modulates the activities of Mre11.

A DNA damage response results in the re-localization of repair proteins that can be visualized using an immunohistochemical assay of Mre11/Rad50/NBS1. Upon IR treatment, Rad50, Mre11 and NBS1 co-localize to the sites of DNA damage, forming discrete IR-induced immunofluorescent foci (IRIF) early in DNA-damage response and remain associated with DSBs until the repair is complete (Maser *et al.*, 1997; Nelms *et al.*, 1998). Interestingly, hMre11-hRad50 IRIF do not form in cells harboring a mutation in *NBS1*, *ATM*, or *BRCA1* genes (see below), highlighting the complex regulation of such DNA damage response in mammalian cells.

The 3′ ssDNA tails resulted from nucleolytic processing of DSBs described above mediate search for a DNA homolog when bound by recombination proteins. Invasion of the homologous DNA results in the formation of heteroduplex DNA which extends its length by DNA strand exchange (Petukhova *et al.*, 1999). These processes are coupled with DNA synthesis to replace the genetic information eliminated during nucleolytic processing of the DSB. Subsequently, Holliday junctions are formed and heteroduplex regions are extended by branch migration of the Holliday junction. Finally, resolution of the Holliday junctions yields mature recombinants with or without crossover in relation to the flanking markers (see Figure 2).

*Rad51-dependent homologous recombination* In *S. cerevisiae*, HR is the predominant pathway for the repair of DSBs. This repair pathway involves the members of the *RAD52* epistasis group, which include *RAD50, RAD51, RAD52, RAD54, RAD55, RAD57, RAD59, MRE11* and *XRS2*. These genes can be divided into three subgroups. *RAD52* is the gene required for all HR pathways and thus *rad52* mutants have the most severe phenotype among all mutants of

the *RAD52* epistasis group (Paques and Haber, 1999). *rad51, rad54, rad55* and *rad57* mutants share common phenotypes and belong to one subgroup. As described above, *mre11, rad50* and *xrs2* mutants share similar phenotypes and these genes form the third subgroup. Biochemical studies have provided a framework for the HR pathways mediated by these proteins.

Eukaryotic Rad51 protein, a homologue of *Escherichia coli* RecA protein, plays a central role in HR and recombinational repair. Mutations in *RAD51* gene result in IR-sensitivity, defects in meiosis and accumulation of DSBs. Similar to *E. coli* RecA, *S. cerevisiae* Rad51 forms nucleoprotein filaments on DNA and promotes homologous pairing and strand exchange *in vitro*. Several groups showed recently that heteroduplex DNA formation mediated by Rad51 is stimulated by RPA (reviewed by Baumann and West, 1998b). However, extensive strand exchange was not observed under standard conditions, indicating the requirement of additional proteins. Indeed, Rad51 nucleoprotein filament assembly is enhanced by Rad52 and Rad55/Rad57 in the presence of RPA (Benson *et al.*, 1998; New *et al.*, 1998; Shinohara *et al.*, 1998; Sung, 1997).

Although yeast *rad51* mutants are viable, knockout of the mouse *Rad51* gene leads to embryonic lethality (Lim and Hasty, 1996; Tsuzuki *et al.*, 1996). Using a tetracyclin-regulated promoter to control the expression of Rad51, Sonoda *et al.* (1998) demonstrated that the absence of Rad51 leads to extensive chromosome breakage, cell cycle arrest (mainly G2/M) and cell death. Furthermore, sister chromatid exchange frequency is greatly reduced in cells lacking Rad51, suggesting a potential role for HR in sister chromatid exchange (Sonoda *et al.*, 1999). The identification of novel Rad51 interaction proteins (see below) as well as the discoveries of multiple cellular processes involving Rad51 will provide a better explanation for the lethal phenotype caused by Rad51 inactivation in mammals.

Both genetic and biochemical data indicate that Rad52 interacts with the Rad51 recombinase (Baumann and West, 1998b). Purified Rad52 protein binds both single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA). *In vitro*, Rad52 enhances Rad51-mediated strand exchange by alleviating the inhibitory effects of RPA (New *et al.*, 1998; Shinohara *et al.*, 1998; Sung, 1997). Consistent with this observation, Rad52 interacts with the 34 kDa subunit of RPA (Shinohara *et al.*, 1998). Interaction of these repair proteins *in vivo* is supported by the observation that Rad52, RPA, Rad55, Rad57 and Rad51 co-localize to the sites of DSB during meiotic recombination (Gasior *et al.*, 1998). Recently, human Rad52 was shown to bind directly to DSBs and, like Ku (see below), protect DNA from exonuclease attack and promote end-to-end association through intermolecular interactions between hRad52 (Van Dyck *et al.*, 1999). Since Ku binds to DSB and mediates the NHEJ pathway, it is proposed that Rad52 plays a similar role but directs the protein bound DNA to the homologous recombination pathway. If this is the case, Rad52 may act immediately following the completion of nucleolytic processing by Mre11/Rad50/Xrs2. In support of this view, it has been demonstrated that murine Rad52 co-localizes with Rad50 IRIF upon DNA damage (Liu *et al.*, 1999). Surprisingly, while both biochemical and cellular studies suggest an important role for Rad52 in HR in yeast, knockout of Rad52 results in a subtle phenotype in the mouse. This raises the possibility that either mammalian Rad52 function may not be similar to that of ScRad52 or that there may be yet to be identified homologs of RAD52 in the mammalian genome.

Rad54 is a member of the SWI2/SNF2 subfamily of ATPase (Eisen *et al.*, 1995). The ATPase activity of Rad54 is completely dependent on dsDNA. Rad51 was shown to interact with Rad54 and this interaction is functionally important for HR (Clever *et al.*, 1997; Jiang *et al.*, 1996). *In vitro*, Rad54 promotes homologous DNA pairing at the expense of ATP hydrolysis. Purified human Rad54 protein unwinds dsDNA upon ATP hydrolysis (Petukhova *et al.*, 1998), thereby promoting the formation and/or stabilization of hRad51-mediated joint molecules (Tan *et al.*, 1999). The important role of Rad54 in HR and sister chromatid exchange has been demonstrated by gene inactivation in both mouse and chicken cells (Essers *et al.*, 1997; Sonoda *et al.*, 1999).

Studies of recombinational proteins have so far indicated that Rad52 and Rad55/Rad57 heterodimer act before joint molecules are formed (Baumann and West, 1998b) whereas Rad54 is required at a later step of HR (Tan *et al.*, 1999). In mammals, additional proteins are required to facilitate the error-free repair process (see below).

*Non-homologous end joining* NHEJ pathway re-joins DNA ends either in a homology-independent or a microhomology-dependent manner. Similar to HR, many players of the NHEJ are highly conserved through evolution. In yeast, ScKu70 and ScKu80 are important for NHEJ and telomere maintenance. However, the Ku70 and Ku80 phenotypes are obvious only in the *rad52* mutant background because HR is the dominant mechanism for DSBR in budding yeast (Siede *et al.*, 1996).

Both HR and Ku-mediated NHEJ have been shown in *Xenopus laevis* (Labhart, 1999). Recent studies demonstrated that NHEJ activities can be re-constituted *in vitro* with two separate chromatographic fractions (Baumann and West, 1998a). Joining is dependent on ligase IV, XRCC4, Ku and DNA-PKcs (Baumann and West, 1998a). The identification of important genes for NHEJ using human, mouse and hamster mutant cells defective in DSBR or V(D)J recombination, together with biochemical studies have led to a better understanding of NHEJ.

*The Ku autoantigen* Ku was first identified as an autoimmune antigen in scleroderma-polymyositis and other autoimmune patients. Ku is a stable heterodimer, consisting of 70 and 86 kDa subunits (Smith and Jackson, 1999). In *S. cerevisiae*, loss of Ku function shortened the telomere length (Boulton and Jackson, 1998) and altered the expression of telomere-located genes, indicating disruption of telomeric chromatin in its absence.

Ku binds to DNA avidly in a sequence-independent fashion. It binds tightly to ds DNA ends with 3′-protruding, 5′-protruding or blunt ends. Ku also binds to DNA nicks, gaps, bubbles and stem-loop structures at DNA ends but not ssDNA ends (Dynan and Yoo,

1998). *In vivo* cross-linking experiments demonstrated that Ku binds to telomeric DNA (Gravel *et al.*, 1998). Furthermore, Ku interacts with Sir4 which is involved in the transcriptional silencing of telomere-adjacent region (Tsukamoto *et al.*, 1997). These biochemical properties of Ku likely underlie Ku's role in telomere maintenance and transcriptional silencing that was first predicted based on genetic studies in yeast.

Ku translocates along the DNA molecule in an ATP-independent manner (Paillard and Strauss, 1991). Ku can also transit from one linear DNA molecule to another if the termini of the DNA ends form base pairing (Bliss and Lane, 1997). Consistent with this observation, Ku was found to stimulate DNA end ligation by DNA ligase I *in vitro* (Ramsden and Gellert, 1998).

A group of human DNA repair genes, called *XRCC* (X-ray repair cross complementation) genes, was identified based on complementation studies using mutant rodent cell lines that are hypersensitive to IR and defective in both DSB rejoining and V(D)J recombination. Ku80 is encoded by *XRCC5* while Ku70 is the protein product of *XRCC6* (Gu *et al.*, 1997a; reviewed by Jeggo, 1998). Studies of Ku80- and Ku70-knockout mice have further confirmed their roles in DSBR.

*DNA-dependent protein kinase (DNA-PK)* DNA-PK is a nuclear serine/threonine kinase that is activated upon binding to DNA ends, nicks, and gaps (Gottlieb and Jackson, 1993). It consists of a regulatory subunit, the Ku70 and Ku80 heterodimer, and the catalytic subunit (DNA-PKcs) of 465 kDa. There are sequence homologies between DNA-PKcs and aforementioned PI3 kinase family members, e.g., ATM and ATR. Although Ku70 and Ku80 have been evolutionarily conserved, a budding yeast counterpart of human DNA-PKcs has not been identified.

The importance of DNA-PKcs in DSBR was initially demonstrated using cells established from severe combined immune deficient (SCID) mice. SCID mice lack mature B and T cells due to defective V(D)J recombination. SCID cells are hypersensitive to IR, suggesting that common factors might be used for V(D)J recombination and DSBR.

*In vitro* experiments have shown that upon binding of dsDNA ends, Ku recruits DNA-PKcs and activates its kinase activity. Atomic-force microscopy study indicates that in the presence of large DNA fragments, DNA-PKcs alone also binds to DNA termini, with Ku70/Ku80 binding adjacent to DNA-PKcs (Yaneva *et al.*, 1997). DNA-PKcs can also hold the ends of two ds DNA molecules (Cary *et al.*, 1997). DNA-PKcs alone exhibits a low level of DNA-dependent kinase activity. Addition of Ku protein stimulates the kinase activity 5–10-fold (Yaneva *et al.*, 1997). Based on the abundant amount of Ku in cells, it is likely that activation of DNA-PK by DSBs *in vivo* involves Ku.

Recent studies of DNA-PK structure by electron crystallography have revealed an open channel and an enclosed cavity with openings that allow entry of ss DNA. Guided by the results, further biochemical studies suggested that activation of the kinase requires both ds and ss DNA (Leuther *et al.*, 1999). Future structural studies of DNA-PK in the presence of Ku should yield additional insights into the regulation of DNA-PK activity. It is also important to identify the cellular substrates of DNA-PK. Many proteins can be phosphorylated by DNA-PK *in vitro*, but its *in vivo* substrates remain elusive (Smith and Jackson, 1999). Studies to date indicate that Ku and DNA-PKcs both participate in V(D)J recombination and DSBR. However, functions such as growth regulation and telomere maintenance appear to be uniquely involving Ku.

### *XRCC4 and DNA ligase IV*

The *XRCC4* gene encodes a nuclear phosphoprotein important for cellular resistance to IR (Li *et al.*, 1995). Since XRCC4 interacts with DNA ligase IV and stimulates its activity fivefold *in vitro* (Critchlow and Jackson, 1998; Grawunder *et al.*, 1997), it appears likely that DNA ligase IV is required for the ligation step in NHEJ and in V(D)J recombination (see Figure 2; Grawunder *et al.*, 1998a). This was confirmed in the mouse knockout model in which the ligase IV gene had been inactivated (Grawunder *et al.*, 1998b). It is worthwhile noting that XRCC4 interacts with DNA ligase IV via the ligase IV carboxyl terminus that contains two tandem BRCT domains.

In addition to genes identified by genetic approaches described above, the tumor suppressor genes, *ATM*, *BRCA1* and *BRCA2* have also been implicated in DSBR. The biochemical properties and function of these tumor suppressors are described below.

### *Tumor suppressor genes implicated in double-strand break repair in metazoans*

*ATM and double-strand break repair* There is accumulating evidence that ATM functions directly in DSBR, in addition to checkpoint regulation. Several groups reported higher residual levels of DSBs persisting in A-T cells after IR. Since these experiments were performed using non-cycling cells (G0/G1), the deficiencies observed in A-T cells could not have been due to defective cell cycle checkpoints *per se*. A-T cells display several abnormalities in the repair of DSBs; (i) higher levels spontaneous intra-chromosomal recombination after IR; (ii) frequent error-prone intra- and extra-chromosomal recombinations; (iii) aberrant NHEJ characterized by large deletions or insertions at the site of joining (Meyn, 1995; see Shiloh, 1997; and references therein). Since the studies described above were performed in transformed A-T cell lines, it is possible that DSB-induced signaling pathways might be de-regulated in these cell lines. Therefore, it is important to study DSBR in ATM-deficient mouse embryonic stem (ES) cells and mouse embryonic fibroblasts (MEF).

In contrast to checkpoint control, little is known about the biochemical basis of defective DSBR in A-T cells. Liu and Weaver (1993) demonstrated a delayed IR-, but not UV-induced phosphorylation of the 34 kDa subunit of RPA in A-T cells. A similar observation was made in yeast, suggesting that RPA phosphorylation in response to DSB is conserved in eukaryotes (Brush *et al.*, 1996). By overexpressing the kinase domain of ATM, Morgan and Kastan (1997) demonstrated that IR-induced RPA phosphorylation is

dispensable for the S phase checkpoint. Since it is unclear whether similar site(s) of RPA are phosphorylated, the role of ATM-dependent phosphorylation of RPA in DSBR remains to be investigated.

Recently, tyrosine phosphorylation of Rad51 was found to be dependent on c-Abl kinase both *in vitro* and *in vivo* (Chen *et al.*, 1999; Yuan *et al.* 1998). c-Abl is a non-receptor tyrosine kinase involved in multiple signaling pathways. Yuan *et al.* (1998) showed that c-Abl can phosphorylate Rad51 on tyrosine-54 *in vitro*. The IR-induced-phosphorylation of Rad51 by c-Abl may inhibit both the binding of Rad51 to DNA and its function in DNA strand exchange reactions. In contrast, Chen *et al.* (1999) demonstrated that tyrosine-315 in Rad51 is most likely an *in vivo* target site phosphorylated by c-Abl after IR. Since phosphorylation of Rad51 by c-Abl enhances its interaction with Rad52 and since IR-induced enhancement of tyrosine phosphorylation of Rad51 was not seen in A-T cells, Chen *et al.* (1999) proposed that ATM and c-Abl facilitate DSBR, at least partly by enhancing the repair protein complex formation. Although these studies provide a plausible mechanism for ATM involvement in DSBR, how the post-translational modification of Rad51 affects its function during HR *in vivo* remains to be established.

ATM signaling to Mre11/Rad50/NBS1 is likely to be important for both NHEJ and HR. IR-induced phosphorylation of these proteins is minimal in A-T cells (Zhao *et al.* unpublished). Furthermore, NBS1 appears to be a downstream target of ATM and can be phosphorylated upon IR in an ATM-dependent manner (Yuan *et al.* in preparation). This observation provides a biochemical link between ATM and proteins essential for NHEJ and HR.

*BRCA1, BRCA2 and double-strand break repair* *BRCA1* and *BRCA2* are two tumor suppressor genes mutated in a significant percentage of patients with familial breast cancer (reviewed by Koller, this issue). Insights into the function of these two proteins are based on studies of knockout phenotypes and identification of proteins that interact with them. These studies indicate that both genes might be involved in DSBR.

BRCA1 has been implicated in the regulation of replication checkpoint (Scully *et al.*, 1997) and transcription-coupled repair (Abbott *et al.*, 1999). Recent evidence suggested that BRCA1 constitutively associates with the Rad50/Mre11/NBS1 complex. However, Mre11/Rad50/NBS1 IRIF form only in the presence of BRCA1 (Zhong *et al.*, 1999). It is likely that other mediators are required in addition to BRCA1 for the assembly of Rad50/Mre11/NBS1 and DNA DSB site-containing complexes.

On the other hand, BRCA2 interacts with Rad51. The BRC repeats located within exon 11 of BRCA2 are necessary and sufficient for binding to Rad51 (Chen *et al.*, 1998; Sharan *et al.*, 1997; Wong *et al.*, 1997). Furthermore, formation of Rad51 IRIF is defective in BRCA2- but not BRCA1-deficient cells, suggesting that BRCA2 might contribute to the assembly of Rad51 repair complex in response to IR-induced DNA damage (Yuan *et al.*, 1999a). The identification of key repair proteins, Rad50 and Rad51, as interacting proteins of the BRCA tumor suppressor proteins provides an explanation for the IR sensitivity of cells harboring mutation in the *BRCA* genes. Future studies of how DSBR pathways are affected in these cells will provide important information on how these newly evolved tumor suppressors modulate DSBR.

*Double-strand break repair proteins in telomere maintenance*

Telomeres are special structures at the ends of chromosomes (Griffith *et al.*, 1999). Telomeres protect chromosome ends from DNA degradation and fusion between chromosome ends. In yeast, a number of genes including *ScKU70, ScKU80, SIR2, SIR3, SIR4 and RAD50/MRE11/XRS2* are required for telomere maintenance. Ku-deletion mutants display telomere shortening (Boulton and Jackson, 1996), loss of telomere silencing and dissociation of telomere from the nuclear periphery while *sir* mutants are mainly defective in telomere silencing (Boulton and Jackson, 1998; Laroche *et al.*, 1998; Nugent *et al.*, 1998). Both *ku* and *sir* mutants are defective in NHEJ. Recent studies demonstrated that in the presence of even a single DSB, Sir and Ku proteins will be partially dissociated from telomere and are diffusely distributed within the nucleus. Interestingly, the re-localization of Ku and Sir requires the checkpoint proteins Rad9 and Mec1 (Martin *et al.*, 1999; Mills *et al.*, 1999). Unlike *ku* or *sir* mutants, *rad50, mre11*, or *xrs2* mutants have shortened telomeres without the loss of telomere silencing. Rad50/Mre11/Xrs2 function epistatically with telomerase and synergistically with Ku and Sir proteins in telomere maintenance (Nugent *et al.*, 1998). Whether telomere maintenance requires the enzymatic or structural role of Rad50/Mre11/Xrs2 is controversial (Boulton and Jackson, 1998; Moreau *et al.*, 1999). Taken together, a number of DSBR proteins are also involved in telomere maintenance through interaction with different telomere binding proteins.

*Homologous recombination vs non-homologous end joining in higher eukaryotes*

As discussed above, there are different genetic requirements for HR and NHEJ. In budding yeast, DSBs are mainly repaired by HR mechanisms. HR processes have been extensively studied in the context of mitotic recombination, meiotic recombination, and mating type switching (Nickoloff and Hoekstra, 1998; Paques and Haber, 1999; Petukhova *et al.*, 1999). In higher eukaryotes, both HR and NHEJ have been demonstrated in *Xenopus laevis* (Carroll, 1998; Labhart, 1999). Earlier studies of hamster mutant cell lines led to the identification of important genes in NHEJ. Only recently, genes important for HR were identified. Both XRCC2 and XRCC3 share sequence similarities with Rad51 (Cartwright *et al.*, 1998; Liu *et al.*, 1998). The *xrcc3* mutant cell line is sensitive to IR, and fails to form Rad51 IRIF, suggesting that XRCC3 might be a component of the Rad51 repair complex (Bishop *et al.*, 1998).

The role of HR in maintaining the genomic stability in mammals is largely unknown. Using specially designed substrates that when integrated into chromosomal DNA will allow the analysis of recombination

products generated after DSBR by either HR or NHEJ, it was shown that HR repair pathways are likely to be equally important in mammalian cells (Liang *et al.*, 1996, 1998). In addition to a repair role, HR activities may also contribute to the loss of heterozygosity (Moynahan and Jasin, 1997; Shulman *et al.*, 1995), which is frequently involved in inactivation of tumor suppressors and formation of tumors. Increasing evidence also indicates the role for HR in sister chromatid recombination (Sonoda *et al.*, 1999). In summary, emerging evidence indicates broader roles for HR pathways in maintaining genomic stability, but the details of most of the processes remain to be elucidated.

### *Cell cycle-dependent double-strand break repair in eukaryotes*

During HR, intact DNA template is used in the repair process. Thus it is likely that HR is a preferred mechanism for repairing DSBs at specific stages of the cell cycle. Hamster mutant cells defective in NHEJ are highly sensitive to IR in G1 and early S phases, but only mildly sensitive in late S and G2 (Lee *et al.*, 1997; Mateos *et al.*, 1994). Rad51-deficient chicken DT40 cells have normal IR-sensitivity in G1 and early S phases but increased sensitivity in late S and G2. In contrast, the Ku70-deficient DT40 cells display increased sensitivity in G1/early S and normal sensitivity in late S/G2 (Takata *et al.*, 1998), confirming the predominant role of NHEJ during G1 and early S and HR during late S and G2. Since it has been hypothesized that Rad52 and Ku are involved in the choice of repair pathways (Van Dyck *et al.*, 1999), it appears to be crucial then to address the activities of Rad52 and Ku during different cell cycle stages. Whether there is a link between DNA repair components and checkpoint molecules leading to specific checkpoint or repair defects also needs to be explored.

### *Mouse models for DSB-induced cell cycle checkpoint and repair in development and tumorigenesis*

While mouse models of defective nucleotide excision repair and mismatch repair have provided crucial information regarding the effects of repair defects on development and tumorigenesis, it is only recently that such models for DSBR were established. We will summarize findings from these mouse models (Table 1).

*Knockout mouse models for Mre11/Rad50/NBS1* Mutant ES cells heterozygous for Mre11 disruption have no apparent growth abnormalities in culture. However, homozygous mutant ES cells are non-viable, suggesting that mammalian Mre11 is an essential gene (Xiao and Weaver, 1997). Similarly, no ES cells homozygous for targeted disruption of Rad50 can be isolated. Homozygous mutations of Rad50 in mice result in early embryonic lethality at E6.5, likely due to defects in proliferation (Luo *et al.*, 1999). In agreement with Rad50's role in DSBR, Rad50$^{-/-}$ embryos are extremely sensitive to IR (Luo *et al.*, 1999). Therefore, mutations in Mre11 and Rad50 cause more severe phenotypes in mammals than in yeast. Since *RAD50* and *MRE11* are not essential genes in yeast, mammalian homologs may have evolved to carry out additional functions. One possibility is that the interaction between this protein complex and recently evolved gene products, such as BRCA1 that plays important roles in both DNA repair and cell cycle checkpoints. As discussed earlier, the Rad50/Mre11/NBS1 complex has both enzymatic and structural functions, knock-in mice carrying defined mutations should provide important information to delineate the functions of Mre11 and Rad50 proteins.

While knockout mice of NBS1 are yet to be reported, NBS1$^{-/-}$ ES cells are viable (R Maser, meeting report; Lavin *et al.*, 1999). All NBS patients studied to date have mutations resulting in the truncation of the *NBS1* gene, mostly at the amino terminus (Varon *et al.*, 1998). Based on the clinical and cellular features of NBS, it is clear that NBS1 is involved in DSBR. How is it that loss of Mre11 and Rad50 results in non-viability of ES cells whereas loss of NBS1 does not? One possibility is that Mre11 is capable of forming a complex with Rad50 in NBS cells (Carney *et al.*, 1998). Mre11 nuclease activities are enhanced by the presence of Rad50 although the combination of the three components results in the highest activities (Paull and Gellert, 1999). These results suggest that Mre11/Rad50 complex is functional, albeit sub-optimal, in the absence of NBS1 and may partially explain the ability of NBS$^{-/-}$ cells to survive while Rad50 or Mre11 deletion results in lethality. In addition, Rad50/Mre11 may have an essential function that is independent of NBS1.

*Rad51, Rad52, and Rad54* As mentioned previously, Rad51, Rad52, and Rad54 are key players in HR. Homozygous Rad51 mutation leads to early embryonic lethality associated with decreased proliferation in mouse embryos. Rad51$^{-/-}$ ES cells are not viable, indicating that Rad51 is essential for cell viability (Lim and Hasty, 1996; Tsuzuki *et al.*, 1996). In addition, Rad51$^{-/-}$ trophoblasts are hypersensitive to IR and suffer multiple chromosome loss (Lim and Hasty, 1996). Interestingly, the survival of Rad51$^{-/-}$ embryos is extended in a p53 mutant background, probably because checkpoint activation due to chromosomal abnormalities is abrogated in the absence of p53. Fibroblasts derived from double mutant embryos fail to proliferate in tissue culture (Lim and Hasty, 1996). The inviability of the mutant ES cells and MEFs may be due to the increased amounts of oxidative DNA damage that occur during *in vitro* cell culture.

Rad52$^{-/-}$ mice are viable and healthy (Rijkers *et al.*, 1998). Moreover, Rad52$^{-/-}$ ES cells display only a moderate reduction in homologous recombination, and are not any more sensitive to DSB-inducing agents or MMS and mitomycin C than wildtype cells (Rijkers *et al.*, 1998). The mild phenotype exhibited by mice deficient in Rad52 may be explained by the existence of additional Rad52 homologs.

Rad54 is a member of the SNF2/SWI2 family of DNA-dependent ATPases which might function in the remodeling of chromatin structure. The interaction between Rad51 and Rad54 is increased upon DSB (Tan *et al.*, 1999). In agreement with this observation, DNA damage-induced foci of Rad51 and Rad54 co-localize. Interestingly, the formation of Rad51 IRIF

**Table 1** Mouse models for DSB-induced cell cycle checkpoint and repair in development and tumorigenesis

| *Protein* | *function* | *knockout phenotype* |
|---|---|---|
| *Homologous recombination and non-homologous end-joining* | | |
| Rad50 | enhancing Mre11's nuclease activity<br>telomere maintenance | early embryonic lethality (~E6.5)<br>decreased proliferation<br>Rad50$^{-/-}$ ES cells not viable |
| Mre11 | nuclease<br>telemere maintenance | Mre11$^{-/-}$ ES cells not viable |
| *Homologous recombination* | | |
| Rad51 | homologous pairing<br>sister chromatid exchange | early embryonic lethality (~E8.5)<br>decreased proliferation<br>embryos hypersensitive to IR<br>Rad51$^{-/-}$ ES cells not viable |
| Rad52 | enhancing the activities of Rad51<br>binding of DSB ends | viable, no growth defects<br>Rad52$^{-/-}$ ES cells show a moderate reduction in homologous recombination frequency |
| Rad54 | ATP-dependent ATPase<br>stimulating Rad51-mediates pairing<br>stabilizing heteroduplex<br>sister chromatid exchange | viable, no growth defects<br>Rad54$^{-/-}$ ES cells show reduced homologous recombination frequency and are sensitive to IR, MMS, and mitomycin C |
| *V(D)J recombination and non-homologous end-joining* | | |
| Ku70 | DNA end joining<br>telomere maintenance | viable but growth retarded<br>arrested B cell development<br>impaired coding-end and signal-end joining<br>T cell Lymphoma |
| Hu80 | DNA end joining<br>telomere maintenance | viable but growth retarded<br>arrested B/T cell development<br>impaired coding-end and signal-end joining |
| DNA-PKcs | Ser/Thr protein kinase<br>DNA end joining | viable, no growth defects<br>arrested B/T cell development<br>impaired coding-end joining |
| XRCC4 and Ligase IV | ligating DNA ends during NHEJ | late embryonic lethality<br>defective B/T cell development<br>impaired coding-end and signal-end joining<br>massive neuronal cell death during neurogenesis |
| *Checkpoint control and DNA repair* | | |
| Atm | Ser/Thr protein kinase<br>phosphorylating p53, CHK2 and NBS1<br>regulating multiple checkpoints<br>telomere maintenance | viable, but growth retarded<br>infertility<br>defective T cell maturation<br>neurologic abnormalities/dysfunction<br>malignant thymic lymphoma |
| Atr | Ser/Thr protein kinase<br>phosphorylating p53<br>S and G2/M checkpoints | postimplantational lethality<br>proliferational defects<br>increased chromosomal breaks |
| Brca1 | Rad50-mediated repair processes<br>G2/M checkpoint | postimplantational lethality (~E7.5)<br>decreased proliferation<br>Brca1$^{-/-}$ ES cells not viable<br>defective G2/M checkpoint<br>genetic instability<br>abnormal centrosome duplication |
| mammary-specific Brca1 conditional mutants | | impaired mammary ductal development<br>mammary tumor formation after 10–13 months |
| Brca2 | Rad51-mediated repair processes | |
| mutant alleles truncating Brca2 prior to the 1st BRC repeats in exon 11 | | postimplantational lethality (~E9.5)<br>decreased proliferation<br>embryos hypersensitive to IR<br>Brca2$^{-/-}$ ES cells not viable |
| mutant alleles disrupting Brca2 after the 3rd or 7th BRC repeats in exon 11 | | embryonic lethality/survival (see text for details)<br>growth retardation<br>infertility<br>thymic lymphoma<br>intact checkpoint activation<br>elevated chromosomal aberrations<br>inefficient repair of DSBs |

requires Rad54 (Tan *et al.*, 1999). As expected, Rad54$^{-/-}$ ES cells show a reduced frequency of HR and are sensitive to IR, MMS, and mitomycin C. Rad54 null mice are viable but surprisingly, have no apparent defects in spermatogenesis and oogenesis (Essers *et al.*, 1997). This raises the possibility of the existence of homologs of Rad54 that may function specifically during meiosis. There is a meiosis-specific Rad51 homolog, Dmc1, which is specifically expressed in testis (Yoshida *et al.*, 1998). Homozygous deletion of Dmc1 in the mouse results in the failure of homologous chromosome synapsis during meiosis (Pittman *et al.*, 1998; Yoshida *et al.*, 1998).

*Knockout mice for DNA-PKcs/Ku70/Ku80* Readers are referred to a recent review for a more detailed description and comparison of mutant mice deficient for different components of the DNA-PK (Smith and

Jackson, 1999). Targeted disruption of any component of DNA-PK (DNA-PKcs, Ku70, and Ku80) results in enhanced IR sensitivity and severe combined immune deficiency due to defects in DSBR and V(D)J recombination (Nussenzweig *et al.*, 1996; Zhu *et al.*, 1996; Gu *et al.*, 1997b; Ouyang *et al.*, 1997; Gao *et al.*, 1998a; Taccioli *et al.*, 1998). In Ku80 and DNA-PKcs mutant mice, the development of both T and B lymphocytes is arrested at an early progenitor stage, $CD4^-$ $CD8^-$ double-negative (DN) and $B220^+$ $CD43^+$, respectively (Nussenzweig *et al.*, 1996; Zhu *et al.*, 1996; Gao *et al.*, 1998a). In Ku70 null mice, however, only B cell development is completely blocked, and T cells develop through the $CD4^+$ $CD8^+$ double-positive (DP) stage and mature into $CD4^+$ $CD8^-$ and $CD4^-$ $CD8^+$ single-positive (SP) cells, although the numbers are significantly reduced (Gu *et al.*, 1997b; Ouyang *et al.*, 1997). The V(D)J recombination defect in these mutant mice lies in the joining step. Contrary to expectations, both hairpin coding ends and blunt full-length signal ends accumulate in the absence of Ku, indicating that Ku is not required to protect V(D)J recombination intermediates (Zhu *et al.*, 1996; Gu *et al.*, 1997b; Han *et al.*, 1997). In Ku80 and Ku70 mutant mice, both coding-end joining and signal-end joining are severely impaired (Nussenzweig *et al.*, 1996; Zhu *et al.*, 1996; Gu *et al.*, 1997b; Ouyang *et al.*, 1997), while only coding-end joining is defective in DNA-PKcs mutant mice (Gao *et al.*, 1998a; Taccioli *et al.*, 1998).

Recent studies have indicated that immunoglobulin heavy chain class switching, which involves the processing of DSB intermediates (Wuerffel *et al.*, 1997), is highly dependent on DNA-PKcs and Ku (Rolink *et al.*, 1996; Manis *et al.*, 1998; Casellas *et al.*, 1998). Moreover, Ku has been shown to participate in the sequence-specific transcriptional repression of a specific promoter and it appears that DNA-PKcs is required for such repression (Giffin *et al.*, 1996).

In addition to their differential effects on coding- and signal-end joint formation, $Ku80^{-/-}$ and $Ku70^{-/-}$ mice can be distinguished from $DNA\text{-}PKcs^{-/-}$ mice based on their growth. Ku80- and Ku70- deficiency results in growth defects (Nussenzweig *et al.*, 1996; Zhu *et al.*, 1996; Gu *et al.*, 1997b; Ouyang *et al.*, 1997), and Ku80-deficient mice show signs of aging early in life and their cells are likely to undergo premature replicative senescence (P Hasty, personal communication). In contrast, $DNA\text{-}PKcs^{-/-}$ cells and mice have apparently normal growth (Gao *et al.*, 1998a; Taccioli *et al.*, 1998). The difference between Ku and DNA-PKcs deficient mice indicates that Ku may have unique functions independent of DNA-PKcs.

Mice deficient in Ku70 undergo T cell development but form lymphomas. This may be due to defective processing of T cell receptor (TCR) gene rearrangement in $Ku70^{-/-}$ cells, which are defective in DSBR, leading to aberrant genetic changes and tumor development. Indeed, $Ku70^{-/-}$ but not $Ku80^{-/-}$ and SCID mice develop spontaneous thymic and disseminated T cell lymphomas at a mean age of 6 months with DP tumor cells (Gu *et al.*, 1997b; Li *et al.*, 1998). Taken together, mice carrying a deletion of an individual component of the DNA-PK complex provide new insights into their shared and distinct functions and lay the foundation of future mechanistic studies.

*DNA ligase IV and XRCC4* DNA ligase IV forms a stable complex with XRCC4 and its activity is stimulated by XRCC4 (Grawunder *et al.*, 1997). The complex of ligase IV and XRCC4 participates in the final step of V(D)J recombination and DNA-end joining (Wilson *et al.*, 1997). Inactivation of either ligase IV or XRCC4 in mice leads to late embryonic lethality (Frank *et al.*, 1998; Gao *et al.*, 1998b). Lymphocyte development is blocked and both coding-end and signal-end joining in V(D)J recombination are defective in these mice (Frank *et al.*, 1998, Gao *et al.*, 1998b). Embryonic fibroblasts (MEFs) derived from these mice proliferate poorly, enter senescence prematurely, and are extremely sensitive to IR (Frank *et al.*, 1998; Gao *et al.*, 1998b). All these phenotypes, except the embryonic lethality, are similar to those seen in Ku-deficient cells, suggesting that Ku, XRCC4 and ligase IV may be involved in the same end-joining process. These results in mammalian cells recapitulate the epistatic relationships established in yeast. However, it should be noted that in yeast Ku is involved in telomere maintenance but ligase IV does not. Interestingly, in addition to defects in lymphogenesis, XRCC4 and ligase IV deficient embryos suffer massive cell death in newly generated, postmitotic neuronal populations (Gao *et al.*, 1998b). It was postulated that increased death of early postmitotic $XRCC4^{-/-}$ and ligase $IV^{-/-}$ neurons is due to increased susceptibility to excess DSBs during neuronal development, which can not be repaired efficiently in the absence of these factors (Gao *et al.*, 1998b). These studies underscore the importance of NHEJ in V(D)J recombination and DSBR as well as the aforementioned roles of Ku, ligase IV, and XRCC4 in cellular proliferation.

*Brca1 and Brca2* BRCA1 and BRCA2 have been shown to interact with Rad50 and Rad51, respectively (see above), implying that they participate in DSBR. Loss of Brca1 leads to embryonic death associated with hypoproliferation in mice (Hakem *et al.*, 1996; Liu *et al.*, 1996; Gowen *et al.*, 1996; Ludwig *et al.*, 1997; Xu *et al.*, 1999b). Brca1 mutants also show decreased expression of mdm2, a negative regulator of p53 activity, and dramatically increased expression of p21, a target for p53 transcriptional activation (Hakem *et al.*, 1996). Deletion of Brca1 exon 11 is associated with G2/M checkpoint defects in MEFs, genetic instability characterized as chromosomal structural aberrations and aneuploidy, and abnormal centrosome duplication, indicating the important role of Brca1 in maintaining genetic stability and inhibiting tumorigenesis (Xu *et al.*, 1999b). Different Brca2 mutant alleles have been generated and have different phenotypes (Sharan *et al.*, 1997; Ludwig, *et al.* 1997; Suzuki *et al*, 1997; Connor *et al.*, 1997; Friedman *et al.*, 1998). Truncation of Brca2 prior to the first BRC repeat in exon 11 leads to early embryonic lethality at E6.5 $\sim$ E9.5 (Sharan *et al.*, 1997; Ludwig *et al.*, 1997; Suzuki *et al.*, 1997). Disruption of Brca2 after the third BRC repeat results in $\sim$50% live birth of homozygous mutants in the mixed genetic background (Friedman *et al.*, 1998). Disruption after the seventh BRC repeat leads to enhanced embryonic survival and results in $\sim$30% live homozygous mutants in the 129/B6/DBA background (Connor *et al.*, 1997). Brca2 mutant mice survive to adulthood, are growth retarded, infertile, and develop lethal thymic lymphomas by 5 months of age (Connor *et al.*, 1997; Friedman *et al.*,

1998). Brca2$^{-/-}$ embryos are hypersensitive to IR (Sharan *et al.*, 1997). MEFs harboring mutations disrupting Brca2 after the third or seventh BRC repeat grow poorly in culture, arrest in G1 and G2/M, have elevated p53 and p21 protein levels, are extremely sensitive to UV and MMS, and are only mildly sensitive to IR (Connor *et al.*, 1997; Friedman *et al.*, 1998; Patel *et al.*, 1998). The G1/S and G2/M DNA damage checkpoints as well as the replication checkpoint are intact in these MEFs (Patel *et al.*, 1998). Moreover, these MEFs display higher spontaneous chromosomal structural aberrations, including chromatid breaks and chromatid exchange (Patel *et al.*, 1998).

The elevated p53 and p21 levels in both Brca1 and Brca2 mutant embryos are presumably a consequence of the increased genetic instability. Since mutations in either p53 or p21 prolong the survival of Brca1$^{-/-}$ and Brca2$^{-/-}$ embryos, it appears that activation of the p53/p21 pathway by spontaneous DNA damage plays a part in the impaired cellular proliferation caused by the lack of Brca1 and Brca2 (Hakem *et al.*, 1997; Ludwig *et al.*, 1997). Indeed, when Brca2$^{-/-}$ MEFs are transduced with retroviruses encoding dominant-negative (DN) p53 mutants, they overcome the growth arrest and lose the checkpoints in response to IR and nocodazole (Lee *et al.*, 1999). Thymic lymphomas from Brca2$^{-/-}$ mice have defective checkpoints and harbor p53 mutations which have been shown to reverse the proliferative defects in Brca2$^{-/-}$ MEFs (Lee *et al.*, 1999). Thus inactivation of p53 cooperates with Brca2 deficiency to promote neoplastic transformation. Recently, the importance of Brca1 in mammary gland development and tumorigenesis was demonstrated by the generation of Brca1 conditional knockout mice using the Cre-loxP system (Xu *et al.*, 1999a). Deletion of Brca1 exon 11 from mammary epithelial cells causes abnormal ductal development accompanied by increased apoptosis. Mammary tumors develop after a long latency period (10–13 months), and are associated with genomic instability, including aneuploidy and chromosomal rearrangements. Detailed analyses of genomic alterations and chromosomal aberrations in the mammary tumors indicate the involvement of p53 dysfunction. Consistent with this notion, loss of p53 accelerates tumor formation in the Brca1 conditional knockout mice resulting in a much shorter latency period (6–8 months).

*Atr* Inactivation of Atr results in early embryonic lethality, and chromosome instability. Furthermore, Atr$^{-/-}$ cells fail to proliferate *in vitro* (EJ Brown, personal communication). Thus, the phenotype of the Atr$^{-/-}$ mice overlaps significantly with those of the Brca1 and Brca2 knockouts. These results together with the biochemical properties of Rad50 (including its interaction with BRCA1) suggest that there may be functional interactions among BRCA1, BRCA2, Rad50, and Atr.

### *Atm in mouse development, V(D)J recombination, and tumorigenesis*

Atm deficient mice reiterate most of the phenotypes of A-T patients, including growth retardation, neuronal abnormalities, immunodeficiency, meiotic defects, and cancer predisposition (Barlow *et al.*, 1996; Elson *et al.*, 1996; Xu *et al.*, 1996).

*Neurogenesis* Unlike A-T patients, no overt ataxia is detected in Atm$^{-/-}$ mice (Barlow *et al.*, 1996; Xu *et al.*, 1996). However, they show impaired performance in specific tests for motor coordination (Barlow *et al.*, 1996), indicating the presence of potential neuronal defects in Atm$^{-/-}$ mice. Atm deficiency causes severe degeneration of dopaminergic nigro-striatal neurons in the CNS of older mice (Eilam *et al.*, 1998). Surprisingly, many regions of the developing neonatal CNS in Atm mutants fail to undergo cell death and show reduced p53 induction after IR (Herzog *et al.*, 1998). Based on this observation, it was hypothesized that Atm deficiency may selectively compromise the death of certain damaged neurons and lead to neuronal deterioration later in life as the damages accumulate.

*T and B cell development* Lymphoid tissues of Atm$^{-/-}$ mice are generally smaller and contain fewer cells when compared to controls. The absolute numbers of DP and SP thymocytes are fewer, resulting in a large reduction of T cells in the peripheral lymphoid organs. However, the development of B cells is not altered significantly in Atm$^{-/-}$ mice (Barlow *et al.*, 1996; Elson *et al.*, 1996; Xu *et al.*, 1996). The abnormal development of T cells in Atm$^{-/-}$ mice could be due to impaired cell cycle checkpoint control during V(D)J recombination which appears to be restricted to the G0/G1 phase (Lin and Desiderio, 1995). In the absence of Atm, lymphocytes might enter S phase before V(D)J recombination is completed, resulting in a reduction of productive V(D)J recombination events and mature lymphocytes. Alternatively, the interaction between Atm and repair proteins, such as the Rad50/Mre11/NBS1 complex (see above), may be required for normal T cell maturation.

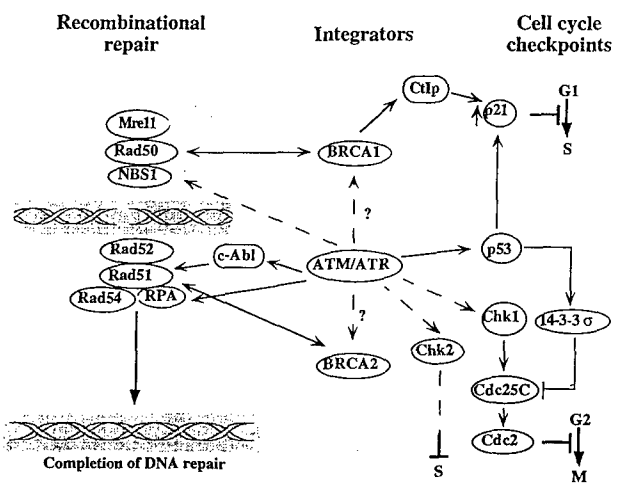


**Figure 3** Integration of DNA repair and checkpoint pathways in mammalian cells. An overview of the relationships among the recombinational repair machinery, tumor suppressors and cell cycle checkpoint regulators during DNA DSB repair. The four tumor suppressors, ATM, BRCA1, BRCA2 and NBS1 are being proposed to function as integrators for repair, gene regulation and activation of checkpoints (see text for details). Physical interactions are indicated by bi-directional arrows (< >). Signaling is indicated by a unidirectional arrow (>): a broken line (- - -) is used where no direct biochemical evidence is available. A question mark (?) denotes a hypothetical pathway. For cell cycle progression, (–|) indicates an inhibition

*Meiosis* Atm mutant mice are infertile due to the lack of mature gametes (Barlow *et al.*, 1996; Elson *et al.*, 1996; Xu *et al.*, 1996). The development of $Atm^{-/-}$ spermatocytes is arrested at the zygotene/pachytene stage of prophase I (Xu *et al.*, 1996). Moreover, aberrant chromosomal synapses in $Atm^{-/-}$ spermatocytes are followed by chromosome fragmentation, leading to nuclear degeneration and cellular death (Xu *et al.*, 1996). Interestingly, Atm deficiency is associated with a failure to properly assemble Atr as well as Rad51 and Dmc1 onto chromosomal axes in leptonema of meiosis I (Barlow *et al.*, 1997, 1998). Meiosis I progresses to later stages and is only partially rescued by p53 or p21 deficiency (Barlow *et al.*, 1997). Moreover, assembly of Rad51 onto chromosomal axes remains defective in these double mutant mice (Barlow *et al.*, 1997). Whether Rad51 mislocalization is directly due to the absence of Atm or a consequence of meiotic failure is not clear. Recently, leptotene/zygotene spermatocytes of Atm-null mice were found to display aberrant telomere clustering and elevated interactions between telomeres and nuclear matrix (Pandita *et al.*, 1999). These observations support a possible role for Atm in telomere maintenance.

*Tumorigenesis* $Atm^{-/-}$ mice develop malignant thymic lymphomas of DP origin by 4 months of age (Barlow *et al.*, 1996; Elson *et al.*, 1996; Xu *et al.*, 1996). However, no increased frequency of other tumor types is seen in Atm mutant mice, possibly due to the early lethality caused by lymphomas. Aberrant chromosomal rearrangements, translocations and insertions, were observed in tumor cells, and in one case, the rearrangement involved the TCR$\beta$ locus (Barlow *et al.*, 1996; Xu *et al.*, 1996). These observations suggest that lymphoma development in the absence of Atm is associated with the processing of DNA DSBs during V(D)J recombination. Indeed, in the RAG1-deficient background, where V(D)J recombination does not occur, no tumors develop in the $Atm^{-/-}$ mice (Liao & Van Dyke, 1999). Because V(D)J recombination is dispensable for thymoma development in $p53^{-/-}$ mice (Nacht and Jacks, 1998; Liao *et al.*, 1998), Atm and p53 appear to suppress thymoma through different mechanisms. Consistent with this notion, Atm/p53 double mutants exhibit a dramatic acceleration of tumor development relative to singly null mutants (Westphal *et al.*, 1997; Xu *et al.*, 1998), indicating a cooperative role between Atm and p53 in tumor suppression.

*Cellular proliferation and checkpoint response* $Atm^{-/-}$ mice are growth retarded. $Atm^{-/-}$ fibroblasts grow poorly in culture, undergo premature senescence, and show inefficient G1/S progression following serum stimulation (Barlow *et al.*, 1996; Elson *et al.*, 1996; Xu *et al.*, 1996). Interestingly, $Atm^{-/-}$ cells show an increased constitutive level of p21, which may account for the observed growth arrest (Xu and Baltimore, 1996). In agreement with this notion, Atm/p21 as well as Atm/p53 double mutant cells proliferate normally and the p21 level is not elevated in Atm/p53 double mutant cells (Xu *et al.*, 1998). Taken together, the activated p53/p21 pathway accounts for the proliferative defects seen in the absence of Atm.

$Atm^{-/-}$ ES cells, MEFs and thymocytes are sensitive to IR, and have defective G1 and S checkpoints in response to IR, correlating with an impaired p53 up-regulation (Barlow *et al.*, 1996; Elson *et al.*, 1996; Xu and Baltimore, 1996; Westphal *et al.*, 1997). However, $Atm^{-/-}$ thymocytes display a partial resistance to IR-induced apoptosis *in vivo* and *in vitro*. The additional loss of p53 renders them completely resistant, a phenotype that is shared by p53-null thymocytes (Xu and Baltimore, 1996; Westphal *et al.*, 1997). This suggests that both Atm-dependent and Atm-independent up-regulation of p53 may mediate the IR-induced apoptotic responses of thymocytes. Therefore, damaged thymocytes survive in the absence of Atm and accumulate more genetic mutations, which eventually lead to neoplasms as $Atm^{-/-}$ mice are prone to the development of thymic tumors.

## Summary and perspectives

Key players in cell cycle checkpoint control and DSB repair machinery are evolutionarily conserved. New family members in these cellular processes have been identified in mammals, e.g., the Rad51 recombinase gene family. Surprisingly, despite the existence of multiple family members, deletion of one of the members often results in embryonic lethality, e.g., Rad51, suggesting that each member of the protein family has a specialized function. Additionally, many newly evolved genes such as *p53* and *BRCA* have critical regulatory functions. Studies to date indicate intricate relationships among the DSBR machinery, tumor suppressors and cell cycle regulatory proteins (Figure 3). Mutation in any of the key players may disrupt the intricate network and thus provide a basis for the pleiotropic and severe phenotypes of the mutant cells.

In addition to nucleolytic processing of damaged DNA, Mre11/Rad50/NBS1 proteins are also involved in cell cycle checkpoint and chromosome homeostasis. While this protein complex exists in both undamaged and IR-treated cells, enhanced phosphorylation of these proteins upon DNA damage (Dong *et al.*, 1999) is likely to be required for recruiting other repair proteins. Recent studies indicate that BRCA1 is required for IR-induced Mre11 IRIF formation (Zhong *et al.*, 1999); on the other hand, BRCA2 is required for the formation of Rad51 IRIF (Yuan *et al.*, 1999a). These results strongly suggest that complex signaling pathways are initiated upon DNA damage to co-ordinate multiple cellular processes. Six Rad genes have been identified in yeast that are required for sensing/processing/signal transduction. It is unclear whether there are biochemical and functional links among hRad1/Rad9/Hus1, Rad17 protein complexes, and Mre11/Rad50/NBS1 in mammals. In addition, how ATM and ATR are activated upon DNA damage and whether and how the kinase cascades link BRCA proteins to the Mre11/Rad50/NBS1 protein complex remain to be addressed.

The presence of multiple cellular substrates is likely to underline the pleiotropic function of ATM. One of its substrates, p53, is a potent transcription activator of the cyclin-dependent kinase inhibitor, p21. The release of the BRCA1-associated co-repressor, CtIp, upon

DNA damage also plays an important role in the induction of p21 (Figure 3; Li *et al.*, 1999). In addition to a crucial role in G1/S checkpoint control, p53 regulates the duplication of the centrosome, an event which is central to the maintenance of a 2N genome in mammalian cells (Fukasawa *et al.*, 1996). Biochemical mechanisms involved in this process remain to be elucidated. Two key checkpoint kinases, Chk2 and Chk1, are subjected to regulation by ATM and ATR (Matsuoka *et al.*, 1998; Dasika *et al.* in preparation). The biochemical basis of S and G2/M checkpoint regulation is largely unknown. These checkpoints are of paramount importance in the maintainance of high fidelity DNA replication and chromosome segregation. Since aneuploidy is one of the hallmarks of tumor cells, delineation of players in the mammalian G2/M checkpoint and spindle assembly checkpoint will provide a better understanding as to how such genomic catastrophes are prevented in normal cells.

Genetic manipulation using knockout technology is time consuming in mammals, but mutant cells, especially conditional mutants generated through the now widely used Cre-lox P system, will serve as invaluable tools in delineating protein networks in DSB repair, cell cycle checkpoint regulation and signaling pathways. Identification of such protein networks will provide a rational basis for drug design that may sensitize cancer cells to radiation and chemotherapy as well as for cancer prevention.

**Abbreviations**

ATM, ataxia-telangiectasia mutated; BRCT, BRCA1 carboxyl terminus; DSB, double-strand break; DSBR, double-strand break repair; FHA, forkhead-associated; HR, homologous recombination; IR, ionizing radiation, IRIF, ionizing radiation-induced immunofluorescent foci; MMS, methyl-methane-sulfonate; NBS, Nijmegen breakage syndrome; NHEJ, non-homologous end joining; RDS, radiation-resistant DNA synthesis; SCID, severe combined immune deficient; SDDC, S phase DNA damage checkpoint; TCR, T cell receptor; XRCC, X-ray repair cross complementation; Mouse homologs are represented in lower case, e.g., Atm.

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# NBS1 and TRF1 Colocalize at Promyelocytic Leukemia Bodies during Late S/$G_2$ Phases in Immortalized Telomerase-negative Cells

## IMPLICATION OF NBS1 IN ALTERNATIVE LENGTHENING OF TELOMERES*

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**Nijmegen breakage syndrome, a chromosomal instability disorder, is characterized in part by cellular hypersensitivity to ionizing radiation. The *NBS1* gene product, p95 (NBS1 or nibrin) forms a complex with Rad50 and Mre11. Cells deficient in the formation of this complex are defective in DNA double-strand break repair, cell cycle checkpoint control, and telomere length maintenance. How the NBS1 complex is involved in telomere length maintenance remains unclear. Here we show that the C-terminal region of NBS1 interacts directly with a telomere repeat binding factor, TRF1, by both yeast two-hybrid and *in vivo* DNA-coimmunoprecipitation assays. NBS1 and Mre11 colocalize with TRF1 at promyelocytic leukemia (PML) nuclear bodies in immortalized telomerase-negative cell lines, but rarely in telomerase-positive cell lines. The translocation of NBS1 to PML bodies occurs specifically during late S to $G_2$ phases of the cell cycle and coincides with active DNA synthesis in these NBS1-containing PML bodies. These results suggest that NBS1 may be involved in alternative lengthening of telomeres in telomerase-negative immortalized cells.**

Telomeres comprise tracts of noncoding chromosomal hexanucleotide repeat sequences that, in combination with specific proteins, prevent degradation, rearrangement, and chromosomal fusion events (1). Telomere length is maintained by the *de novo* addition of telomere repeats by telomerase (2). In mammals, telomerase expression is ubiquitous in embryonic tissues and down-regulated in somatic adult tissues. There are, however, exceptions such as in regenerative tissues or tumor cells (3).

Recombination can lengthen telomeres in the absence of telomerase. For example, when the yeast telomerase RNA component, TLC1, is deleted, telomeres become shortened and most cells die (4). However, gene conversion mediated by the RAD52 pathway subserves telomere lengthening in rare surviving cells (5). Genetic studies in yeast have also implicated the Rad50-Mre11-Xrs2 complex in telomere length maintenance, aside from its additional roles in homologous and nonhomologous recombinational repair, DNA damage assessment, and/or cell cycle checkpoint regulation (6). The Rad50-Mre11-Xrs2 complex has been proposed to function in the preparation of DNA ends for telomerase-mediated replication and is therefore implicated in telomerase-dependent telomere length maintenance, rather than in protection of telomeric ends.

In mammalian cells, Mre11 and Rad50, together with NBS1 (p95) form a complex (7–9) comparable in mass to a similar assemblage in *Saccharomyces cerevisiae* containing Rad50, Mre11, and Xrs2. The *NBS1* gene mutated in Nijmegen breakage syndrome, a chromosomal instability disorder, encodes a 95-kDa protein (NBS1) with two functional modules found in cell cycle checkpoint proteins, a forkhead-associated domain and an adjacent BRCA1 C-terminal (BRCT) repeat (10). Rad50 is a coiled coil SMC (for structural maintenance of chromosomes)-like protein with ATP-dependent DNA binding activity (11). Mre11 has been proposed to have both structural (end-holding) and catalytic activities including double-stranded DNA 3′ to 5′ exonuclease and single-stranded endonuclease activity (9, 12–15). Despite their similar size (95 kDa), NBS1 and Xrs2p are quite dissimilar in sequence, although it remains possible that they will be functional analogues or early related homologues (16).

The function of NBS1 is unknown although there is speculation that it might recognize signals from a DNA damage-sensing complex that could be in the form of phosphorylation of serine or threonine residues that are, in turn, recognized by the forkhead-associated domain in NBS1 (16). Mre11 colocalizes to subnuclear regions containing DNA breaks within 30 min after irradiation of normal human diploid fibroblasts (17). In NBS[1] cells, a deficiency of NBS1 is correlated with an inability to form Mre11-Rad50 nuclear foci in response to ionizing radiation (8). Together, these observations point to a major role for the Mre11-Rad50-NBS1 complex in repair of DNA double-strand breaks. The NBS1 protein is essential for Mre11 phosphorylation upon DNA damage (18). In addition, NBS1 function has been linked to ATM by the observation that phosphorylation of NBS1 in response to radiation exposure is ATM-dependent (19–21). Whether the mammalian Rad50-

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[1] The abbreviations used are: NBS, Nijmegen breakage syndrome; PML, promyelocytic leukemia; ATM, ataxia telangiectasia mutated; CPRG, chlorophenol red-β-D-galactopyranoside; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; DAPI, 4′,6′-diamino-2-phenylindole; mAB, monoclonal antibody; GST, glutathione *S*-transferase; BrdUrd, bromodeoxyuridine.

Mre11-NBS1 complex also plays a role in the maintenance of telomere length has not yet been demonstrated. To address this issue, we have undertaken to identify cellular interacting partners of NBS1 by yeast two-hybrid screening. Here we report the identification of a telomere repeat binding factor, TRF1, as an interaction partner of NBS1. We show that NBS1 and Mre11 colocalize with TRF1 in PML nuclear bodies in telomerase-negative immortalized cells during $G_2$ phase of the cell cycle. Significantly, the NBS1/TRF1 foci undergo active BrdUrd incorporation during late S/$G_2$ transition, thus suggesting a novel role for NBS1 in telomere lengthening in telomerase-negative immortalized cell lines.

## EXPERIMENTAL PROCEDURES

*Yeast Two-hybrid Interaction System*—Full-length NBS1 cDNAs (minus the first 12 amino acids) were fused to the DNA-binding domain of GAL4 in the pAS2–1 vector and used as the bait in two-hybrid screens using human lymphocyte cDNA library. Detailed screening procedures were described previously (22). Similarly, a cDNA fragment containing Rad50 (amino acid 753 to 1312), Mre11 (amino acid 1–680), or BRCA1 (amino acid 1–1315) was fused to the DNA-binding domain of GAL4 in the pAS2–1 vector. To map the binding regions, various truncated forms of TRF1 and NBS1 were generated and fused to the GAL4 activation domain of pGAD10 and DNA-binding domain of pAS2–1, respectively, and cotransformed into Mav203 with full-length NBS1 and TRF1, respectively. β-Galactosidase activity was quantified with chlorophenol red-β-D-galactopyranoside (CPRG) as the substrate (22).

*Cell Lines, Culture Conditions, Synchronization, and BrdUrd Labeling*—T24, a human bladder carcinoma cell line, MCF7, a human breast cancer cell line, HeLa, a human cervical carcinoma cell line, Saos2, a human osteosarcoma cell line, and IMR90, a human fetal lung primary fibroblast are from ATCC (America Type Culture Collection). SV40-immortalized human fibroblast cell lines, VA13, GM847, and LM217, were kindly provided by J. Shay and J. P. Murnane. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (10% $CO_2$). Cell synchronization by double thymidine block in $G_1$/S boundary was performed as described with modification (23). Cells blocked at $G_1$/S transition were released and fixed at different time points (0~13 h). For an additional arrest at early $G_2$ by Hoechst 33342, cells were released from double thymidine block for 2 h, incubated with Hoechst 33342 at the final concentration of 0.2 μg/ml for 11 more h, washed with PBS, fixed, and processed for staining (24). For BrdUrd pulse labeling, cells were released from double thymidine block for 13 h, labeled with BrdUrd for 30 min with a cell proliferation kit (Amersham Pharmacia Biotech, RPN20), fixed, and stained as described.

*DNA Coimmunoprecipitation Assay*—The procedure was performed essentially as described (25). Briefly, cell nuclear extracts were prepared as described previously (26). A telomeric DNA-containing $TTAGGG^{11}$ repeat sequence was labeled with $^{32}$P-dCTP by Klenow filling and purified in 5% polyacrylamide gel. For each reaction, 50 μg of nuclear extract was incubated with the telomere probe (30, 000 cpm) in a binding buffer (20 mM HEPES, pH 7.9, 150 mM KCl, 1 mM $MgCl_2$, 5% glycerol, 4% Ficoll, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) at room temperature for 30 min. The protein-DNA complex was then immunoprecipitated by specific antibody and protein A beads and washed 3 times with binding buffer. The complex was digested by proteinase K and extracted with phenol/chloroform. The telomere probe was then precipitated by ethanol and electrophoresed in 5% native polyacrylamide gel and autoradiographed.

*Immunostaining*—This immunostaining procedure was adapted from the previous published work of Durfee *et al.* (27). Briefly, cells were grown on coverslips in tissue culture dishes and collected at various time points as indicated (random populated or synchronized). The cells were washed with PBS and fixed for 30 min in 3.7% formaldehyde in PBS plus 0.1% Triton X-100. Cells were then permeated with 0.05% Saponin at room temperature for 30 min, followed by washing five times with PBS. After being blocked with 10% goat serum in PBS/0.5% Nonidet P-40 at room temperature for 30 min, cells were incubated with primary antibodies at 4 °C for overnight. After five PBS washes, cells were incubated with FITC or Texas Red-conjugated secondary antibodies (Southern Biotechnology Associates, Inc.) for 30 min. Cells were then washed extensively in PBS/0.5% Nonidet P-40, further stained with DAPI (1 μg/ml in $H_2O$, Fisher), and mounted in Permafluor (Lipshaw-Immunonon, Inc). Immunofluorescence microscopy was performed with a Nikon Eclipse TE300 immunofluorescence microscope. Images captured were processed with Adobe Photoshop.

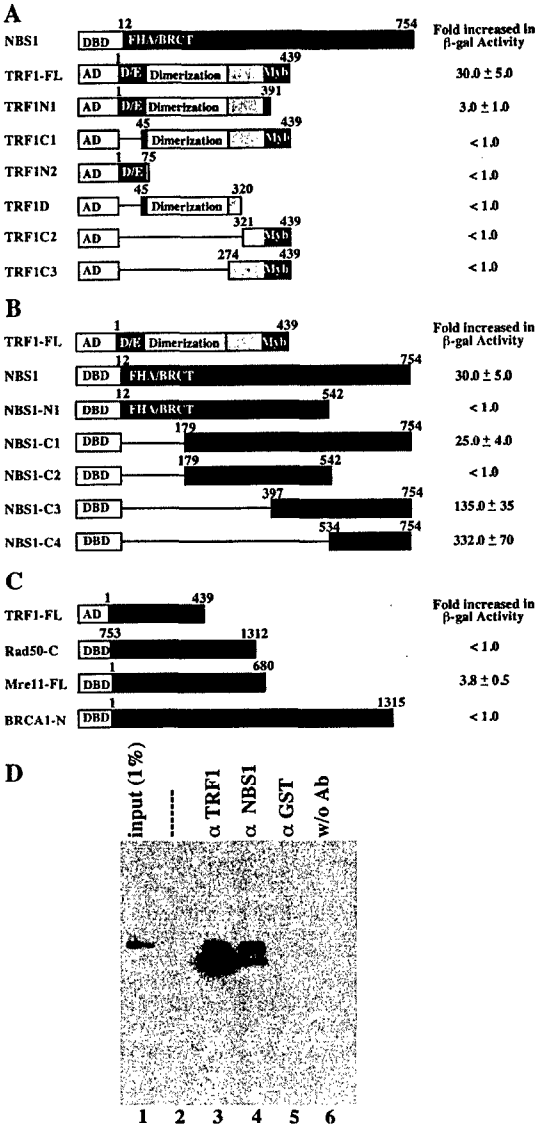


FIG. 1. **NBS1 interacts with TRF1.** *A–C*, NBS1 interacts with TRF1 in a yeast two-hybrid assay. β-Galactosidase activities were quantified using CPRG as the substrate. *A*, a near full-length NBS1 cDNA was fused to the GAL4 DNA-binding domain (*DBD*) of pAS2–1. Six truncated TRF1 fragments were fused to the GAL4 activation domain (*AD*) of pGAD10 and cotransformed into Mav203 with pAS2–1-NBS1, respectively. Only TRF1N1 showed detectable but low binding activity. *B*, various NBS1 fragments were fused to the GAL4 DNA-binding domain of pAS2–1 and cotransformed together with pGAD10-TRF1-FL into Mav203, respectively. The results showed that NBS1-C4 (amino acid 534–754) is responsible for binding to TRF1. With this fragment, the β-galactosidase activity was increased by 10-fold, when compared with the near full-length NBS1. *C*, Mre11, Rad50, and BRCA1 showed no or very little binding activity with TRF1 in the same system. The N-terminal region of Rad50 and C-terminal region of BRCA1 were removed, because they exhibit transactivation activity in yeast two-hybrid assays. Full-length Mre11 showed low binding activity with TRF1. *D*, NBS1 associates with telomere repeat sequences in an *in vivo* DNA coimmunoprecipitation assay. A $TTAGGG^{11}$ telomeric DNA fragment radioactively labeled by $^{32}$P was coprecipitated specifically with NBS antibodies (*lane 4*) and TRF1-antibodies (*lane 3*, positive control) but not GST antibodies (*lane 5*, negative control). *Lane 1* shows 1% input of labeled DNA, whereas *lane 6* shows a precipitation reaction performed in the absence of antibodies.

*Antibodies*—Rabbit polyclonal antibodies specific for NBS1 and Mre11 were obtained from Novus Biologicals (Littleton, CO). PML mAB PG-M3 was from Santa Cruz Biotechnology (Santa Cruz, CA). 12D7 mAB specific for Mre11 has been described before (28). Mouse α-TRF1

polyclonal antibodies were obtained by using bacterially expressed and purified GST-TRF1 fusion protein as antigen.

*Isolation of GM847 Cell Clones with Inducible Expression of the GFP-TRF1*—To establish cell clones that express a GFP-TRF1 fusion protein, we have used the inducible expression system controlled by a *tet*-responsive promoter. A cDNA fragment containing GFP-TRF1 fusion protein was subcloned into an expression plasmid (pUHD10–3) driven by a core cytomegalovirus promoter linked to a *tet* operator heptad (pUHD10–3-GFP-TRF1) (29). This plasmid was cotransfected into GM847 cells with pCHTV, bearing a hygromycin-resistance gene and a cytomegalovirus-controlled tetracycline repressor-VP16 fusion transcription unit, and *hygro*-resistant clones were subsequently isolated. Several cell clones that express the GFP-TRF1 fusion protein upon removal of tetracycline were obtained.

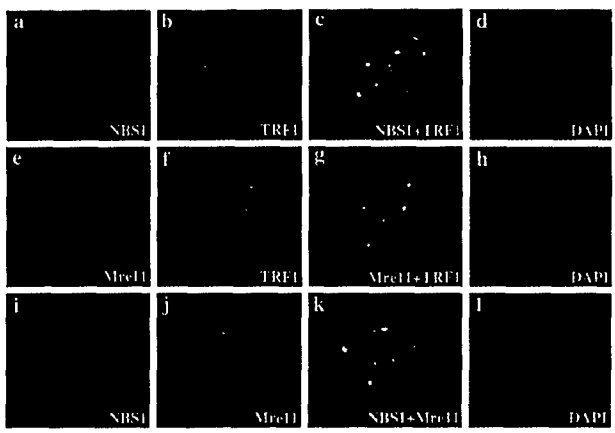


FIG. 2. **NBS1 and Mre11 colocalize with TRF1 in GM847 cells.** Actively growing GM847 cells were fixed and immunostained using a conventional protocol as described. NBS1 colocalizes with TRF1 (*a–d*). Mre11 colocalizes with TRF1 (*e–h*). NBS1 also colocalizes with Mre11 (*i–l*). For *a* and *b*, cells were costained with a rabbit polyclonal NBS1 antibody and a mouse polyclonal TRF1 antibody. A rabbit polyclonal Mre11 antibody co- stained cells with anti-TRF1 (*e* and *f*). In *i* and *j*, cells were costained with rabbit NBS1 antiserum and 12D7, a monoclonal Mre11 antibody. The first antibodies were detected with a goat anti-rabbit antibody conjugated with Texas Red and a goat anti-mouse antibody conjugated with FITC. *c*, *g*, and *k* are the merged images. DNA was stained with DAPI, shown in *blue* (*d*, *h*, and *l*).

## RESULTS AND DISCUSSION

To explore the potential biological function(s) of NBS1, we used near full-length NBS1 as the bait in a yeast two-hybrid screen to recover interacting proteins encoded by a human lymphocyte cDNA library. One of the clones thus isolated corresponded to a near full-length cDNA for the telomeric DNA-binding protein TRF1. TRF1 consists of an acidic N terminus followed by a dimerization domain and a Myb-like DNA-binding domain at its C terminus (30). To determine the specific domain(s) of TRF1 required for binding to NBS1, various regions of TRF1 fused to the GAL4-transactivation domain were individually tested for their respective abilities to interact with NBS1 fused to the GAL4-DNA-binding domain by yeast two-hybrid assay. As shown in Fig. 1*A*, none of the isolated TRF1 fragments exhibited significant binding activity, suggesting that full-length TRF1 is required for efficient binding to NBS1. Reciprocally, a series of NBS1 deletion mutants fused to the GAL4-DNA-binding domain were tested for their abilities to interact with full-length TRF1. Interestingly, the C-terminal region of NBS1 (amino acids 534 to 754) is required for TRF1 binding, and its binding activity appears much stronger than that of the near full-length clone (Fig. 1*B*). It is possible that one or more inhibitory motifs reside N-terminally of amino acid 534 within NBS1 and that the NBS1-TRF1 interaction is enhanced by release of this inhibitory function by post-translational modification such as phosphorylation. In this regard, we note that there are multiple potential phosphorylation sites in this region, some of which are subject to regulation by the ATM kinase during the DNA damage response (19–21).

Because NBS1 forms a complex with Mre11, Rad50, and BRCA1 (28), we next tested whether TRF1 binds to any of these proteins in a yeast two-hybrid assay. As shown in Fig. 1*C*, TRF1 exhibits very little binding activity toward any one of these three proteins. These results suggest that TRF1 may bind to the Rad50-Mre11-NBS1 triplex through NBS1.

To determine whether the complex of TRF1 and NBS1 exists in human cells, telomeric DNA repeats were radioactively labeled

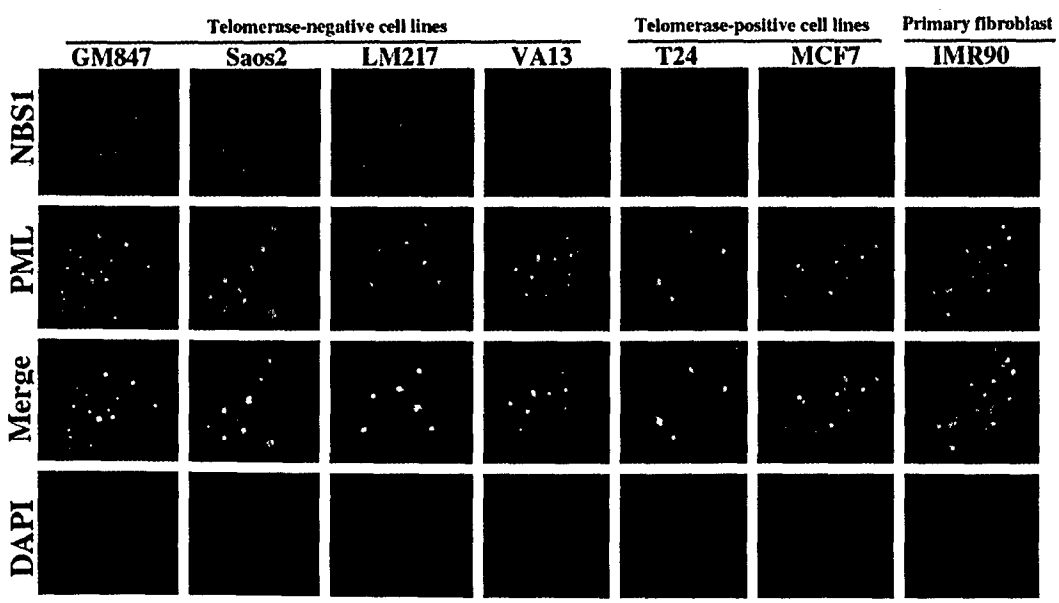


FIG. 3. **NBS1 colocalizes with PML bodies in telomerase-negative immortal cell lines but not in telomerase-positive immortal cell lines or primary fibroblasts.** Actively growing cells were fixed and costained for NBS1 with a rabbit NBS1 antibody (*row 1*) and for PML with a monoclonal PML antibody (*row 2*). In telomerase-negative immortal cells (GM847, Saos2, LM217, and VA13), NBS1 foci were detectable in about 5% of cells within a randomly growing culture; these NBS1 foci colocalize with PML bodies. NBS1 foci were not detected or evident in telomerase-positive immoral cell lines (T24 and MCF7) or a primary cell line (IMR90). PML bodies were readily detected in all cells tested. Cells in *row 1* were stained with Texas Red and in *row 2* with FITC. *Row 3* corresponds to merged images of NBS1 and PML. *Row 4* corresponds to DAPI staining, which indicates nuclei. Note that these NBS1 foci, whose number usually ranges from 2 to 15, are generally larger and more distinctive than ionizing radiation-induced foci. In VA13, NBS1 foci are smaller than those in the other telomerase-negative immortal cell lines.

and incubated with nuclear extracts. The labeled telomeric DNA repeats were specifically coimmunoprecipitated by antibodies against either TRF1 or NBS1 but not control antibodies against GST (Fig. 1*D*). These results further suggest that NBS1 binds to TRF1 in human cells and, furthermore, that the complex has the ability to bind the telomeric DNA repeats.

It has been well documented that the Rad50-Mre11-NBS1 triplex is involved in DNA double-strand break repair and, moreover, that specific nuclear foci positive for Rad50-Mre11-NBS1 are formed after ionizing radiation (8). Previous reports have suggested that, in the absence of DNA damage, no evident NBS1/Mre11 foci can be detected. Interestingly, we found that NBS1/Mre11 foci could be detected in about 5% of an asynchronously growing culture of human GM847 cells even in the absence of DNA damage treatment. These DNA damage-independent NBS1/Mre11 foci colocalize with TRF1 by immunostaining (Fig. 2), further supporting an *in vivo* association of NBS1 and TRF1 in human cells.

Yeager *et al.* (31) reported that TRF1 is associated with PML bodies in several telomerase-negative immortalized cell lines. Telomere length maintenance in this type of cells requires a telomerase-independent mechanism, previously designated as alternative lengthening of telomeres (ALT) (32). Coincidentally, the cell lines in which we observed DNA damage-independent NBS1/Mre11 foci are deficient in telomerase activity and immortalized through the ALT pathway. We therefore reasoned it likely that NBS1 might associate with PML bodies in these ALT cells. To determine whether this is the case, we examined a panel of telomerase-negative and -positive cell lines for the colocalization of NBS1 with PML bodies. As shown in Fig. 3, NBS1 colocalized with PML bodies in telomerase-negative immortalized cell lines including human GM847, Saos2, LM217, and VA13. However, in either telomerase-positive immortalized cell lines, such as T24 and MCF7, or human primary fibroblasts (IMR90), whereas PML bodies were detected, neither NBS1 foci nor NBS1 colocalization with PML bodies could be detected (Fig. 3).

Because only a small fraction of cells within a randomly growing telomerase-negative cell culture contain NBS1/Mre11 foci, it is likely that NBS1 foci formation is cell cycle stage-specific, perhaps during $G_2$ phase. To test this possibility, cells synchronized at the $G_1$/S boundary by a double thymidine block were released into the cell cycle and then fixed at specific time points post-release for immunostaining with NBS1-specific antibodies. As shown in Fig. 4, NBS1 foci formation in telomerase-negative immortalized cells occurs during late S/$G_2$ phase. This cell cycle-specific formation of NBS1 foci was not observed in telomerase-positive cell lines. To more accurately establish the point at which NBS1 foci formation occurs during S/$G_2$ phase, cells released from a double thymidine block were subsequently treated with Hoechst 33342, which specifically arrests cells at $G_2$ phase. A significantly higher percentage of NBS1 foci-containing cells were observed in ALT cell lines but not in T24, MCF7, or HeLa cells (Fig. 4). These results suggest that NBS1 specifically translocates to PML bodies in ALT cells at $G_2$ phase.

Telomere repeat sequences have been found in TRF1-containing PML bodies (31) and, as demonstrated above, NBS1 is localized to such PML bodies. It is therefore very likely that these PML bodies represent sites for maintaining telomere length in ALT cells. To test this possibility, a GFP-TRF1-expressing GM847 cell line was enriched for late S/$G_2$ phase cells, pulse-labeled with BrdUrd for 30 min, and immunostained with an anti-BrdUrd antibody. GM847 cells were also subjected to an identical procedure and immunostained with anti-BrdUrd and anti-NBS1 antibodies. As shown in Fig. 5, the PML bodies in a

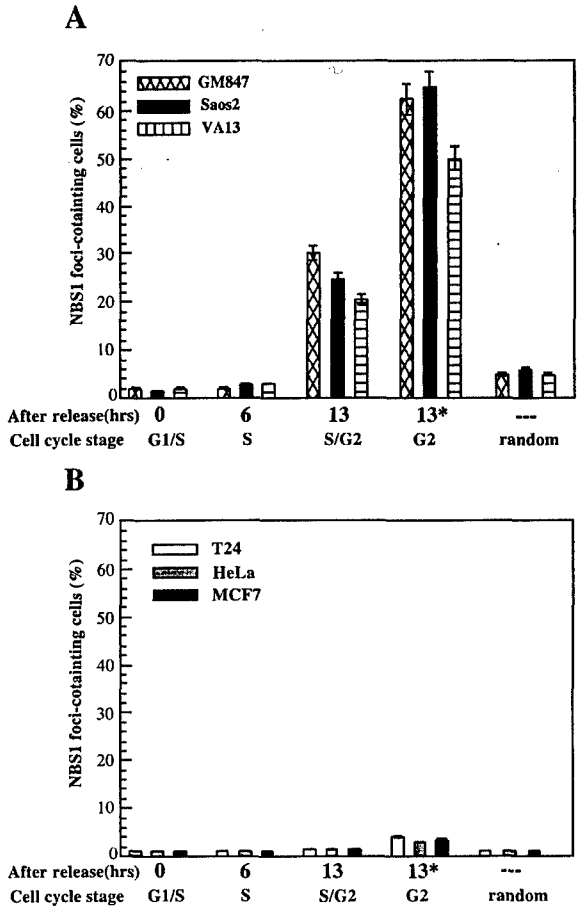


FIG. 4. **Cell cycle-dependent NBS1 foci formation in immortalized telomerase-negative cells.** Cells from a randomly growing population, or from the specified time points following release from a double thymidine block, were fixed and processed for immunostaining. The asterisk (*) shows an additional block by Hoechst 33342, in addition to the double thymidine treatment, which enriches $G_2$ cells more efficiently than double thymidine block alone. Cells were stained for NBS1. The percentage of NBS1 foci-containing cells are shown as Y coordinates in *A* and *B*. For every time point and for each cell line, at least 180 cells were scored, and the results are summarized from two or three independent experiments. In telomerase-negative immortal cells (GM847, Saos2, and VA13), NBS1 foci are most prominent at the late S/early $G_2$ boundary (*A*), whereas in telomerase-positive immortal cells (T24, MCF7, and HeLa), there is no significant NBS1 foci formation at any time point, compared with a random cell population (*B*). Therefore, NBS1 foci formation is cell cycle-dependent. This is also true for Mre11 (data not shown).

fraction of GM847 cells, as indicated by NBS1 immunostaining or GFP-TRF1 immunofluorescence, underwent active BrdUrd incorporation at late S/$G_2$ phase. By contrast, no BrdUrd incorporation could be observed in MCF7 cells following a similar labeling procedure. These results imply that the telomere lengthening process in ALT cells that requires DNA synthesis occurs in PML bodies at $G_2$ phase of the cell cycle.

NBS1 is an apparent multi-functional protein, with demonstrated roles in DNA double-strand break repair and S-phase checkpoint control (8, 19). The interaction between TRF1 and the NBS1 complex suggests that NBS1 may be involved in telomere length maintenance in ALT cells. A homologous recombination-mediated mechanism has been proposed for telomere length maintenance in telomerase-negative cells in organisms ranging from human to yeast (32). Interestingly, Rad51, Rad52, RPA, and NBS1/Mre11 are localized to PML bodies (see Ref. 31 and this report). Given that multiple key players in homologous recombination are localized to PML

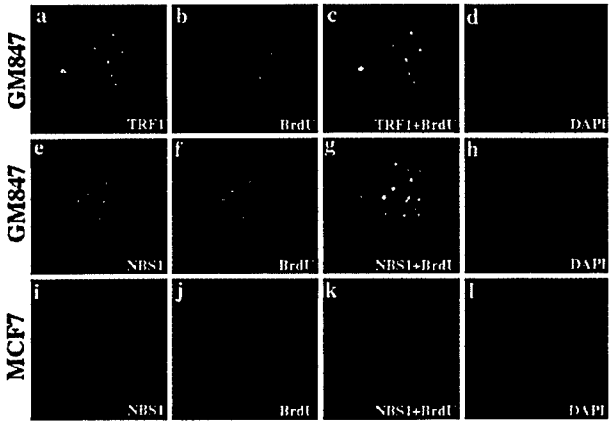


FIG. 5. **TRF1/NBS1 foci undergo active BrdUrd incorporation in late S/$G_2$ phase, suggestive of telomere lengthening.** All cells were subjected to a double thymidine block and then labeled with BrdUrd for 30 min at 13 h post-release. Subsequently cells were fixed and stained as described. A GFP-TRF1-inducible clone of GM847 was stained with a monoclonal BrdUrd antibody (*a–d*). A fraction of cells (5–10%) showed foci-like BrdUrd staining (*b*) that colocalize with TRF1 (*a*). In GM847 cells (*e–h*), this type of BrdUrd foci (*f*) also colocalize with NBS1 foci (*e*) and PML bodies by deduction. However, these BrdUrd foci were not observed in the telomerase-positive cell line MCF7 (*I–l*), which barely incorporated BrdUrd (*j*) and had relatively homogeneous NBS1 staining (*i*). *Panels b* and *f* indicate staining with Texas Red, and *e* and *i* with FITC. Panels *c*, *g*, and *k* indicate merged images. Panels *d*, *h*, and *l* are stained with DAPI.

bodies in telomerase-negative immortalized cells, it is reasonable to speculate that active DNA synthesis in these nuclear domains in late S/$G_2$ may reflect a telomere-maintenance process potentially involving a homologous recombination. However, the mechanistic details of how these factors work in concert remains to be investigated.

It has been reported that yeast Rad50-Mre11-XRS2 is important for telomere maintenance in a telomerase-dependent manner, perhaps by making DNA ends available for telomerase to replicate (33). The equivalent mammalian complex, Rad50-Mre11-NBS1, may share a similar activity, although it remains to be demonstrated. Electron microscopic analysis has revealed that mammalian telomeres end in a large duplex loop (34). Binding of TRF1 and single-strand-binding protein suggested that t loops be formed by invasion of the 3′ telomeric overhang into the duplex telomeric repeat array. It is possible that the association between TRF1 and NBS1 provides a bridge between the telomere repeat sequences and the nuclease complex of Mre11 that could make telomere ends available for replication. However, it remains to be shown that the NBS1-Mre11-Rad50 triplex is located at the telomere ends for this purpose.

NBS is a disorder characterized in part by an aging phenotype, and NBS1-deficient cells show accelerated telomere shortening *in vitro*. The link between NBS1 and TRF1 in telomere length maintenance provides a plausible explanation for these phenotypes.

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# Sequence-Specific Transcriptional Corepressor Function for BRCA1 through a Novel Zinc Finger Protein, ZBRK1

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## Summary

**BRCA1 has been implicated in the transcriptional regulation of DNA damage–inducible genes that function in cell cycle arrest. To explore the mechanistic basis for this regulation, a novel human gene, *ZBRK1*, which encodes a 60 kDa protein with an N-terminal KRAB domain and eight central zinc fingers, was identified by virtue of its interaction with BRCA1 in vitro and in vivo. ZBRK1 binds to a specific sequence, GGGxxx CAGxxxTTT, within *GADD45* intron 3 that supports the assembly of a nuclear complex minimally containing both ZBRK1 and BRCA1. ZBRK1 represses transcription through this recognition sequence in a BRCA1-dependent manner. These results thus reveal a novel corepressor function for BRCA1 and provide a mechanistic basis for the biological activity of BRCA1 through sequence-specific transcriptional regulation.**

## Introduction

Potential insight into the molecular basis for the caretaker function of BRCA1 has been provided by studies that implicate this tumor suppressor in both the repair of damaged DNA and the regulation of transcription (Miki et al., 1994; reviewed by Chen et al., 1999; Welcsh et al., 2000). Several lines of evidence support a direct role for BRCA1 in DNA damage repair. First, BRCA1-deficient cells are hypersensitive to ionizing radiation (IR) and characterized by defects in the repair of both oxidative DNA damage by transcription-coupled processes and chromosomal double-strand breaks by homologous recombination (Gowen et al., 1998; Moynahan et al., 1999; Zhong et al., 1999). Second, Brca1 mutant mouse embryo fibroblasts are characterized by genetic instability through improper regulation of centrosome duplication and defective $G_2$/M checkpoint control (Xu et al., 1999). Third, BRCA1 functionally interacts with the hRad50–hMre11–NBS1 complex that participates in both the DNA damage response and the repair of DNA double-strand breaks (Zhong et al., 1999). Finally, IR-induced phosphorylation of BRCA1 by ATM and hCds1/Chk2 appears to be critical for proper execution of the cellular DNA damage response (Cortez et al., 1999; Lee et al., 2000).

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A significant body of experimental evidence also implicates BRCA1 in the regulation of transcription. First, the carboxyl terminus of BRCA1 exhibits an inherent transactivation function that is sensitive to cancer-predisposing mutations (Chapman and Verma, 1996; Monteiro et al., 1996; Haile and Parvin, 1999). Second, BRCA1 has been identified as a component of the RNA polymerase II holoenzyme (Scully et al., 1997a; Anderson et al., 1998). Third, BRCA1 has been reported to interact with a variety of transcriptional activators or coactivators, such as p53 and CBP/p300, or corepressors, including CtIP/CtBP and histone deacetylases (HDACs) (Reviewed by Chen et al., 1999; Welcsh et al., 2000). Finally, BRCA1 has been reported to regulate transcription of genes that encode activities involved in cell cycle arrest, including *p21* and *GADD45* (Somasundaram et al., 1997; Harkin et al., 1999).

Thus, while BRCA1 likely ensures global genome stability through its dual participation in transcription and DNA double-strand break repair processes, a number of important issues remain to be resolved. For example, it is not presently clear how BRCA1-mediated transcriptional regulation is functionally linked to its role in DNA damage repair and cell cycle checkpoint control. Furthermore, it is not presently known how BRCA1 mediates gene-specific transcriptional regulation, since BRCA1 itself exhibits no sequence-specific DNA binding activity.

The recent identification of *GADD45* as a target gene transcriptionally induced by BRCA1 overexpression is of interest in this regard (Harkin et al., 1999). GADD45 has been implicated in a variety of growth regulatory processes, including activation of DNA damage–induced G2/M checkpoints (Wang et al., 1999) and maintenance of genome stability (Hollander et al., 1999). The regulatory mechanisms governing *GADD45* gene transcription have in part been revealed by the identification of discrete regulatory elements within its proximal promoter and intron 3 sequences and transcription factors, including p53 and CEB/P$\alpha$, capable of activating *GADD45* gene transcription (Kastan et al., 1992; Hollander et al., 1993; Constance et al., 1996). However, a more complete understanding of the regulatory events underlying its inducible control awaits a description of how BRCA1 regulates *GADD45* gene transcription.

Here we identify and characterize a novel zinc finger and *BRCA1*-interacting protein with a KRAB domain, designated ZBRK1. ZBRK1 binds to a specific consensus sequence within intron 3 of the *GADD45* gene through which it functions as a transcriptional repressor. Significantly, we find that this repression activity is mediated by BRCA1.

## Results

### Isolation of ZBRK1, which Contains a KRAB Domain and Eight Zinc Finger Motifs

*ZBRK1* was isolated as a strong positive clone, BRAP12, in a yeast two-hybrid screen for proteins associated

**A**

```
                                  M I Q A Q E S I T L E  11
D V A V D F T W E E W Q L L G A A Q K D L Y R D V M L E  39
N Y S N L V A V G Y Q A S K P D A L F K L E Q G E Q P W  67
T I E D G I H S G A C S D I W K V D H V L E R L Q S E S  95
L V N R R K P C H E H D A F E N I V H C S K S Q F L L G 123
Q N H D I F D L R G K S L K S N L T L V N Q S K G Y E I 151
K N S V E F T G N G D S F L H A N H E R L H T A I K F P 179
A S Q K L I S T K S Q F I S P K H Q K T R K L E K H H V 207
C S E C G K A F I K K S W L T D H Q V M H T G E K P H R 235
C S L C E K A F S R K F M L T E H Q R T H T G E K P Y E 263
C P E C G K A F L K K S R L N I H Q K T H T G E K P Y I 291
C S E C G K G F I Q K G N L I V H Q R I H T G E K P Y I 319
C N E C G K G F I Q K T C L I A H Q R F H T G K T P F V 347
C S E C G K S C S Q K S G L I K H Q R I H T G E K P F E 375
C S E C G K A F S T K Q K L I V H Q R T H T G E R P Y G 403
C N E C G K A F A Y M S C L V K H K R I H T R E K Q E A 431
A K V E N P P A E R H S S L H T S D V M Q E K N S A N G 459
A T T Q V P S V A P Q T S L N I S G L L A N R N V V L V 487
G Q P V V R C A A S G D N S G F A Q D R N L V N A V N V 515
V V P S V I N Y V L F Y V T E N P                      532
```

**B**

```
                    A Box                                  B Box
CONSENSUS     VTF DVAV F  EEW  LD  QR LYR VMLENY  LVSL G    KPDL   LEQ   W

ZNF7        2EVVTFGDVAVHFSREEWQCLDPGQRALYREVMLENHSSVAGLAGFLVFKPELISRLEQGEEPW
ZNF90       2GPLEFRDVAIEFSLEEWHCLDTAQQNLYRDVMLENYRHLVFL.GIVVTKPDLITCLEQGKKPF
ZNF85       2GPLTFRDVAIEFSLKEWQCLDTAQRNLYRNVMLENYRNLVFL.GITVSKPDLITCLEQGKEAW
ZNF141      2ELLTFRDVAIEFSPEEWKCLDPDQQNLYRDVMLENYRNLVSL.GVAISNPDLVTCLEQRKEPY
ZNF45       6EAVTFKDVAVVFSEEELQLLDLAQRKLYRDVMLENFRNVVSV.GHOST.PDGLPQLEREEKLW
ZNF10      12TLVTFKDVFVDFTREEWKLLDTAQQILYRNVMLENYKNLVSL.GYQLTKPDVILRLEKGEEPW
ZNF177     12GLLTFQEVFVDFSQEEWGLLDPAQKNLYKDVMLE.FRNLASV.GYQLCRHSLISKVDQEQLKT
ZNF91      12GLLTFRDVAIEFSPEEWQCLDTAQQNLYRNVMLENYRNLAFL.GIALSKPDLIPYLEQGKEPW
ZNF157     24GSVSFEDVAVDFTRQEWHRLDPAQRTMHKDVMLETYSNLASV.GLCVAKPEMIFKLERGEELW
ZNF140      4GSVTFRDVAIDFSQEEWKWLQPAQRDLYRCVMLENYGHLVSL.GLSISKPDVVSLLEQGE.PW

ZBRK1       6ESITLEDVAVDFTWEEWQLLGAAQKDLYRDVMLENYSNLVAV.GYQASKPDALFKLEQGEQPW
```

Figure 1. *ZBRK1* cDNA Encodes a Novel Protein with a KRAB Domain and a Zinc Finger Domain

(A) The ZBRK1 protein sequence. The KRAB domain is underlined, and the Cys and His residues of the C2H2-type zinc fingers are shadowed.

(B) Amino acid sequence alignment of the ZBRK1 KRAB domain with KRAB domains of other zinc finger proteins (Friedman et al., 1996). Conserved residues are indicated by bold letters.

with BRCA1 (Chen et al., 1996). RNA blot analysis using the original 1.2 kb cDNA as a probe revealed a single 2.4 kb mRNA that was expressed in breast epithelial cell lines HBL100, MCF10A, and breast cancer cell lines T47D, MB231, and MCF7 (data not shown). A full-length cDNA was subsequently isolated that encodes a novel human protein of 532 amino acids bearing eight consecutive Krupple-type C2H2 zinc finger motifs within its central region (Figure 1A). The N-terminal region shares significant homology with KRAB domains (Margolin et al., 1994; Witzgall et al., 1994) that have been identified in many zinc finger proteins (Figure 1B). The presence within its predicted coding sequence of these consensus functional motifs (zinc fingers and a KRAB domain) suggests that *ZBRK1* encodes a transcription factor.

### ZBRK1 Interacts with BRCA1 In Vitro

To define the ZBRK1 interaction region(s) on BRCA1, in vitro translated ZBRK1 was tested for its ability to bind to different polypeptide fragments of BRCA1 fused to GST (Figures 2A and 2B) as described (Li et al., 1998; Zhong et al., 1999). ZBRK1 bound specifically to GST-BRCA1Bgl, containing BRCA1 amino acid residues 341–748 (aa 341–748), but not to GST-BRCA1N (aa 1–302), GST-BRCA1M (aa 762–1315), or GST-BRCA1C (aa 1316–1863) (Figure 2C).

To confirm this result, different fragments of BRCA1 fused in-frame with the Gal4 DNA-binding domain (Zhong et al., 1999) were individually tested for their respective abilities to interact with full-length ZBRK1 fused to the Gal4 transactivation domain (Figure 2A) by yeast two-hybrid assay (Durfee et al., 1993). Corresponding β-galactosidase activities revealed binding of ZBRK1 to both BRCA1Bgl (aa 341–748), as well as BRCA1-3.5 (aa 1–1142), the original bait used in the yeast two-hybrid screen from which ZBRK1 was first isolated (Chen et al., 1996) (Figure 2D). Fragments corresponding to two regions other than BRCA1Bgl within BRCA1-3.5, BRCA1N (aa 1–302) and BRCA1StuI/PstI (aa 513–914), exhibited little binding activity. These results confirm those of the in vitro binding assays and thus reveal that ZBRK1 binds to BRCA1 aa 341–748.

To map the regions on ZBRK1 required for binding to BRCA1, the BRCA1Bgl region (aa 341–748), carrying a FLAG epitope, was translated in vitro and subsequently tested for binding to different regions of ZBRK1 fused with GST (Figures 2E and 2F). BRCA1Bgl bound to full-length ZBRK1 and to ZBRK1C1 (aa 319–532), but not to ZBRK1N, (aa 1–168) nor to ZBRK1Zn, corresponding to the zinc-finger domain (aa 169–439) (Figure 2G). Interestingly, ZBRK1C2, corresponding to the C-terminal 94 amino acids of ZBRK1 (aa 439–532), was not sufficient for binding to BRCA1, implying that ZBRK1 sequences encoding the last four zinc fingers are also required for BRCA1 binding. Therefore, a C-terminal region corresponding to amino acids 319–532 is required for ZBRK1 to bind to BRCA1.

### ZBRK1 Is a 60 kDa Cellular Protein that Interacts with BRCA1 In Vivo

To identify the cellular protein encoded by ZBRK1, we raised mouse polyclonal antibodies specific for ZBRK1 residues 68–208, a less conserved region among KRAB domain–containing zinc finger proteins. These antibod-

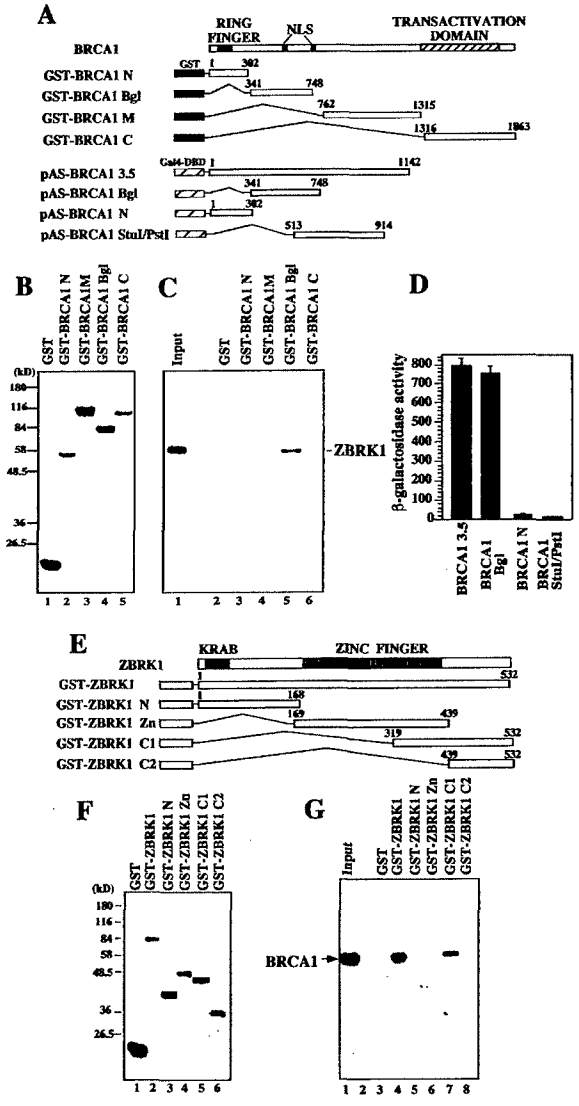


Figure 2. Specific Interaction between ZBRK1 and BRCA1

(A) Schematic diagram of the full-length BRCA1 protein and different BRCA1 polypeptide fragments fused to GST or the Gal4 DNA-binding domain.

(B) Purified GST-BRCA1 fusion proteins were visualized by SDS–PAGE and Coomassie blue staining. Comparable amounts of each fusion protein were used for binding reactions shown in (C).

(C) Specific binding of $^{35}$S-methionine-labeled in vitro–translated ZBRK1 to GST-BRCA1Bgl (lane 5) but not to other BRCA1 polypeptide fragments, as detected by SDS–PAGE and subsequent autoradiography. Lane 1 represents the total input of in vitro–translated ZBRK1 used in each binding reaction.

(D) The indicated fragments of BRCA1, expressed as Gal4 DNA-binding domain fusion proteins, were tested for interaction with ZBRK1 fused to the Gal4 transactivation domain in yeast two-hybrid assays. β-galactosidase activities were quantified as described (Durfee et al., 1993).

(E) Schematic diagram of the full-length ZBRK1 and different polypeptide fragments of ZBRK1 fused to GST.

(F) Comparable amounts of each fusion protein were used for binding reactions shown in (G).

(G) Specific binding of in vitro–translated BRCA1 Bgl fragment to the full-length ZBRK1 (lane 4) and the ZBRK1C1 region (lane 7). Lane 1 represents the total input of in vitro–translated BRCA1 Bgl fragment used in each binding reaction.

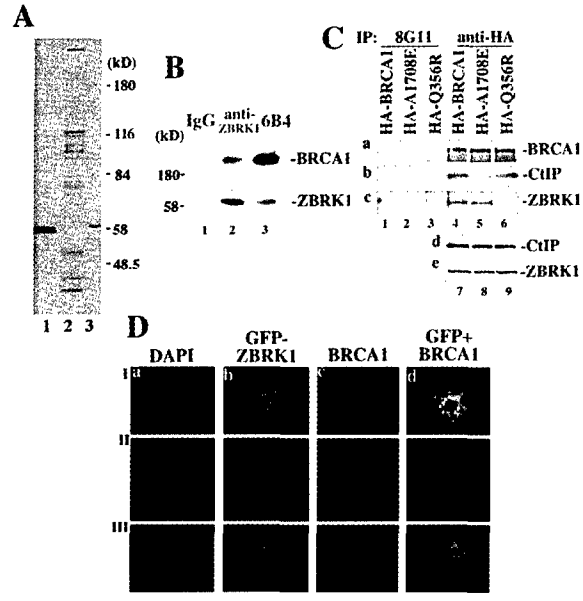


Figure 3. Detection of Cellular ZBRK1 Protein and In Vivo Interaction of ZBRK1 and BRCA1

(A) Detection of cellular ZBRK1 protein. Lysate from $^{35}$S-methionine-labeled T24 cells ($1.6 \times 10^7$) was subjected to immunoprecipitation once by preimmune sera (lane 2) or twice by anti-ZBRK1 antibodies (lane 3). In vitro translated ZBRK1 is indicated in lane 1.

(B) In vivo interaction between ZBRK1 and BRCA1. T24 cell ($1.6 \times 10^7$) lysate was immunoprecipitated by rabbit IgG (lane 1), anti-ZBRK1 antibodies (lane 2), or anti-BRCA1 mAb 6B4 (lane 3). Immunoprecipitated proteins were eluted by boiling in SDS sample buffer and separated by SDS-7.5% PAGE followed by immunoblot analysis with 6B4 to detect BRCA1 (upper panel) or with anti-ZBRK1 antibodies to detect ZBRK1 (lower panel).

(C) Specific coimmunoprecipitation of ZBRK1 and BRCA1. Myc-GFP-ZBRK1 was cotransfected with either HA-tagged wild-type BRCA1 (lanes 1, 4, and 7), or BRCA1 mutants, A1708E (lanes 2, 5, and 8), or Q356R (lanes 3, 6, and 9). Lysates were immunoprecipitated with anti-GST mAb 8G11 (lanes 1–3) or anti-HA (Santa Cruz) polyclonal antibodies (lanes 4–6). The immune complexes were separated by SDS-7.5% PAGE followed by immunoblot analysis with anti-BRCA1 mAb 6B4 to detect BRCA1 (a), anti-CtIP mAb to detect CtIP (b), and anti-GFP mAb (Clontech) to detect GFP-tagged ZBRK1 (c). Equivalent amount of CtIP or GFP-tagged ZBRK1 was present in the lysates (lanes 7–9) as determined by immunoblot analysis with anti-CtIP mAb (d) and anti-GFP mAb (e).

(D) Nuclear distribution patterns of ZBRK1 and BRCA1. Three representative U2OS cells stably transfected with GFP-ZBRK1 shown in I, II, and III, respectively, were stained with DAPI (row a) and anti-BRCA1 mAb Ab-1 (Oncogene Research Products) followed by Texas red–conjugated secondary antibody (row c), as described elsewhere (Zhong et al., 1999). Row b shows the images of GFP signals, and row d shows the merged images of GFP signals and BRCA1 staining.

ies were subsequently used to immunoprecipitate ZBRK1 from $^{35}$S-methionine-labeled cell lysates of human bladder carcinoma T24 cells. As shown in Figure 3A, anti-ZBRK1 antibodies immunoprecipitated a 60 kDa protein that was absent from a parallel immunoprecipitate of the same cells using preimmune serum. This 60 kDa protein exhibits an electrophoretic mobility upon SDS–PAGE similar to that of the product translated in vitro from the *ZBRK1* cDNA and thus appears to represent the cellular ZBRK1 protein.

To test whether BRCA1 and ZBRK1 bind to each other in vivo under physiological conditions, we performed

coimmunoprecipitation analysis of the endogenous proteins in T24 cells. As shown in Figure 3B, both ZBRK1 and BRCA1 were specifically and reciprocally coprecipitated with each other, while rabbit IgG did not precipitate either protein from an equivalent amount of cell lysate. This result demonstrates directly that ZBRK1 is associated with BRCA1 in vivo.

To determine the specificity of this interaction in vivo, we performed coimmunoprecipitation analyses from human osteosarcoma U2OS cells cotransfected with GFP-tagged ZBRK1 and either HA-tagged wild-type BRCA1 or, alternatively, either of two familial breast cancer–derived BRCA1 mutants. One of these, Q356R, carries a mutation within the ZBRK1-binding region of BRCA1, while the second, A1708E, carries a mutation that disrupts CtIP binding activity (Li et al., 1999). ZBRK1 and CtIP were both coimmunoprecipitated along with wild-type BRCA1 using HA epitope–specific antibodies (Figure 3C). Mutant BRCA1-A1708E bound to ZBRK1, but not to CtIP, while mutant BRCA1-Q356R bound to CtIP, but not to ZBRK1. These results thus reveal that BRCA1 and ZBRK1 interact specifically in cells and, furthermore, that this interaction is compromised by a clinically relevant BRCA1 missense mutation identified in familial breast cancer.

Because BRCA1 and ZBRK1 interact in vivo, it was of interest to examine the subcellular distribution of these two proteins. To this end, immunofluorescence analysis was performed on U2OS cells stably expressing GFP-ZBRK1. Within an asynchronously growing culture, approximately 37%–45% of cells exhibited distinct BRCA1 immunoreactive foci against a background of homogeneous BRCA1 nuclear staining (Figure 3D, row c, I and II), consistent with previous observations (Scully et al., 1997b). GFP-ZBRK1 exhibited exclusively a homogeneous distribution throughout the nucleus and did not form foci (row b). In those cells that did not exhibit BRCA1 foci, ZBRK1 and BRCA1 exhibited a similar nuclear distribution pattern. Neither BRCA1 nor GFP-ZBRK1 was localized to nucleoli. Therefore, protein complexes containing both BRCA1 and ZBRK1 are likely to be distributed throughout nuclei, but not specifically localized within either nucleoli or nuclear foci that are more likely relevant to the function of BRCA1 in DNA repair (Scully et al., 1997b; Zhong et al., 1999).

## Identification of a Consensus ZBRK1 DNA-Binding Sequence

The zinc finger domain of ZBRK1 conforms to a classic C2H2 zinc finger domain with sequence-specific DNA-binding potential. To identify a binding sequence for ZBRK1, we employed a method for selection and amplification of DNA-binding sites (Blackwell and Weintraub, 1990). Purified GST-ZBRK1Zn was incubated with a random pool of double-stranded 55-mer oligonucleotides, each of which carried 16 base fixed-end sequences flanking 23 central bases of random sequences. Specific GST-ZBRK1Zn-DNA complexes were resolved by EMSA, and bound oligonucleotides were recovered and subjected to PCR amplification using primers corresponding to the 16 fixed bases at both ends of the 55-mer. As shown in Figure 4A, the affinity of GST-ZBRK1Zn for 55-mer pools selected by EMSA following the second, fourth, and sixth rounds of amplification increased with repeated selection. The PCR products from the final round were subcloned and sequenced. A consensus sequence of GGGxxxCAGxxxTTT was derived from sequence alignment of individual subclones (Figure 4B).

To test the binding specificity of ZBRK1 to this consensus sequence, GST-ZBRK1Zn was analyzed by EMSA for its ability to bind to an oligonucleotide containing the consensus sequence GGGxxxCAGxxxTTT (wild-type probe; Wt) or, alternatively, an oligonucleotide identical in length but different in sequence (Mutant probe; Mut) (Figure 4C). Consistently, GST-ZBRK1Zn

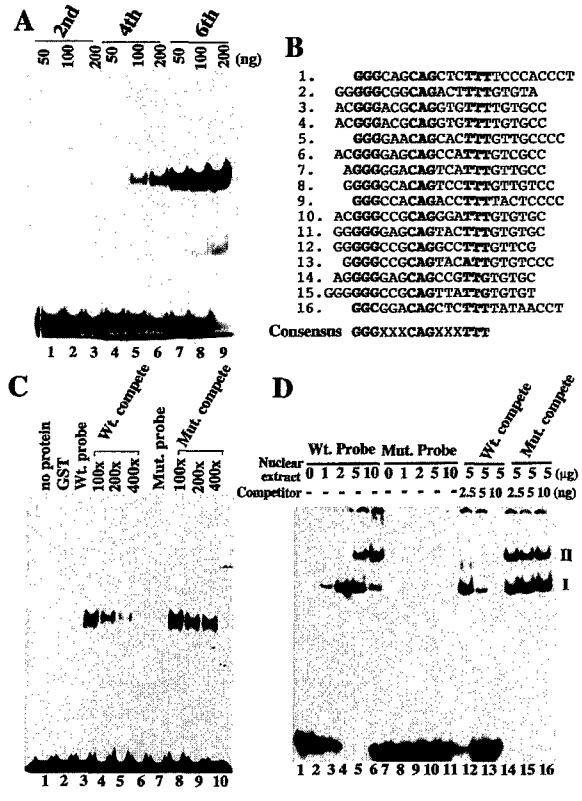


Figure 4. ZBRK1 Binds to a Specific DNA Sequence

(A) Selection and amplification of a ZBRK1-binding site. Equivalent amounts of PCR-amplified DNA derived from each of the second, fourth, and sixth rounds of the SAAB assay were subjected to EMSA using 50, 100, or 200 ng of recombinant GST-ZBRK1Zn protein.

(B) Alignment of individual DNA sequences selected by SAAB assay. The deduced consensus ZBRK1 DNA-binding sequence is indicated below the individually aligned sequences.

(C) Competition EMSA assays. EMSA was performed with Wt. probe, corresponding to the consensus ZBRK1 binding sequence, and either no added protein (lane 1), GST alone (lane 2), or GST-ZBRK1Zn (lanes 3–6 and lanes 8-10). Mut probe, corresponding to a double-stranded oligonucleotide identical in length but differing in sequence from the Wt. probe, was incubated with GST-ZBRK1Zn (lane 7). A 100-, 200-, or 400-fold molar excess of unlabeled Wt. probe (lanes 4–6) or Mut. Probe (lanes 8–10) was added to binding reactions as indicated.

(D) Nuclear complex formation on Wt. probe. EMSA was performed with the indicated amounts of T24 cell nuclear extract and radioactively labeled Wt. probe (lanes 1–5 and 11–16) or Mut Probe (lanes 6–10). A molar excess of unlabeled Wt. probe (lanes 11–13) or Mut probe (lanes 14–16) was added to binding reactions as indicated. DNA–protein complexes I and II are indicated.

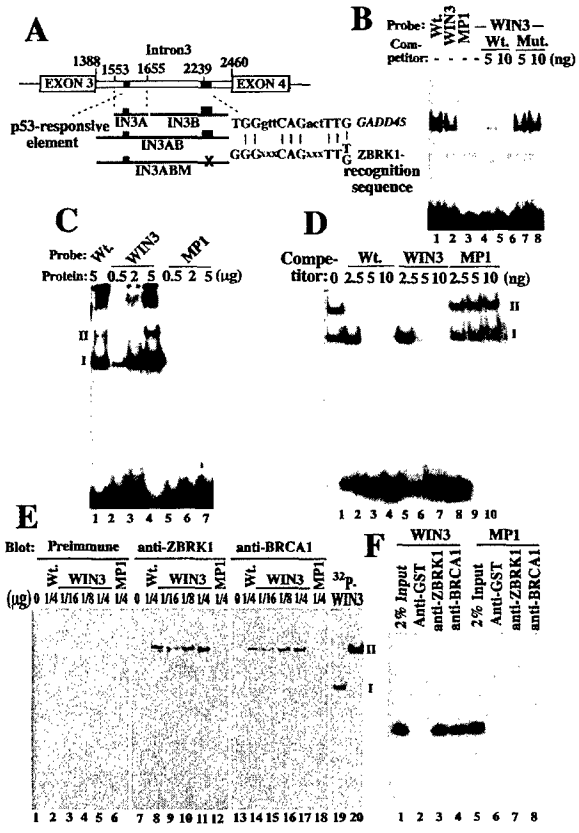


Figure 5. The ZBRK1 Recognition Sequence in *GADD45* Intron 3 Binds to a Nuclear Complex Containing Both ZBRK1 and BRCA1

(A) Schematic diagram of the *GADD45* intron 3 gene region. The p53 responsive element is indicated by a small black box, and the putative ZBRK1 recognition sequence is indicated by a large black box. The consensus ZBRK1 recognition sequence is aligned with *GADD45* intron 3 sequences.

(B) GST-ZBRK1Zn binds to the *GADD45* intron 3–derived ZBRK1 recognition sequence. EMSA performed with 50 ng GST-ZBRK1Zn (lanes 1–3) or GST (lane 4) and radioactively labeled Wt (lane 1), WIN3 (lanes 2 and 4–8), or MP1 (lane 3) probes. Binding reactions included either no specific competitor DNA (lanes 1–4) or the indicated amounts of unlabeled Wt (lanes 5 and 6), or Mut (lanes 7 and 8) probes.

(C) Nuclear complex formation on the *GADD45* intron 3–derived ZBRK1 recognition sequence. EMSA was performed with the indicated amounts (μg) of T24 nuclear extract and radioactively labeled Wt (lane 1), WIN3 (lanes 2–4), or MP1 (lanes 5–7) probes.

(D) Competitive EMSA assays. EMSA was performed with 5 μg T24 nuclear extract and radioactively labeled WIN3 probe. Binding reactions included either no specific competitor DNA (lane 1) or the indicated amounts of unlabeled Wt (lanes 2–4), WIN3 (lanes 5–7), or MP1 (lanes 8–10) probes.

(E) WIN3 probe binds a nuclear complex containing ZBRK1 and BRCA1. Fifty micrograms of nuclear extract in each of lanes 1–18 was incubated with no probe (lanes 1, 7, and 13), the Wt. probe (lanes 2, 8, and 14), indicated amounts of the WIN3 probe (lanes 3–5, 9–11, and 15–17), or the MP1 probe (lanes 6, 12, and 18). Binding reactions were resolved by electrophoresis on a native polyacrylamide gel, and DNA–protein complexes were subsequently analyzed by Western transfer and immunoblotting with anti-ZBRK1 antibodies (lanes 7–12). The blot was stripped of antibodies and sequentially immunoblotted with mouse preimmune serum (lanes 1–6) and anti-BRCA1 mAb 6B4 (lanes 13–18). Control binding reactions using 2 or 50 μg of nuclear extract and radioactively labeled WIN3 probe were run on the same gel to mark the electrophoretic positions of DNA–protein complexes I and II (lanes 19 and 20).

(F) Coimmunoprecipitation of the WIN3 probe with ZBRK1 and BRCA1. Equivalent amounts of nuclear extract were incubated with radioactively labeled WIN3 (lanes 1–4) or MP1 (lanes 5–8) probes, followed by immunoprecipitation with anti-GST mAb 8G11 (lanes 2 and 6), anti-ZBRK1 antibodies (lanes 3 and 7), or anti-BRCA1 mAb 6B4 (lanes 4 and 8). Lanes 1 and 5 indicate 2% of the total amount of each probe used in binding reactions.

bound to the Wt probe (lane 3), but not to the Mut probe (lane 7). The specificity of GST-ZBRK1Zn binding to its consensus sequence was further revealed by testing the ability of a molar excess of unlabeled Wt or Mut probe, respectively, to compete for the binding of GST-ZBRK1Zn to the Wt probe. The results of these competition EMSA assays indicated that the Wt probe, but not the Mut probe, could effectively compete for GST-ZBRK1Zn binding to its Wt consensus sequence (Figure 4C), thereby suggesting that the ZBRK1 zinc fingers comprise a sequence-specific DNA-binding domain.

### A Nuclear Protein Complex Binds to the ZBRK1 Recognition Sequence

Should the consensus ZBRK1 binding sequence that we derived represent a biologically relevant motif, it should support the assembly of a DNA–protein complex with one or more cellular proteins. To test this notion, $^{32}$P-labeled Wt or Mut probes were used in an EMSA with nuclear extract derived from cultured cells. A DNA–protein complex (complex I) was formed on the Wt probe (Figure 4D, lanes 1–5) but not on the Mut probe (lanes 6–10). Increased nucleoprotein complex formation was observed when the amount of input nuclear extract was increased. Interestingly, a more slowly migrating complex (complex II) appeared as more nuclear extract was used. Consistently, a molar excess of the unlabeled Wt probe could effectively compete for the formation of both complexes I and II (Figure 4D, lanes 11–13). Formation of neither complex I nor II could be efficiently competed by the mutant probe (Figure 4D, lanes 14–16). Immunoblot analysis following EMSA using procedures described for Figure 5E (below) confirmed the presence of ZBRK1 in complex II. These results thus reveal the presence in nuclear extract of one or more activities containing endogenous ZBRK1 capable of binding to the ZBRK1 consensus sequence.

### *GADD45* Intron 3 Harbors a ZBRK1 Recognition Sequence

Recently, the *GADD45* gene was identified as a major transcriptional target of BRCA1 (Harkin et al., 1999). Because ZBRK1 interacts with BRCA1 in vivo, we reasoned that ZBRK1 might also participate in the regulation of *GADD45* gene transcription. On this basis, we searched the *GADD45* gene and identified within intron 3 a sequence, TGGxxxCAGxxxTTG, which conforms closely to the ZBRK1 consensus sequence (Figure 5A). Interestingly, intron 3 also harbors a functionally important p53 response element residing upstream of this potential ZBRK1-binding site, and the two elements are separated by a nonconserved AT-rich region within intron 3 (Hollander et al., 1993). Significantly, the potential ZBRK1-binding site lies within a region that exhibits

considerable sequence conservation between species (Hollander et al., 1993).

To determine whether ZBRK1 can bind to this potential ZBRK1-binding sequence, GST-ZBRK1Zn was tested by EMSA for its ability to bind to the WIN3 probe, which is identical in sequence to the potential ZBRK1-binding site. As shown in Figure 5B, the WIN3 probe supported the formation of a DNA–protein complex (lane 2) indistinguishable in its electrophoretic mobility from the complex formed on the Wt probe, which contained the consensus ZBRK1 binding sequence (lane 1). Interestingly, GST-ZBRK1Zn did not bind to the MP1 probe that corresponds to a sequence (GATxxxCAGxxxTTT) present in the proximal promoter region of *GADD45* that differs by two nucleotides from the consensus ZBRK1 recognition sequence (lane 3). The binding of GST-ZBRK1Zn to the WIN3 probe could be effectively competed by the addition of a molar excess of unlabeled Wt probe, but not by an equivalent molar excess of the Mut probe (lanes 5–8). These results thus identify the putative ZBRK1 recognition sequence within *GADD45* intron 3 to be an authentic ZBRK1-binding site.

### A Nuclear Protein Complex Including ZBRK1 and BRCA1 Binds to the ZBRK1 Recognition Sequence in *GADD45* Intron 3

Next, we determined by EMSA whether the WIN3 probe could serve as a platform for the assembly of proteins present in a crude nuclear extract. As shown in Figure 5C, the WIN3 probe supported the formation of DNA–protein complexes (lanes 2–4) indistinguishable in their electrophoretic mobilities from the complexes formed on the Wt probe (lane 1). By contrast, the MP1 probe did not support the formation of a stable DNA–protein complex (lanes 5–7). Nucleoprotein complex formation on the WIN3 probe was specifically competed by the addition of a molar excess of unlabeled Wt or WIN3 probe, but not by an equivalent molar excess of the MP1 probe (Figure 5D).

To test whether the nuclear complex formed on the WIN3 probe contains both ZBRK1 and BRCA1, we performed EMSA followed by immunoblot analysis (Mueller et al., 1990) using antibodies specific for both ZBRK1 and BRCA1. Parallel binding reactions were assembled using 50 μg of nuclear extract with no probe (Figure 5E; lanes 1, 7, and 13), the Wt. probe (lanes 2, 8, 14), the WIN3 probe (lanes 3–5, 9–11, and 15–17), or the MP1 probe (lanes 6, 12, and 18). Resultant DNA–protein complexes were resolved by native gel electrophoresis and subsequently subjected to Western transfer and immunoblot analysis using mouse preimmune sera (lanes 1–6), anti-ZBRK1 antibodies (lanes 7–12), or anti-BRCA1 mAb 6B4 (lanes 13–18). Control EMSA reactions using two different amounts of nuclear extract and radioactively labeled WIN3 probe were loaded onto the same gel to mark the electrophoretic positions of DNA–protein complexes I and II (lanes 19 and 20). Both ZBRK1 and BRCA1 were detected in complex II in a dose-dependent manner (lanes 9–11 and 15–17). Neither ZBRK1 nor BRCA1 could be detected when EMSA reactions were performed using either no probe or the MP1 probe. Additionally, no immunoblot signal was detected using preimmune sera (lanes 1–6). Because immunodetection required the use of preparative amounts of nuclear extract for individual binding reactions, most of the DNA–protein complex formed on the WIN3 probe represented complex II (compare lane 20 with lane 19). Consequently, we cannot exclude the possibility that complex I also contains BRCA1 and ZBRK1.

To demonstrate further that ZBRK1 and BRCA1 reside within the same complex, DNA coimmunoprecipitation assays (Yew et al., 1994) were performed. Radioactively labeled WIN3 or MP1 probes were incubated with nuclear extract, followed by immunoprecipitation of protein–DNA complexes with antibodies specific for either ZBRK1, BRCA1, or, as a negative control, GST. Immunoprecipitated protein–DNA complexes were deproteinized, and the radioactively labeled probe was subsequently resolved by native gel electrophoresis and detected by autoradiography. As shown in Figure 5F, the WIN3 probe, but not the MP1 probe, was specifically coimmunoprecipitated by both anti-ZBRK1 and anti-BRCA1 antibodies. By contrast, neither the WIN3 probe nor the MP1 probe was immunoprecipitated by anti-GST mAb. Collectively, these results thus reveal the *GADD45* intron 3 ZBRK1 recognition sequence to be a platform for the specific assembly of a nuclear complex containing both ZBRK1 and BRCA1.

### ZBRK1 Is a Sequence-Specific Transcriptional Repressor

The presence within ZBRK1 of both zinc finger and KRAB domains indicated that it might function as a sequence-specific transcriptional repressor. To test this possibility, a plasmid containing four copies of the ZBRK1 consensus binding site upstream of the HSV TK promoter driving expression of a CAT reporter gene (pBLcat-E) was engineered and transfected into human osteosarcoma Saos2 cells. The resultant CAT activity was compared with that of an otherwise identical reporter template lacking the four ZBRK1-binding sites (pBLcat). As shown in Figure 6A, the ZBRK1 recognition sequences conferred a 4-fold repression on pBLcat reporter activity. Subsequently, an expression plasmid carrying the *ZBRK1* cDNA driven by the CMV promoter (pCHPL-ZBRK1) was individually cotransfected along with each of the aforementioned CAT reporter templates. Expression of exogenous ZBRK1 caused an additional 2-fold repression of CAT activity from the reporter carrying ZBRK1-binding sites but had no effect on the control CAT reporter. These results thus reveal that the repression activity of ZBRK1 is mediated through its cognate recognition sequence.

### ZBRK1 Confers Repression through Intron 3 of the *GADD45* Gene

Intron 3 of the *GADD45* gene, from +1388 to +2460 (where +1 represents the transcription start site), represents a critical regulatory locus that coordinately controls transcription in conjunction with *cis*-acting elements in the proximal promoter region (Hollander et al., 1993). The p53 response element in the *GADD45* gene lies within an intron 3 fragment extending from +1553 to +1655 (herein designated as IN3A; Figure 5A). The ZBRK1-binding site (WIN3) resides in a fragment extending from +1656 to +2460 (herein designated as

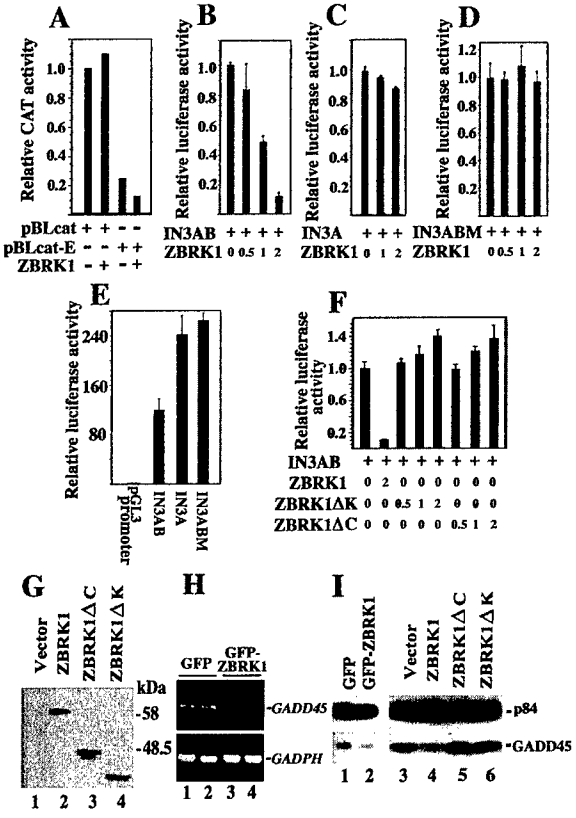


Figure 6. ZBRK1 Is a Transcriptional Repressor through *GADD45* Intron 3

(A) pBLcat or pBLcat-E (containing four copies of the ZBRK1 recognition sequence) was transfected into Saos2 cells either with (+) or without (−) pCHPL-ZBRK1. Resultant CAT activities are expressed relative to the level of CAT activity observed with pBLcat vector–transfected cells.

(B–E) U2OS cells were transfected as indicated with 0.5 μg of pGL3p-IN3AB (B and E), pGL3p-IN3A (C and E), pGL3p-IN3ABM (D and E), or pGL3p (E) along with the indicated amounts (micrograms of DNA) of pCEPF-ZBRK1.

(F) U2OS cells were transfected with pGL3p-IN3AB (0.5 μg) along with the indicated amounts (micrograms of DNA) of either pCNF-ZBRK1, pCNF-ZBRK1ΔK, or pCNF-ZBRKΔC. In (A)–(F), relative CAT or luciferase activities are calculated as the fold increases in CAT or luciferase activities relative to those observed in cells transfected by reporter and CMV vector alone (first lane in each panel). CAT or luciferase activities were normalized to β-galactosidase activity obtained by cotransfection of 1 μg of SV40-β-gal vector as described previously (Li et al., 1999).

(G) Expression of Flag-tagged wild-type ZBRK1 and ZBRK1 mutant derivatives in U2OS cells transiently transfected with pCNF, pCNF-ZBRK1, pCNF-ZBRK1ΔC, and pCNF-ZBRK1ΔK. Flag-tagged proteins were detected by immunoblot analysis using anti-Flag mAb M2 (Sigma).

(H) Semiquantitative RT–PCR analysis of endogenous *GADD45* (upper panel) or *GADPH* (lower panel) mRNAs in U2OS cells stable-expressing GFP (lanes 1 and 2) or GFP-ZBRK1 (lanes 3 and 4). RT–PCR reactions were run in duplicate.

(I) Expression of endogenous GADD45 protein in U2OS cells stable-expressing GFP (lane 1) or GFP-ZBRK1 (lane 2), or transiently transfected with either pCNF, pCNF-ZBRK1, pCNF-ZBRK1ΔC, or pCNF-ZBRK1ΔK (lanes 3–6, respectively). GADD45 (lower panel) or nuclear matrix protein p84 (upper panel, included as an internal loading control [Li et al., 1999]) were detected by immunoblot analysis using rabbit anti-GADD45 antibody H-165 (Santa Crutz), and anti-p84 mAb 5E10, respectively.

IN3B; Figure 5A). Intron 3 in its entirety is designated as IN3AB (Figure 5A).

To address whether the WIN3 sequence represents a functional ZBRK1 response element in its natural context, we constructed pGL3p-IN3AB by inserting IN3AB upstream of the *SV40* promoter driving expression of a luciferase reporter. This reporter template was cotransfected into U2OS cells along with increasing amounts of the ZBRK1-expressing plasmid, pCEPF-ZBRK1. ZBRK1 repressed reporter activity in a dose-dependent manner (Figure 6B). Consistent with a functional requirement for its cognate recognition sequence, ZBRK1 had no effect on the activity of a reporter template containing only the IN3A region (Figure 6C) or on the activity of the IN3ABM reporter template in which the WIN3 sequence was mutated (Figure 6D). Consistently, the reporter activity of IN3AB is lower than that of either IN3A or IN3ABM (Figure 6E). Thus, the *cis*-acting DNA sequence within *GADD45* intron 3 responsible for conferral of ZBRK1-directed transcriptional repression is the ZBRK1-binding site located in the IN3B region.

### ZBRK1 Repression Activity Is Mediated Both by Its KRAB Domain and BRCA1-Binding Domain

To understand the mechanism(s) by which ZBRK1 represses transcription, we first sought to determine whether the KRAB domain of ZBRK1 is required. To this end, we engineered ZBRK1ΔK, a deletion derivative of ZBRK1 that carries an N-terminal truncation of the KRAB domain. ZBRK1ΔK was cotransfected along with the IN3AB reporter template into U2OS cells. ZBRK1ΔK failed to repress transcription from the IN3AB reporter template (Figure 6F), indicating that the KRAB domain on ZBRK1 is important for its repression activity. By implication, the KRAB domain–interacting corepressor KAP-1/KRIP-1/TIFβ (Friedman et al., 1996; Kim et al., 1996; Le Douarin et al., 1996; Moosmann et al., 1996) is likely to contribute to ZBRK1-directed repression, although future studies will be required to confirm this prediction.

To assess the requirement of its C-terminal BRCA1-interacting domain for ZBRK1 repression activity, we engineered ZBRK1ΔC, a ZBRK1 deletion derivative lacking its C-terminal 94 amino acid residues. ZBRK1ΔC retains the ZBRK1 KRAB domain and its entire DNA-binding zinc finger domain, but it lacks the C-terminal region of its BRCA1-binding domain. This deletion derivative can therefore bind to its cognate ZBRK1 recognition sequence (Figures 4A and 4C), but it cannot bind to BRCA1 (Figure 2G). Interestingly, ZBRK1ΔC also failed to repress transcription from the IN3AB reporter (Figure 6F).

To test whether the endogenous *GADD45* gene is a target of ZBRK1 regulation, we examined expression of the endogenous *GADD45* gene in U2OS cells stably expressing GFP-ZBRK1 and compared this expression to that in cells stably expressing GFP alone. Results from semiquantitative RT–PCR analysis (De Toledo et al., 1995) indicated that the *GADD45* mRNA level was decreased by approximately 5- to 6-fold in cells expressing GFP-ZBRK1, while the mRNA level of GAPDH was unchanged (Figure 6H). A concordant decrease in the GADD45 protein level was also observed in these

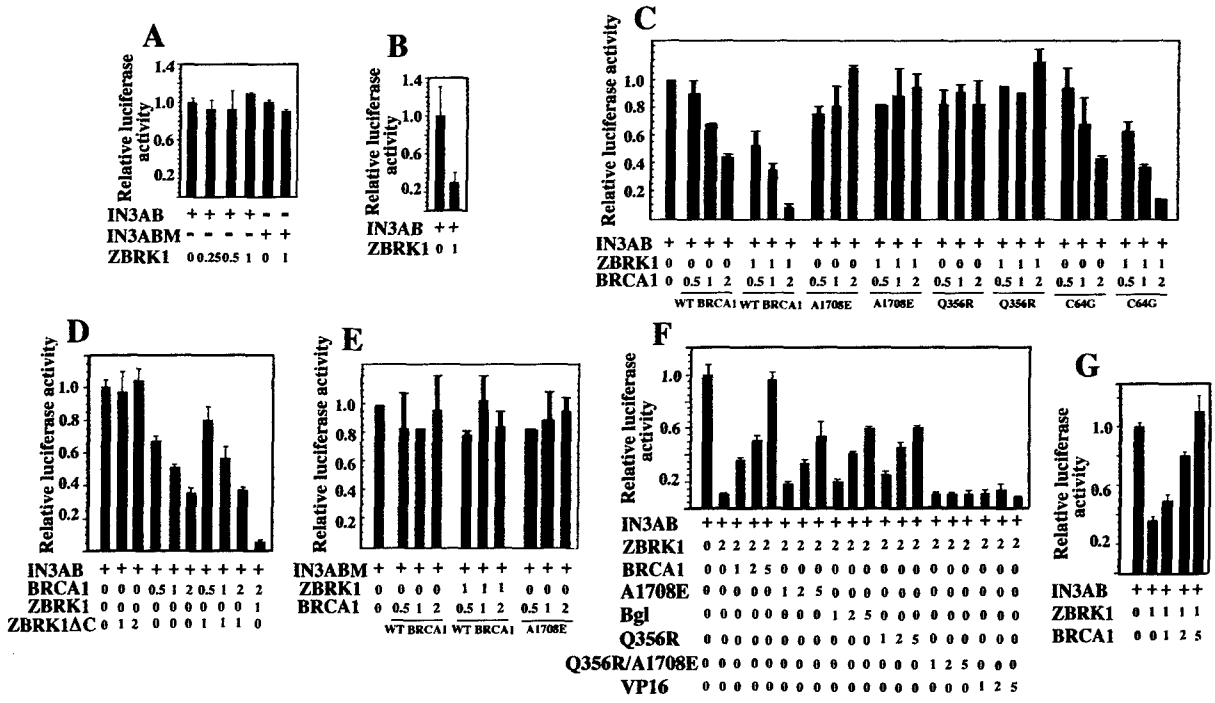


Figure 7. BRCA1 Functions as a ZBRK1 Corepressor through *GADD45* Intron 3

(A) Brca1-deficient (*Brca1*$^{-/-}$; *p53*$^{-/-}$) MEFs were transfected with 2.5 μg of pGL3p-IN3AB or pGL3p-IN3ABM along with the indicated amounts (micrograms of DNA) of pCNF-ZBRK1.

(B) Brca1-proficient (*p53*$^{-/-}$) MEFs were transfected with 2.5 μg of pGL3p-IN3AB along with the indicated amounts (micrograms of DNA) of pCNF-ZBRK1.

(C–E) Brca1-deficient (*Brca1*$^{-/-}$; *p53*$^{-/-}$) MEFs were transfected as indicated with 2.5 μg of pGL3p-IN3AB (C and D) or pGL3p-IN3ABM (E) along with the indicated amounts (micrograms of DNA) of pCNF-ZBRK, pCNF-ZBRK1ΔC, pcDNA3.1-BRCA1, pcDNA3.1-BRCA1A1708E, pcDNA3.1-BRCA1Q356R, or pcDNA3.1-BRCA1C64G.

(F) U2OS cells were transfected with pGL3p-IN3AB (0.5 μg) along with the indicated amounts (micrograms of DNA) of pCNF-ZBRK, pcDNA3.1-BRCA1, pcDNA3.1-BRCA1A1708E, pcDNA3.1-HA-Bgl expressing the ZBRK1-binding region in BRCA1, or pVP16 expressing the VP16 transactivation domain (Li et al., 1999).

(G) *p53*$^{-/-}$ MEFs were transfected with 2.5 μg of pGL3p-IN3AB along with the indicated amounts (micrograms of DNA) of pcDNA3.1-BRCA1 and pCNF-ZBRK1. In (A)–(G), relative luciferase activities are calculated as described in the previous figure.

cells (Figure 6I, lanes 1 and 2). Consistent with these results, the endogenous GADD45 protein level was also decreased by approximately 1- to 2-fold in cells transiently transfected with a wild-type ZBRK1 expression vector when compared to cells transfected with vector alone (Figure 6I, lanes 3–6). By contrast, transient expression of either ZBRK1ΔC or ZBRK1ΔK not only failed to repress but, in fact, moderately activated endogenous *GADD45* expression (Figure 6I). We consider it likely that ZBRK1ΔC and ZBRK1ΔK, both of which retain DNA binding activity, can compete with endogenous ZBRK1 for occupancy of ZBRK1-binding sites and thereby confer dominant-negative effects. Taken together, these results indicate that its KRAB domain and BRCA1-binding domain are both required for ZBRK1-directed repression of *GADD45* transcription.

### BRCA1 Mediates the Repression Activity of ZBRK1

The requirement for its BRCA1-binding domain for repression activity is consistent with a functional requirement for BRCA1 in ZBRK1-directed repression. To test this possibility, we examined the ability of ZBRK1 to function as a repressor in Brca1-deficient mouse embryo fibroblasts (MEFs) (*Brca1*$^{-/-}$; *p53*$^{-/-}$). As shown in Figure 7A, ZBRK1 exhibited no repression activity on the IN3AB reporter template in Brca1-deficient MEFs. By contrast, ZBRK1 repression activity was readily observed in Brca1-proficient MEFs (*p53*$^{-/-}$) (Figure 7B). Similar results were obtained in human BRCA1-mutated HCC1937 cells (data not shown).

Significantly, ectopic expression of wild-type BRCA1 in Brca1-deficient MEFs conferred repression on transcription from the IN3AB reporter template (Figure 7C), presumably through endogenous murine ZBRK1 (data not shown). Interestingly, cotransfection of human ZBRK1 along with wild-type BRCA1 provided a synergistic repressive effect on transcription (Figure 7C). Similar to wild-type BRCA1, ectopic expression of a BRCA1 derivative harboring a mutation in the N-terminal ring finger domain (C64G) conferred repression on transcription from the IN3AB reporter template (Figure 7C). By contrast, BRCA1 derivatives harboring mutations in either the C-terminal BRCT domain (A1708E and Y1853term) or the ZBRK1-binding region (Q356R) exhibited no repression activity (Figure 7C and data not shown). These results thus identify the regions important for BRCA1-mediated ZBRK1 repression to include the ZBRK1-binding region and the C-terminal BRCT domain.

Table 1. Potential ZBRK1 Binding Motifs Identified in BRCA1-Targeted Genes

| BRCA1-Targeted Genes[a] | Potential ZBRK1 Binding Sequences | Start, End Positions[b] | Genbank Accession No. |
|---|---|---|---|
| | GGGxxxCAGxxxTTT | Consensus | |
| GADD45 | TGGgttCAGactTTG | +2239, +2253 | L24498 |
| GADD153 | CGGaaaCAGcagCTT | −520, −506 | S40707 |
| Ki-67 | TGGtctCAGtccCTT | −757, −733 | X94762 |
| | GGGacgCAGtccTGT | −781, −757 | |
| Bax | GGGcctCTGagcTTT | +6, +20 | AH00538 |
| p21 | GGTcacCAGactTCT | −893, −879[c] | U24170 |
| | TGGttgCAGcagCTT | −767, −781[c] | |
| | AGGacaCAGcacTGT | −288, −302[c] | |
| EGR1 | AGGgaaCAGcctTTC | −930, −916 | AJ243425 |
| Amphiregulin | GGTtgtCAGagtTTG | −280, −266 | AH002608 |
| Prothymosin | CGGcgcCAGaagCTT | −13, −27 | S56449 |
| TIMP-1 | TGGcacCAGggtTGT | −842, −856 | Y09720 |
| TIMP-2 | GGGacgCAGtttTAT | −67, −81 | S68860 |
| Topo IIa | GCGagtCAGggaTTG | −79, −65 | X66794 |

[a] Genes that are activated or repressed by overexpression of BRCA1 as described previously (Harkin et al., 1999; MacLachlan et al., 2000).
[b] Positions are relative to transcriptional start sites except those indicated.
[c] Positions are relative to the TATA box.

In contrast to full-length ZBRK1, ZBRK1ΔC failed to synergistically repress transcription when cotransfected with BRCA1 (Figure 7D). Furthermore, in the presence of ectopically expressed wild-type BRCA1, ZBRK1 did not repress transcription from the IN3ABM reporter template, confirming that BRCA1-mediated repression is specific for ZBRK1 (Figure 7E). Collectively, these experiments thus reveal a role for BRCA1 as a transcriptional corepressor of ZBRK1.

### BRCA1 Overexpression Relieves ZBRK1-Directed *GADD45* Intron 3–Mediated Repression

Recently, *GADD45* was identified as a gene induced in a p53-independent manner following BRCA1 overexpression (Harkin et al., 1999). Our identification of BRCA1 as a ZBRK1 corepressor led us to question whether this induction could derive from relief of BRCA1-mediated *GADD45* repression. To explore this possibility, we examined whether overexpression of BRCA1 in BRCA1-proficient cells could overcome ZBRK1-directed *GADD45* repression. As shown in Figure 7F, ectopic expression of BRCA1 could efficiently override ZBRK1-mediated repression of transcription from the IN3AB reporter template in a dose-dependent manner. To examine the p53 dependency of this BRCA1-mediated effect, we performed the same experiment in p53-deficient MEFs and obtained similar results (Figure 7G). This observation suggests that induction of IN3AB reporter gene transcription by BRCA1 overexpression is independent of BRCA1-mediated coactivation of p53.

To explore the mechanism for this derepression activity, we tested several BRCA1 mutants for their respective derepression capabilities. BRCA1-Bgl, a deletion derivative retaining only the ZBRK1-binding region, as well as missense mutants BRCA1-A1708E and BRCA1-Q356R, could override ZBRK1-directed repression, albeit less effectively than wild-type BRCA1 (Figure 7F). By contrast, no derepression activity was conferred by the double mutant BRCA1-Q356R/A1708E.

Relief of *GADD45* repression by BRCA1 overexpression could derive from either or both of two alternative processes. First, derepression could derive from competitive displacement by ectopically expressed BRCA1 of an endogenous BRCA1-containing repressor complex from DNA-bound ZBRK1. Alternatively, ectopically expressed BRCA1 could titrate other critical corepressors from DNA-bound ZBRK1. If relief of repression derives from competitive displacement, then BRCA1 mutant A1708E, which can bind to ZBRK1 but not to the CtIP/CtBP corepressor complex or HDACs (Li et al., 1999; Yarden and Brody, 1999), would be expected to exhibit derepression activity. On the other hand, if relief of repression derives from repressor titration, then BRCA1 mutant Q356R, which can bind to CtIP/CtBP and HDACs but not to ZBRK1 (Figure 3C), would also be expected to exhibit derepression activity. The observation that both of these BRCA1 single mutants retain derepression activity suggests that both competitive displacement and repressor titration contribute to the derepression activity of wild-type BRCA1. Consistent with this interpretation is the lack of depression activity exhibited by the double mutant BRCA1-Q356R/A1708E, which can bind neither ZBRK1 nor the corepressors CtIP/CtBP or HDACs (Figure 7F).

To eliminate the possibility that BRCA1-mediated *GADD45* derepression occurs simply as a consequence of elevated levels of an activation domain, we overexpressed the potent Herpes simplex virus VP16 activation domain and observed no effect on ZBRK1 repression from the IN3AB reporter (Figure 7F). Collectively, these results suggest that BRCA1 overexpression can lead to induction of *GADD45* transcription through the relief of ZBRK1-directed repression and, furthermore, provide a plausible molecular explanation for the apparent transcriptional activation of *GADD45* gene transcription by BRCA1 overexpression in the absence of p53 (Harkin et al., 1999).

While derepression by BRCA1 overexpression may accurately reflect some aspect of its function in vivo, more physiologically relevant mechanisms for derepression are likely to involve alterations in the phosphorylation and/or protein interaction status of BRCA1. In this

regard, DNA damage–induced dissociation of a CtIP-CtBP corepressor complex from BRCA1 could relieve ZBRK1 repression of *GADD45* transcription, thereby leading to *GADD45* induction (Li et al., 2000).

## Discussion

An overriding question concerning the role of BRCA1 in transcription control is how it confers gene-specific regulation in the absence of sequence-specific DNA binding activity. The identification herein of a novel DNA-binding transcription repressor and BRCA1 interaction partner, ZBRK1, suggests a means by which BRCA1 may be physically tethered and functionally linked to specific regulatory loci. Importantly, we demonstrate that genetic ablation of *Brca1* precludes, while its ectopic expression restores, transcriptional repression by ZBRK1. Our results thus reveal a new role for BRCA1 as a corepressor, a prospect consistent with previous reports demonstrating that BRCA1 can not only repress transactivation mediated by the estrogen receptor or c-Myc (Wang et al., 1998; Fan et al., 1999), but also interact with the CtIP/CtBP corepressor and HDAC complexes that mediate transcription repression (Li et al., 1999; Yarden and Brody, 1999). Collectively, these observations suggest that BRCA1 could function more broadly as a corepressor of sequence-specific DNA-binding transcriptional repressors other than ZBRK1.

The interaction between ZBRK1 and BRCA1 is functionally relevant, since mutagenic disruption of cognate interaction surfaces on either protein abrogates ZBRK1 repression activity. Furthermore, familial breast cancer–derived BRCA1 missense mutations that disrupt its interaction with ZBRK1 or BRCT-binding proteins abrogate its corepressor activity, thereby suggesting that its corepressor function may be important for the biological activity of BRCA1 in tumor suppression and/or cell growth and differentiation.

BRCA1 has been proposed to regulate the expression of genes linked to several different aspects of cellular physiology, including cell cycle checkpoint control, proliferation, and differentiation (Harkin et al., 1999; MacLachlan et al., 2000). ZBRK1, through eight Krupple-type zinc fingers, binds to a compositionally flexible recognition sequence, GGGxxxCAGxxxTTT. Interestingly, sequences closely conforming to this recognition sequence lie within the putative regulatory regions of a subset of BRCA1 target genes (Table 1), thus raising the possibility that ZBRK1 and BRCA1 function coordinately to regulate a common class of genes with roles in these cellular processes.

Among these, we have identified *GADD45* to be target of coordinate regulation by both ZBRK1 and BRCA1. Our findings are consistent with a model in which ZBRK1 and its associated corepressors, KAP-1 and BRCA1, largely silence *GADD45* gene transcription in the uninduced state. In response to an appropriate inducing signal(s), *GADD45* would be liberated from this repression, thereby providing the potential for a rapid and robust increase in gene transcription. This model is consistent not only with the observed elevated basal level of endogenous *GADD45* mRNA in BRCA1 mutant HCC1937 cells, but the correspondingly small induction of *GADD45* mRNA in response to MMS in these same cells (Harkin et al., 1999). It must be emphasized that derepression as an operative mechanism in control of *GADD45* gene transcription must be coordinated with additional mechanisms of true activation, since BRCA1 can stimulate transcription, albeit modestly, from reporter templates that do not contain a ZBRK1 response element but that do contain p53 response elements (Harkin et al., 1999). Hence, in the natural setting, *GADD45* induction may reflect the concerted effects of both derepression and true activation. Significantly, BRCA1 appears to play a critical role in both of these transcriptional processes, wherein it functions both as a corepressor and a coactivator (Li et al., 1999; Yarden and Brody, 1999; Pao et al., 2000). Its dual transcriptional activities are likely to contribute to the differential regulation by BRCA1 of a broad spectrum of cellular genes necessary to mediate its tumor suppressor function.

## Experimental Procedures

### Cell Culture and Generation of *Brca1*$^{-/-}$ *p53*$^{-/-}$ MEFs

Mammalian cells were cultured in the DMEM containing 10% fetal calf serum. For isolation of *Brca1*$^{-/-}$ *p53*$^{-/-}$ and *p53*$^{-/-}$ MEFs, *Brca1*$^{+/-}$ mice (Liu et al., 1996) were crossed with *p53*$^{+/-}$ mice (Jackson Laboratory) to create *Brca1*$^{+/-}$ *p53*$^{+/-}$ mice. The resultant F1 mice were then crossed with each other, and 9.5 day embryos were used to prepare MEFs. The *Brca1*$^{-/-}$ *p53*$^{-/-}$ and *p53*$^{-/-}$ MEFs were obtained from the embryos of the same F2 generation.

### RNA Blot and RT–PCR Analysis

Approximately 15 μg of total RNA from indicated cells was subjected to semiquantitive reverse transcription–polymerase chain reaction (RT–PCR) analysis following a procedure previously described for studying *GADD45* expression (De Toledo et al., 1995).

### Plasmid Construction

pCEPF-ZBRK1, pCNF-ZBRK1, and pCHPL-ZBRK1, for expressing ZBRK1 in mammalian cells, were constructed, respectively, by subcloning the full-length *ZBRK1* cDNA from pBSK-ZBRK1 into pCEPF (pCEP-Flag; Chen et al., 1996), a pCEP4-based vector (Invitrogen), or into pCNF, a pcDNA3.1-based vector (Invitrogen), or into the pCHPL vector (Li et al., 1998), thereby placing it under control of CMV promoter. pCNF-ZBRK1 expresses ZBRK1 with an N-terminal Flag tag. pCNF-ZBRK1ΔK was constructed by translationally fusing a HincII fragment from the *ZBRK1* cDNA with the N-terminal Flag tag in pCNF. pCNF-ZBRK1ΔC was constructed by introducing a nonsense mutation into the *ZBRK1* cDNA at sequences corresponding to codon 439 in pCNF-ZBRK1. pCHPL-GFP-ZBRK1, which expresses an N-terminal GFP fusion with the full-length ZBRK1, was created by inserting the full-length *ZBRK1* cDNA into pCHPL-GFP, which expresses green fluorescent protein under control of CMV promoter (Li et al., 1998). pcDNA3.1 vector was used to express the wild-type BRCA1 or its mutant derivatives as previously described (Li et al., 1999; Zhong et al., 1999). pcDNA3.1-HA-Bgl, which expresses a HA epitope–tagged Bgl fusion protein was constructed by translationally fusing the ZBRK1-binding region (aa 341–748) in the *BRCA1* cDNA with an N-terminal HA epitope tag.

The pGL3p-IN3AB reporter plasmid was generated by subcloning a 1.1 kb BamHI/PstI fragment from intron 3 of the *GADD45* gene from pHG45 (a gift from A. J. Fornace, Jr.), into the pGL3-promoter vector (Promega). pGL3p-IN3ABM was created by site-directed mutagenesis of the nine-nucleotide ZBRK1 consensus binding sequence in pGL3p-IN3AB. pGL3p-IN3A is equivalent to pI-3 (a gift from D. A. Haber). pBLcat-E was created by subcloning four copies of the consensus ZBRK1 recognition sequence upstream of the chloramphenicol acetyltransferase (CAT) reporter gene in the pBLcat2 vector (Luckow and Schutz, 1987).

### Transfection, Immunoprecipitation, and Immunoblot Analysis

For CAT or luciferase reporter assays, Saos2 and U2OS cells ($2 \times 10^5$) or MEFs ($6 \times 10^4$) were transfected by conventional calcium phosphate/DNA coprecipitation methods or by lipofectin-based methods, respectively, as described (Li et al., 1999). For generating stable cell lines expressing GFP or GFP-ZBRK1, U2OS cells were selected with 200 μg/ml hygromycin 48 hr after transfection.

Double immunoprecipitation, coimmunoprecipitation, and immunoblot analysis were performed as previously described (Li et al., 1999). For Figure 3B, anti-ZBRK1 antibodies, anti-BRCA1 mAb 6B4, and rabbit IgG were first coupled with protein G– or protein A–Sepharose beads by dimethylpimelimidate as described (Harlow and Lane, 1988).

### Generation of Oligonucleotide Library and SAAB

55-mer single-stranded oligonucleotides (5′-GCACTAGCGGATC CGT-N23-CGAAGCTTGGTCACGC-3′) bearing 16-nucleotide fixed-end sequences flanking 23 central random nucleotides were synthesized. A $^{32}$P-labeled double-stranded oligonucleotide library was generated by primer extension with the reverse primer (5′-GCG TGACCAAGCTTCG). $^{32}$P-α-dCTP was incorporated in the reaction, and the product was purified by electrophoresis on a 15% polyacrylamide gel, followed by elution and precipitation. Selection and amplification of binding sites (SAAB) was performed as previously described (Blackwell and Weintraub, 1990). DNA binding reactions were performed in the buffer containing 25 mM HEPES (pH 7.5), 50 mM KCl, 4 mM $MgCl_2$, 10% glycerol, 25 μM $ZnSO_4$, 250 μg/ml BSA, 1 mM DTT, 250 μg/ml poly dI/dC, and protease inhibitors.

### Preparation of Nuclear Extract, EMSA, and DNA Coimmunoprecipitation

Nuclear extracts were prepared from T24 or MCF7 cells as described (Carter et al., 1990). For EMSA, Wt probe was obtained by annealing two complimentary synthesized oligonucleotides, 5′-GATCCACGG GACGCAGGTGTTTTGTGCCG-3′ and 5′-GATCCGGCACAAAACAC CTGCGTCCCGTG-3′. Mut probe was obtained by annealing two complimentary synthesized oligonucleotides, 5′-GATCCACCTCAC GTTCGTGCACTGTGCCG-3′ and 5′-GATCCGGCACAGTGCACGAA CGTGAGGTG-3′. WIN3 probe was obtained by annealing two complimentary synthesized oligonucleotides, 5′-TGGGTTGCATGGGTT CAGACTTTGCAATG-3′ and 5′-TACACATTGCAAAGTCTGAACCCA TGCAA-3′. MP1 probe was obtained by annealing two complimentary synthesized oligonucleotides, 5′-GATCCATGGAGTAGGCA GAAATTTCACCA-3′ and 5′-GATCTGGTGAAATTTCTGCCTACTCC ATG-3′. Each of these double-stranded probes had overhangs at both ends, which were filled in with α-$^{32}$P-dCTP by Klenow enzyme. In each reaction, 60000 cpm of $^{32}$P-labeled probe was mixed with GST-ZBRK1Zn fusion protein or nuclear extract in 40 μl DNA-binding buffer as described in the above SAAB assay. One hundred nanograms of poly dI/dC was added to each reaction. After 30 min of incubation at room temperature, the mixture was loaded onto a 5% polyacrylamide gel in running buffer containing 20 mM HEPES, (pH 7.5), 0.1 mM EDTA.

The DNA immunoprecipitation assay was performed essentially as described (Yew et al., 1994) with slight modifications. Six hundred thousand cpm $^{32}$P-labeled WIN3 or MP1 probe was incubated with 60 μg of nuclear extract for 30 min at room temperature in 200 μl DNA-binding buffer with 50 ng/μl of poly dI-dC as described above. Antibody-protein A Sepharose beads were added to the DNA-binding reaction and incubated for 1 hr at room temperature. The reaction was washed twice with DNA-binding buffer, deproteinized, and resolved by electrophoresis in 5% polyacrylamide gel, followed by autoradiography.

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# Expression of BRC Repeats in Breast Cancer Cells Disrupts the BRCA2-Rad51 Complex and Leads to Radiation Hypersensitivity and Loss of $G_2$/M Checkpoint Control*

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**BRCA2 is a breast tumor suppressor with a potential function in the cellular response to DNA damage. BRCA2 binds to Rad51 through its BRC repeats. In support of the biological significance of this interaction, we found that the complex of BRCA2 and Rad51 in breast cancer MCF-7 cells was diminished upon conditional expression of a wild-type, but not a mutated, BRC4 repeat using the tetracycline-inducible system. Cells expressing a wild-type BRC4 repeat showed hypersensitivity to γ-irradiation, an inability to form Rad51 radiation-induced foci, and a failure of radiation-induced $G_2$/M, but not $G_1$/S, checkpoint control. These results strongly suggest that the interaction between BRCA2 and Rad51 mediated by BRC repeats is critical for the cellular response to DNA damage.**

*BRCA2* was cloned based on an analysis of mutations in families predisposed to breast cancer showing that a large percentage of the kindred had alterations within this locus (1, 2). The expression pattern of *BRCA2* is remarkably similar to that of *BRCA1* (3–5), with highest levels in the testis, thymus, and ovaries (5). At the cellular level, expression is regulated in a cell-cycle dependent manner and peak expression of *BRCA2* mRNA is found in S phase (6). These results suggest BRCA2 may participate in regulating cell proliferation.

Recent studies indicate that BRCA2 is important for the cellular response to DNA damage. *Brca2*-null mouse embryos are nonviable at a very early stage of development and blastocysts derived from these embryos are very sensitive to γ-irradiation (7). Mouse embryonic fibroblasts predicted to express BRCA2 that is C-terminally truncated at amino acid 1492 also demonstrated sensitivity to DNA damaging agents, particularly methyl methanesulfonate and UV light (8). Furthermore, Capan-1, a human pancreatic cancer line, that expresses a 220-kDa C-terminally truncated BRCA2 protein, is hypersensitive to a panel of DNA damaging agents (9). Importantly, ectopic expression of wild-type, but not mutated, BRCA2 in Capan-1 cells restores resistance to treatment with MMS (10). These results provided convincing evidence that BRCA2 plays a critical role in the DNA repair process.

Interestingly, BRCA2 was shown to interact with Rad51 (7, 10–12), a key protein in DNA recombinational repair. Human *Rad51* encodes a 40-kDa protein with a structure related to the *Escherichia coli* recombination protein RecA (13) and mediates homologous DNA pairing and strand exchange (14, 15). Similar to *mBrca2*, inactivation of mouse *Rad51* results in an embryonic lethal phenotype, indicating that Rad51 protein is essential for development (16, 17). Beyond serving as a DNA repair protein through its interactions with other Rad proteins including Rad52 and Rad54 (18), how Rad51 may participate in cell growth and development remains unclear.

While an association between BRCA2 and Rad51 is well documented, there is, nonetheless, some discrepancy concerning the regions of BRCA2 that bind to Rad51. It was reported that the C-terminal region of mouse Brca2 binds to mouse Rad51 (7). By contrast, we and others have previously shown that the BRC repeats located in exon 11 (amino acid 1009–2083) of human BRCA2 bind to Rad51 (10, 12). There are eight repeats in BRCA2 designated as BRC1 to BRC8 (Fig. 1A) (19, 20). BRC1, BRC2, BRC3, BRC4, BRC7, and BRC8 are highly conserved and bind to Rad51, whereas BRC5 and BRC6 are less well conserved and do not bind to Rad51 (10, 12). Whether the interaction between BRCA2 and Rad51 has biological significance remains completely unknown. In an effort to investigate this issue, we have used the tetracycline binary gene control system for conditional expression of a BRC4 repeat in breast cancer MCF-7 cells. In this communication, we have found that upon expression of a wild-type, but not a mutated, BRC4 repeat, the interaction between BRCA2 and Rad51 was reduced. Cells expressing a wild-type BRC4 repeat showed hypersensitivity to γ-irradiation, an inability to form radiation-induced Rad51 nuclear foci, and a failure of radiation-induced $G_2$/M checkpoint control. These results strongly suggest that the BRC repeats of BRCA2 are important for mediating the cellular response to DNA damage.

## EXPERIMENTAL PROCEDURES

*Mutagenesis and Selection for BRC Mutants*—The 39-amino acid BRC1 repeat (residue 1003–1042 of BRCA2) (2) in pBSK (Stratagene, La Jolla, CA) was randomly mutagenized by a biased-pool PCR[1] method (21) using T3 and T7 primers. Deoxynucleotide triphosphates used in PCR reaction were 10 mM dGTP, dCTP, dTTP, and 2 mM dATP. The resulting PCR fragments were digested with *Nco*I and *Xho*I and cloned into pAS1 vector (22) to generate a BRC1 mutant library (pAS1/BRC1-ML). These plasmid DNAs were transformed along with pGAD-Rad51 into the yeast Mav203 strain (MATα, leu2–3, 112, trp1–901, his3d200, ade2–101, gal4d, gal80d, SPAL10::URA3, GAL1::lacZ, HIS3UAS GAL1::HIS3@LYS2. can1R, cyh2R) and selected for 5-fluoroorotic acid-resistant colonies (23). The recovered pAS1/BRC DNAs were further sequenced to determine the mutations. β-Galactosidase activities (U) was measured using chlorophenyl-red-β-D-galactopyranoside as substrate for interactions in the yeast two-hybrid system as described (22).

*Isolation of Cell Clones with Inducible Expression of the BRC Re-*

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[1] The abbreviations used are: PCR, polymerase chain reaction; GFP, green fluorescence protein; DAPI, 4′,6-diamidino-2-phenylindole; BrdUrd, bromodeoxyuridine; Tet, tetracycline.

TABLE I

*Identification of mutations in the first and fourth BRC repeats of BRCA2 that disrupt binding to Rad51*

A randomly mutagenized pool of cDNAs encoding BRC1 repeats (amino acid 1003–1042) was cloned into the pAS1 vector and co-transformed along with pGAD-Rad51 into Mav203 cells. Four clones with DNA inserts that showed no detectable β-galactosidase activity in a yeast two-hybrid assay were isolated. Mutations with amino acid changes resulted from single nucleotide changes. Rad51 binding activity using the BRC4 in pAS1 vector was also tested in the assay. Note that this repeat has 3-fold higher activity compared with the BRC1 repeat. The T to A mutation identified above in the BRC1 repeat that abrogates Rad51 binding, and a familial mutation identified in BRC4 (G1529R) was introduced into the BRC4 and tested in the yeast-two hybrid assay. Both of the mutations significantly reduced, but the G to R mutation did not completely eliminate, Rad51 binding in this assay.

| BRC consensus | I I L D<br>F*X*TASGK*X X* S*XXX*L*X*K*XXXX X*<br>V V F E | Relative β-galactosidase activity |
|---|---|---|
| BRC1 | FRTASNKEIKLSEHNIKKSKMFFKD | 7 |
| BRC1-M1 | A | <1 |
| BRC1-M2 | G | <1 |
| BRC1-M3 | S | <1 |
| BRC1-M4 | RG | <1 |
| BRC4 | FHTASGKKVKIAKESLDKVKNLFDE | 22 |
| BRC4-M5 | A | <1 |
| BRC4-M6 | R | 4 |
| vector | | 1 |

*peat*—To generate cell clones that express a GFP-BRC4 fusion protein, we employed the tetracycline-inducible expression system controlled by a *tet*-responsive promoter (24). A pUHD10-3-based plasmid was used to construct pUHD10-3/GFP-BRC plasmids that will express chimeric proteins containing GFP with a nuclear localization signal and a myc epitope fused to either a wild-type BRC4 or a mutated BRC4-M5. These two plasmids were separately co-transfected into MCF-7 cells with the second plasmid, pCHTV, which contains a hygromycin resistance gene and a cytomegalovirus-controlled tetracycline repressor-VP16 fusion transcription unit. Cell clones resistant to hygromycin were subsequently isolated and several of them were shown to express the wild-type or mutant GFP-BRC4 upon removal of tetracycline. The expression of GFP-BRC4 was further confirmed by immunoprecipitation with α-myc 9E10 monoclonal antibody (25) and immunoblotting analysis with a monoclonal α-GFP antibody (CLONTECH, Palo Alto, CA). Two stable lines of MCF-7 cells, WT-8 and MT-11, that conditionally express wild-type BRC4 and BRC4-M5 mutant, respectively, were established.

*Immunoprecipitations and Western Blotting*—Immunoprecipitations were performed as described previously (10). Co-immunoprecipitations were performed similarly but with lysis buffer containing 180 mM NaCl. Antibodies specific for BRCA2 (10), human Rad51, Ab-1 (Oncogene Science, Cambridge, MA) and myc 1-9E10 monoclonal antibody (25) were used for the immunoblotting analysis according to standard procedure (10).

*Immunostaining Rad51 Foci*—Procedures for immunostaining were adapted from Zhong *et al.* (26). Briefly, cells in Dulbecco's modified Eagle's medium with or without tetracycline (1 μg/ml) grown on coverslips to 60–70% confluence were irradiated with 12 Gy using a Mark I, model 68A irradiator. After 6 h, cells were fixed in 4% formaldehyde with 0.1% Triton X-100. α-Rad51 antibody (Ab-1) diluted 1:1000 in 10% goat serum was added onto the cells and then visualized with goat anti-rabbit antibody conjugated to Texas Red. Cells were further stained with 4′,6-diamidino-2-phenylindole (DAPI) and mounted in Permafluor (Lipshaw-Immunonon, Pittsburgh, PA). Rad51 foci-positive cells were counted and recorded using a standard fluorescence microscope.

*Clonogenic Survival Assay*—Cells (WT-8 and MT-11) were seeded in identical plates at 5000 cells/plate in medium with tetracycline (1 μg/ml). Expression of the wild-type or mutant GFP-BRC4 repeat was induced by removing tetracycline 24 h after seeding. Twenty four hours after induction of GFP-BRC4 expression, cells were then γ-irradiated with 3 Gy. After incubation for 14 days, cells were fixed and stained with 2% methylene blue in 50% of ethanol for colony counting. Averages and standard deviations were determined from eight plates. Statistical analyses were performed with the programs InStat and InPlot (GraphPad Inc., San Diego, CA).

*Cell Cycle Checkpoint Analysis*—The $G_1$/S checkpoint was determined according to the procedures described (27). Briefly, cells in logarithmic growth were mock-exposed or γ-irradiated (12 Gy). After 24 h, cells were labeled with 10 μM BrdUrd for 4 h and fixed for BrdUrd staining using a Cell Proliferation Kit (Amersham Pharmacia Biotech). BrdUrd-positive cells were quantified, and expressed as a fraction of the total cells. For the $G_2$/M checkpoint, cells were irradiated to 3 Gy, fixed with 4% paraformaldehyde at indicated time and stained with DAPI for counting mitotic cells in prophase, metaphase, anaphase, and telophase (28). Alternately, cells were irradiated with 4–16 Gy and processed for analysis of mitotic cells after 1 h.

## RESULTS AND DISCUSSION

To systematically address the biological consequence of the interaction between BRCA2 and Rad51, amino acid residues of the first BRC repeat, BRC1, that are critical for Rad51-binding were first examined. BRC1 was subjected to biased PCR mutagenesis (21), and the mutated cDNAs were translationally fused to the GAL4 DNA-binding domain in the yeast vector, pAS1 (22), to generate a library of $2 \times 10^6$ individual clones referred to as pAS/BRC1-ML. A reverse two-hybrid screen with negative selection was used to isolate clones that fail to bind Rad51 as described previously (23). Several mutations in BRC1 were identified that significantly reduced Rad51 binding in a yeast two-hybrid assay (Table I). BRC1-M1 is a mutation that changes a conserved threonine residue to alanine. BRC1-M2 and -M3 are changes in nonconsensus amino acids, and BRC1-M4 carries a double mutation at the two C-terminal BRC1 residues, the last residue of which is conserved. Interestingly, a familial mutation, G1529R, has been previously found in BRC4 (Breast Cancer Information Core). Specific Rad51 binding activity by BRC4 was also tested and found to be approximately three times stronger than BRC1 (Table I). Two BRC4 mutations, BRC4-M5, an analogous mutation to BRC1-M1, in which the conserved threonine at the third position is changed to an alanine, and BRC4-M6, which contains the G1529R mutation, were constructed and found to have reduced Rad51-binding (Table I). These results suggest that, despite their sequence conservation, the ability of BRC repeats to bind Rad51 varies, and is dependent on specific residues.

To determine the functional importance of the interactions between the BRC repeats of BRCA2 and Rad51, two stable lines of MCF-7 cells, WT-8 and MT-11, that conditionally express wild-type BRC4 and mutant BRC4-M5, respectively, were established (Fig. 1*B*). Tetracycline-responsive expression of the GFP-BRC4 fusion proteins in these two lines was clearly demonstrated by immunoprecipitation with α-myc antibodies and immunoblotting with α-GFP or α-Rad51 antibodies (Fig. 1*C*, *top panel* compare *lanes 2* and *4* with *1* and *3*). Rad51 is detected in the immunoprecipitates of wild-type, but not GFP-BRC4-M5 (Fig. 1*C*, compare *lanes 4* with *2*), indicating that the

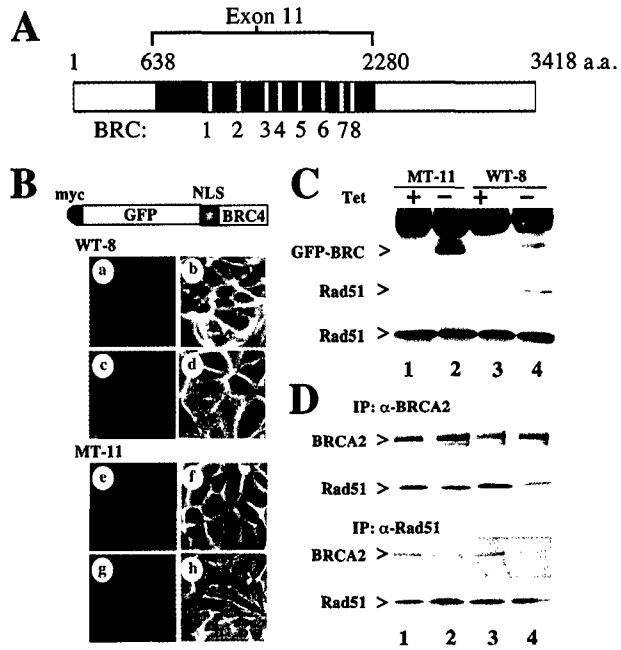


FIG. 1. **Conditional expression of wild-type and mutated GFP-BRC4 fusions in MCF7 cells.** *A*, diagram of BRCA2 showing exon 11 and the eight BRC repeats. *B*, the *top panel* is a schematic drawing illustrating the GFP-BRC4 fusion containing a BRC4 cDNA fragment translationally fused to a myc epitope-GFP-nuclear localization signal cassette in a modified pUHD10-3 plasmid, pUHD10-3/GFP-BRC. The *lower panel* shows cell clones expressing the wild-type GFP-BRC4 (WT-8) (*panels a–d*) and mutated GFP-BRC4-M5 (MT-11) (*panels e–h*) were visualized by GFP autofluorescence (*panels a, c, e,* and *g*) after incubation in the presence (*panels a, b, e,* and *f*) or absence (*panels c, d, g,* and *h*) of tetracycline (*Tet*). Fluorescence overlaid with phase-contrast images (*panels b, d, f,* and *h*) show nuclear localization of these fusion proteins. *C*, co-immunoprecipitation of GFP-BRC4 with Rad51 in cells. Cells expressing wild-type (WT-8) or mutated GFP-BRC4 (MT-11) were immunoprecipitated with α-myc antibody (*top two panels*), immunoblotted with either α-GFP to detect the GFP-BRC4 fusions or α-Rad51 (indicated in the *left margin*). Immunoprecipitation and Western blotting with α-Rad51 antibody (*bottom panel*) determined the relative levels of endogenous Rad51. *D*, expression of wild-type GFP-BRC4 in WT-8 cells reduces the complex formation between BRCA2 and Rad51. Thirty-six hours after induction of GFP-BRC4 expression, cell lysates were co-immunoprecipitated with either α-BRCA2 (*top panel*) or α-Rad51 antibody (*bottom panel*). The resulting immune complexes were analyzed by immunoblot analysis with either α-BRCA2 or α-Rad51 antibody, indicated on the *left margin*.

GFP fusion with wild-type BRC4 binds to Rad51 in cells.

Importantly, expression of wild-type, but not the BRC4-M5 mutant, significantly reduced BRCA2 in the Rad51 immunoprecipitates and, in the reciprocal experiment, reduced Rad51 in the BRCA2 immunoprecipitates (Fig. 1*D*, compare *lane 4* with *lane 2*). These data strongly suggest that conditional expression of a wild-type, but not a mutated, BRC4 repeat effectively disrupts the interaction between BRCA2 and Rad51.

The important role of Rad51 in recombinational DNA double-strand break repair (13) suggests that disruption of the interaction between BRCA2 and Rad51 may have an adverse effect on the ability of cells to respond to DNA damage. To test this possibility, both WT-8 and MT-11 cells cultured either with or without tetracycline, were mock-exposed or γ-irradiated (3 Gy), and cell survival was determined by clonogenic assay. Induction of the expression of a wild-type BRC4 repeat (WT-8, −Tet) significantly reduced cell survival rate when compared with the uninduced (WT-8, +-Tet) or to either the induced (MT-11, −Tet) or uninduced (MT-11, +Tet) mutant BRC4 ($p < 0.0001$) (Table II).

To further explore DNA damage response phenotypes of these two cell lines, the appearance of radiation-induced

TABLE II

*Clonogenicity of WT-8 and MT-11 cells after γ-irradiation*

Actively growing WT-8 and MT-11 cells (5000) were either induced (−Tet) or uninduced (+Tet) to express GFP-BRC4. About 24 h later, cells were either mock-exposed or γ-irradiated (3 Gy) and cultured for 14 days. Survival rates by colony formation (>50 cells/colony) were determined by counting the number of colonies per plate. Averages and S.D. were calculated from eight plates. Survival rates were calculated by dividing the number of colonies in the mock-exposed control by the number from exposed cells. Note that the expression of wild-type, but not mutated BRC4 repeat, significantly reduced cell survival in this assay ($p < 0.0001$).

| | 0 Gy | 3 Gy | Survival rate, mean ± S.D. ($n = 8$) |
|---|---|---|---|
| WT-8 (+Tet) | 405 ± 14 | 189 ± 12 | 46.8 ± 7.9% |
| WT-8 (−Tet) | 358 ± 17 | 72 ± 2 | 20.1 ± 2.0% |
| MT-11 (+Tet) | 371 ± 10 | 173 ± 10 | 46.8 ± 8.0% |
| MT-11 (−Tet) | 379 ± 15 | 163 ± 7 | 43.2 ± 5.2% |

Rad51-containing foci was examined. Under uninduced conditions (+Tet), both clonal lines formed Rad51 foci after γ-irradiation (Fig. 2*A*). However, WT-8 cells induced to express a wild-type GFP-BRC4 repeat exhibited a reduction in the appearance of Rad51 foci compared with MT-11 cells induced to express the GFP-BRC4-M5 mutated repeat (Fig. 2*A* for representative field and Fig. 2*B* for quantification). These data suggest that the interaction between BRCA2 and Rad51 is crucial for the formation of Rad51 repair foci and, furthermore, that exogenous expression of BRC repeats can interfere with this activity.

Increased sensitivity to ionizing radiation may result from defects in the DNA repair machinery or in the molecules essential for cell cycle checkpoint control. When normal mouse embryo fibroblasts are exposed to γ-irradiation, their transit through the cell cycle is arrested at either one of two points (27, 29). The $G_1$/S checkpoint, dependent on p53 and p21 (29–32), prevents the replication of damaged DNA. The $G_2$/M checkpoint prevents segregation of damaged chromosomes (33). To test for a potential role of BRCA2-Rad51 interactions in DNA damage-induced cell cycle checkpoint control, cells expressing the GFP-BRC4 repeat were assayed for $G_1$/S and $G_2$/M checkpoint integrity in response to γ-irradiation. As shown in Fig. 3*A*, WT-8 and MT-11 cells, under all conditions, demonstrated nearly identical numbers of BrdUrd-incorporated cells, indicating that the expression of the BRC repeats did not significantly impair $G_1$/S checkpoint control in response to γ-irradiation.

In contrast, when cells were assayed for mitotic figures at variable times after γ-irradiation, the number of cells in mitosis was not significantly decreased in WT-8 cells induced to express a wild-type BRC4 repeat (Fig. 3*B*, *panel a*). However, MT-11 cells induced to express a GFP-BRC4-M5 mutated repeat, as well as uninduced cells, demonstrated significant reductions in mitotic figures (Fig. 3*B*, *panel a*). In a parallel experiment, cells were irradiated with 4–16 Gy. In the population induced to express a wild-type BRC4, the percentage of mitotic cells was significantly higher than that of cells either uninduced or induced to express mutant GFP-BRC4-M5 (Fig. 3*B*, *panel b*). These results suggest that cellular expression of a wild-type BRC4 repeat interferes with the radiation-induced $G_2$/M checkpoint.

It was reported that mouse embryo fibroblasts, with a genotype *Brca2*$^{tm1Cam}$ and predicted to express a C-terminally truncated BRCA2 at amino acid 1492, have intact cell cycle checkpoint responses (8). This truncated BRCA2 protein possesses the first three BRC repeats (34). The abrogation of embryonic lethality by *Brca2*$^{tm1Cam}$ (8) compared with other truncating mutations that delete exon 11 of all BRC repeats (7) strongly suggests that the remaining 3 BRC repeats in the

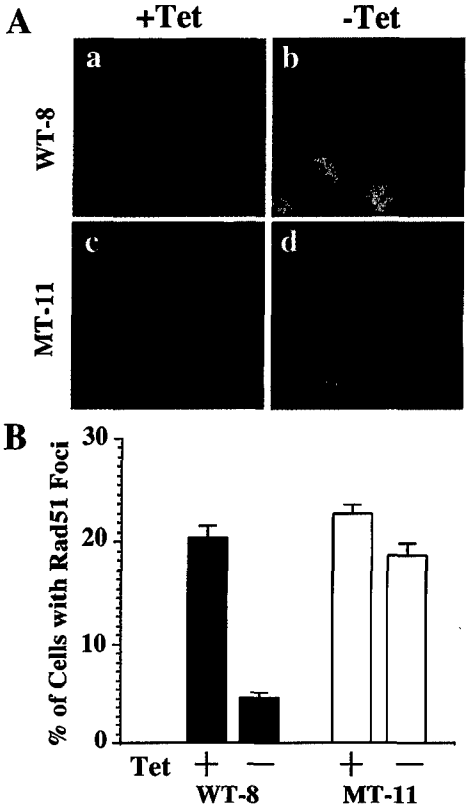


FIG. 2. **Radiation-induced Rad51 foci were diminished in cells expressing wild-type GFP-BRC4.** *A*, WT-8 and MT-11 cells (indicated in the *left margin*) were cultured in the presence (+) or absence (−) of tetracycline (*Tet*) for 48 h and then exposed to 12-Gy γ-radiation. Irradiated cells after 6 h were immunostained with α-Rad51 and Texas Red-conjugated secondary antibody. Expression of wild-type and mutant GFP-BRC4 was visualized by GFP autofluorescence. *Panels a* and *c* show the radiation-induced Rad51 foci overlaid with DAPI in the uninduced cells (+*Tet*). *Panels b* and *d* show the radiation-induced Rad51 foci overlaid with GFP autofluorescence in the induced cells (−*Tet*). Note the radiation-induced Rad51 foci were diminished in the wild-type GFP-BRC4 expressing cells (*panel b* compared with *panel d*). *B*, images containing 250 cells were captured by computer, and the number of cells containing at least 10 foci were recorded and plotted as a percentage of total cells. The plots were generated from two independent experiments.

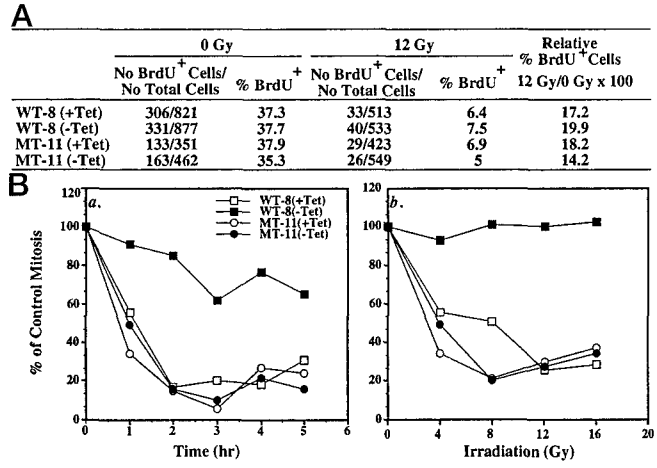


FIG. 3. **Cells expressing GFP-BRC4 show intact $G_1$/S and defective $G_2$/M checkpoints.** *A*, $G_1$/S checkpoint in γ-irradiated cells. WT-8 and MT-11 cells were incubated with (+) or without (−) tetracycline for 24 h and then mock-exposed (0 Gy) or γ-irradiated (12 Gy). Cells were labeled 24 h later with BrdUrd for 4 h and immunostained with α-BrdUrd antibody. The percentage of BrdUrd-positive cells was quantified and expressed as a fraction of the total number of cells. Note that the relative percentage of BrdUrd-positive cells decreases significantly in cells expressing both wild-type (WT-8) and mutated (MT-11) GFP-BRC4 following radiation, indicative of intact $G_1$/S checkpoint in these cells. *B*, defective $G_2$/M checkpoint in cells expressing wild-type GFP-BRC4. Mitotic cells were counted at 1–5 h after exposure to 3 Gy γ-radiation (*panel a*) or at 1 h after exposure of 4–16 Gy (*panel b*). Mitotic indices of mock-exposed cells were used as controls. Over 2000 cells were counted at each exposure and time point.

*Brca2*$^{tm1Cam}$ truncated protein are partially functional. The functional importance of the BRC repeats is supported by our results demonstrating that the expression of GFP-BRC4 repeat in cells results in hypersensitivity to radiation and a failure in $G_2$/M checkpoint control. The increased radiosensitivity in cells expressing the BRC4 repeat indicates that the complex of BRCA2 and Rad51 is important for the mechanics of the repair process. This notion is compatible with the known role of Rad51 in recombination repair. In this capacity, BRCA2 may facilitate Rad51 function in strand exchange by modulating formation of the Rad51-DNA nucleoprotein filament and/or pairing and strand-exchange steps of DNA double strand break repair (13).

The failure of radiation-induced $G_2$/M checkpoint control in cells expressing BRC4 repeat indicates that BRCA2 may have a role in this task. It is possible that the formation of the BRCA2 and Rad51 complex could be important for radiation-induced $G_2$/M checkpoint control. However, the BRCA2-Rad51 complex is formed independently of DNA damage (10–12). Other $G_2$/M checkpoint proteins may be required to participate in the BRCA2-Rad51 complex. Interestingly, mouse embryo fibroblasts expressing exon 11-deleted Brca1 also exhibit defects in radiation-induced $G_2$/M checkpoint control (28) and BRCA2 apparently interacts with BRCA1 (35). It is possible, therefore, that the BRCA2-Rad51 complex may interact with BRCA1 to establish $G_2$/M checkpoint control. Alternatively, the BRC repeats may mediate separate interactions with cell cycle checkpoint proteins when induced by DNA damage signaling.

Regardless of which possibility is operative in cells, the data presented here support and extend the model originally proposed (10), that BRC repeat interactions with Rad51 are important for the cellular DNA damage response. The observation that *mRad51*$^{-/-}$ mouse embryos are not viable and ES cells with the same genotype cannot survive (16, 17), coupled with the observation that *Brca2*$^{tm1Cam}$ cells are viable but radiation-sensitive (8), suggests that Rad51, through interactions with BRCA2, may have functions in addition to DNA repair. Like, BRCA1, it appears that BRCA2 functions in a pathway that bifurcates into one that is important for repair of genetic lesions and another important for restraining cell division until repair is complete. It is possible that each of the BRC repeats, or certain group of the repeats, may have separate and distinct functions within each pathway. Nevertheless, the fact that expression of one BRC repeat can disrupt Rad51-BRCA2 interactions, interfere with the $G_2$/M checkpoint, and can make cells radiation-sensitive suggests that it could be used to radiosensitize and/or chemosensitize resistant tumors.

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# Hec1p, an Evolutionarily Conserved Coiled-Coil Protein, Modulates Chromosome Segregation through Interaction with SMC Proteins

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**hsHec1p, a *Homo sapiens* coiled-coil-enriched protein, plays an important role in M-phase progression in mammalian cells. A *Saccharomyces cerevisiae* protein, identical to Tid3p/Ndc80p and here designated scHec1p, has similarities in structure and biological function to hsHec1p. Budding yeast cells deleted in the sc*HEC1/NDC80* allele are not viable, but this lethal phenotype can be rescued by hs*HEC1* under control of the endogenous sc*HEC1* promoter. At the nonpermissive temperature, significant mitotic delay, chromosomal missegregation, and decreased viability were observed in yeast cells with temperature-sensitive (ts) alleles of hs*HEC1*. In the hs*hec1-113* ts mutant, we found a single-point mutation changing Trp395 to a stop codon, which resulted in the expression of a C-terminally truncated 45-kDa protein. The binding of this mutated protein, hshec1-113p, to five identified hsHec1p-associated proteins was unchanged, while its binding to human SMC1 protein and yeast Smc1p was ts. Hec1p also interacts with Smc2p, and the binding of the mutated hshec1-113p to Smc2p was not ts. Overexpression of either hs*HEC1* or sc*HEC1* suppressed the lethal phenotype of *smc1-2* and *smc2-6* at nonpermissive temperatures, suggesting that the interactions between Hec1p and Smc1p and -2p are biologically significant. These results suggest that Hec1 proteins play a critical role in modulating chromosomal segregation, in part, through their interactions with SMC proteins.**

Chromosome segregation during the cell division cycle results from the cooperation of many complex mechanisms. The physical segregation of pairs of sister chromatids into two daughter cells is modulated precisely during M phase. The ultimate goal of this process is to ensure high-fidelity transmission of replicated DNA to offspring. Each step during M-phase progression is coordinated by a group of structural and regulatory proteins. Many of these proteins are highly conserved in all eukaryotes from *Saccharomyces cerevisiae* to *Homo sapiens*.

The structural proteins required for the reorganization of chromosomes after replication have been identified and found to be essential for chromosome segregation. During this chromosomal reorganization process, linkage of duplicated DNA molecules, termed sister chromatid cohesion, is established, and paired sister chromatids undergo condensation. Sister chromatid cohesion and chromosome condensation are required for proper spindle attachments and chromosome movements. Moreover, the separation of paired chromatids is triggered by the dissolution of sister chromatid cohesion at the metaphase-anaphase transition. All of these steps must be precisely regulated to ensure the faithful transmission of chromosomes (19, 21, 24, 40, 41, 45, 53, 54, 56–59). A better understanding of the structural components involved in these chromatin reassembly processes has been achieved by the identification and characterization of the SMC (structural maintenance of chromosomes) protein family. SMC proteins, with members highly conserved from yeast to human, may be classified into four major subfamilies, SMC1- to SMC4-type proteins (24, 40, 56). SMC1- and SMC3-type proteins have been suggested to be essential for sister chromatid cohesion (21, 24, 41, 45, 56). On the other hand, chromosome condensation depends on SMC2- and SMC4-type proteins (24, 25, 27, 40, 53, 56, 57).

Proteins associated with kinetochore assembly are also essential for faithful chromosome segregation (33). Kinetochore proteins are responsible for attachments and movements of chromosomes along the microtubules of the mitotic spindle. Besides centromere-binding proteins, the components of microtubules, spindle pole bodies, microtubule-based motor proteins, and other microtubule-binding proteins are also required for the proper chromosome movement (2, 31, 42).

The structural dynamics of chromosomes must be coordinated with the cell cycle regulatory machinery to execute the precise order of mitotic events. Ubquitin-dependent proteolysis appears to be a major regulatory mechanism modulating M-phase progression as well as other mitotic events. Yeast genes including *CDC16*, *CDC23*, and *CDC27*, which encode components of the anaphase-promoting complex (34), and *SUG1/CIM3* and *CIM5*, which encode the 26S proteasome subunits (4), play pivotal roles in the completion of mitosis (16, 34). The anaphase-promoting complex has been demonstrated to be essential for the degradation of mitotic cyclins (34, 38). Moreover, it controls the onset of sister chromatid separation and the metaphase-anaphase transition by degradation of specific regulators of chromosome transmission such as Pds1p, Cut2p, and Ase1p (10, 15, 30, 36, 39). *cim3* and *cim5* mutants have been shown to arrest yeast cells at $G_2$/M phase, and a defect in the degradation of mitotic cyclins has also been proposed for these mutants (16).

Through our investigation of the molecular events of mitosis and the origin of the chromosomal abnormalities observed in malignant cells, a novel human nuclear protein, hsHec1p, was previously identified. hsHec1p was originally isolated as a retinoblastoma protein (Rb)-associated protein (13), and it apparently plays an important role in chromosome segregation in mammalian cells (8). Our studies showed that upon inactivation of hsHec1p by microinjection of hsHec1p-specific antibodies, chromosome congression is severely disturbed. Moreover,

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TABLE 1. Yeast strains used

| Strain | Genotype | Source |
|---|---|---|
| YPH499 | *MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1* | P. Hieter |
| YPH501 | *MAT***a**/α *ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 trp1-Δ63/trp1-Δ63 his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ* | P. Hieter |
| WHL101 | *MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1* sc*hec1*Δ::*URA3* hs*HEC1* (YCpPA-HSHEC1::*TRP1*) | This study |
| WHL102 | *MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1* sc*hec1*Δ::hs*HEC1*::*URA3* | This study |
| WHL103 | *MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1* sc*hec1*Δ::*URA3* sc*HEC1* (YCpPA-SCHEC1::*TRP1*) | This study |
| WHL113 | *MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1* sc*hec1*Δ::*URA3* hs*hec1-113* (YCpPA-hshec1-113::*TRP1*) | This study |
| WHL1713 | *MAT*α *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1* sc*hec1*Δ::hs*hec1-113*::*URA3* (CFIII *HIS3 SUP11*) | This study |
| YPH1017 | *MAT*α *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1* (CFIII *HIS3 SUP11*) | P. Hieter |
| WHL9702 | *MAT***a**/α *lys2-801/lys2-801 ura3-52/ura3-52 ade2-101/ade2-101 trp1-Δ63/trp1-Δ63 his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1* sc*hec1*Δ::*URA3*/sc*hec1*Δ::*URA3* hs*HEC1* (YcpPA-HSHEC1::*TRP1*) (CFIII *HIS3 SUP11*) | This study |
| WHL9713 | *MAT***a**/α *lys2-801/lys2-801 ura3-52/ura3-52 ade2-101/ade2-101 trp1-Δ63/trp1-Δ63 his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1* sc*hec1*Δ::*URA3*/sc*hec1*Δ::*URA3* hs*hec1-113* (YcpPA-hshec1-113::*TRP1*) (CFIII *HIS3 SUP11*) | This study |
| CH2080 | *MAT*α *lys2-801 ade2-101 trp1-Δ63 leu2-Δ1* | C. Holm |
| CH2082 | *MAT***a** *lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1* (CFIII *TRP1 SUP11*) | C. Holm |
| WHL2003 | *MAT***a**/α *lys2-801/lys2-801 ade2-101/ade2-101 leu2-Δ1/leu2-Δ1* sc*HEC1*/sc*HEC1* (CFIII *TRP1 SUP11*) | This study |
| 1-1bAS172 | *MAT* × *lys2 his3 leu2 smc1-Δ2*::*HIS3 ura3*::*smc1-2* | D. Koshland |
| 2aAS283 | *MAT* × *ura3 lys2 ade2 his3 leu2-Δ1 smc2-6* | D. Koshland |

hsHec1p has been found to interact with a group of proteins important for $G_2$/M progression (9), including sb1.8, the human SMC1 protein (49); p45/Trip1, the S8 subunit of the human 26S proteasome (4) and the human homolog of *S. cerevisiae* Sug1p/Cim3p; MSS1, the S7 subunit of the human 26S proteasome (4) and the human homolog of *S. cerevisiae* Cim5p; and NEK2, a human homolog of NIMA kinase (14). Collectively, these results have provided circumstantial evidence suggestive of a role for hsHec1p as a regulator of M-phase progression.

To elucidate how hsHec1p is involved in M-phase progression, a functional homolog of hsHec1p was identified in the budding yeast *S. cerevisiae*. This protein, previously isolated as Tid3p/Ndc80p (12, 61) and designated scHec1p here, can be complemented by hsHec1p. By expressing temperature-sensitive (ts) mutants of human hs*HEC1* in yeast strains in which sc*HEC1* has been deleted, we demonstrate that Hec1p functions in chromosomal segregation, at least in part, through interactions with SMC proteins.

## MATERIALS AND METHODS

**Strains, reagents, and media.** Yeast strains are described in Table 1. Chemicals and medium components were purchased from Sigma and Difco Laboratories. Standard media were made as described elsewhere (47). *S. cerevisiae* strains used in this study were grown in complete medium (YPD; 1% yeast extract, 2% peptone, 2% dextrose) or in supplemented minimal medium (SMM) lacking appropriate amino acids. To induce the *GAL1* promoter, yeast cells were grown at 25°C to log phase in complete medium or SMM containing 2% glucose and then shifted to the same medium containing 2% galactose. For all the other purposes, the yeasts were cultured in YPD or SMM containing 2% glucose. Yeast transformation, plasmid, and genomic DNA isolation have been described elsewhere (51).

**Cloning and disruption of sc*HEC1*.** The open reading frame (ORF) of sc*HEC1* and its upstream promoter region were amplified by PCR using yeast genomic DNA isolated from YPH499 as the template. The 2.0-kb DNA fragment of the coding region was subcloned into pBluescript SK (Stratagene), generating pBSK-SCHEC1. The 500-bp fragment of the promoter region with *Hin*dIII-*Eco*RI sites, together with a *Eco*RI/blunt DNA fragment containing the *URA3* gene, were inserted into pBSK-SCHEC1 to replace the 1.5-kb sc*HEC1* coding region between *Hin*dIII and *Eco*RV, creating plasmid pBSK-SCHKO. The *Xho*I-*Bam*HI fragment from pBSK-SCHKO containing the target allele, in which amino acids (aa) 1 to 549 of the sc*HEC1* ORF were replaced by the *URA3* gene, was transformed into diploid strain YPH501 to disrupt the sc*HEC1* allele. $Ura^+$ prototrophs were selected, sporulated, and dissected. To confirm the gene disruption, genotyping by PCR methods with primers outside the targeted region and primers inside of the *URA3* genes were performed.

**Replacement of the sc*HEC1* gene with hs*HEC* cDNA.** The same strategy was used to disrupt the sc*HEC1* allele in the haploid strain YPH499. To complement this sc*HEC1* disruption, YCpLac22 (17) was inserted by using the 500-bp fragment of the sc*HEC1* promoter region flanked with *Eco*RI-*Bam*HI sites at ends; the 2.0-kb DNA fragment containing the complete ORF of sc*HEC1* was inserted in the *Bam*HI site. Meanwhile, YCpLac22 was inserted by using the same 500-bp fragment of the sc*HEC1* promoter but flanked with *Eco*RI-*Hin*dIII sites, and the 2.0-kb hs*HEC1* cDNA (8) was inserted in the *Hin*dIII site. The resultant constructs (YCpPA-SCHEC1 and YCpPA-HSHEC1, respectively) with the selection marker, *TRP1*, were transformed into YPH499 together with the sc*HEC1* disruption construct. $Trp^+$ prototrophs were selected and genotyped by PCR to confirm the gene disruption and replaced into the given haploid strains. To replace the sc*HEC1* ORF with hs*HEC1* cDNA directly on the chromosome, pBSK-FB was created by insertion of the *Hin*dIII fragment of hs*HEC1* full-length cDNA driven by the sc*HEC1* promoter on plasmid pBSK-SCHKO. The *Kpn*I-*Not*I fragment from pBSK-FB was transformed into YPH499, and $Ura^+$ prototrophs were selected.

2μm or CEN plasmids (pGAL1-SCHEC1 or pGAL1-HSHEC1) with *LEU2* selection, containing the *GAL1*-inducible promoter and the same expressing genes as in YCpPA-SCHEC1 and YCpPA-HSHEC1, or the vector (pGAL1) containing the *GAL1* promoter alone, were constructed from a modified form of pGAD10 (Clontech, Palo Alto Calif.) or pOC29 (10). They were transformed into 1-1bAS172, 2aAS283, or YPH499 in the assay for suppressing the *smc1-2* or *smc2-6* mutants.

**Generation of conditional mutant alleles of human hs*HEC1* in yeast.** The ts mutant alleles were isolated as described elsewhere (1). The 1.8-kb *Bam*HI-*Sal*I fragment of hs*HEC1* cDNA on plasmid YCpPA-HSHEC1 was treated with 1 M hydroxylamine at 75°C for 90 min to mutate the hs*HEC1* cDNA randomly and then ligated to the YCpPA-HSHEC1 vector linearized by *Bam*HI-*Sal*I. Approximately $10^6$ ampicillin-resistant *Escherichia coli* DH5α transformants were obtained from this ligation reaction. Mutagenized plasmid DNA was extracted from them and used to transform YPH499 together with the sc*HEC1* disruption fragment. About 5,000 transformants of $Trp^+$ $Ura^+$ prototrophs were selected on SMM plates. Each plate was duplicated; one was cultured at 25°C, and the other was cultured at 37°C. Eleven clones grew at 25°C but not at 37°C. The plasmids from these mutant clones were recovered and retransformed to verify the ts phenotype. One of the ts alleles of hs*hec1*, hs*hec1-113*, was sequenced with an Applied Biosystems model 377A sequencer. Yeast strains with an integrated hs*hec1-113* allele on the sc*HEC1* locus were generated by transforming the same *Kpn*I/*Xho*I DNA fragment carrying the hs*hec1-113* mutation.

**Cell growth and synchronization.** For the growth property studies shown in Fig. 1 and 2, a 24-h culture grown in YPD or SMM at 25°C was diluted with fresh YPD or SMM containing 2% glucose at a starting density of $4 \times 10^6$ cells/ml. Then cells were grown at 25°C with vigorous shaking. Change to the nonpermissive temperature occurred during log phase or immediately after release from synchronization. Aliquots of the culture were removed at the indicated times for determining growth densities, colony formation on solid plates, and individual cell morphology. Growth density was measured by spectrophotometric methods and converted to cell number according to standards measured on a hemacytometer.

Synchronization of yeast cells at $G_1$, at early S phase, or at metaphase was performed as follows. Cells were inoculated into YPD or SMM at $4 \times 10^6$ cells/ml, grown at 25°C for 6 h, and then treated either with α-factor at 5 μg/ml, hydroxyurea at 0.1 M, or nocodazole at 20 μg/ml for the periods of time indicated. Synchronized cultures were released from cell cycle arrest by quick spin followed by washing.

**Immunostaining and fluorescence microscopy.** Cell preparation for immunostaining was modified from the standard procedures already described (48). Aliquots of cultured yeast containing approximately $10^7$ cells were fixed by the addition of 16% EM-grade formaldehyde to a final concentration of 4% for 20 min, followed by incubation with yeast fixation buffer (40 mM potassium phosphate [pH 6.5] containing 0.5 M $MgCl_2$ and 4% formaldehyde) for 24 h. Fixed

cells were washed twice with solution A (40 mM potassium phosphate [pH 6.5], 0.5 M $MgCl_2$, 1.2 M sorbitol). Cells were spheroplasted in 1 ml of solution A containing 10 μl of 2-mercaptoethanol and 55 μl of Glusulase (Du Pont NEN) for 2 h at 36°C with gentle agitation. Cells were washed twice in solution A and resuspended in 0.2 ml of solution A. Twenty microliters of this suspension was spotted into a gelatin-coated cover slide. The immunostaining procedures were as described elsewhere (8). Microtubules were detected by monoclonal antitubulin antibody YOL134 (Accurate Scientific) diluted 1:50, followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-rat immunoglobulin G (Fisher) diluted 1:100. hsHec1p or hshec1-113p was detected by anti-hsHec1p monoclonal antibody (MAb) 11A5 (1:250) or affinity-purified rabbit polyclonal anti-hsHec1p antibodies (1:250 to 1:500), and scHec1p was detected by mouse polyclonal anti-scHec1p antibodies (1:250), followed by FITC- or Texas red-conjugated secondary antibodies as indicated. The cells were further stained with 1 μg of 4′,6-diamidino-2-phenylindole (DAPI) per ml in phosphate-buffered saline (PBS) containing 1 mM *p*-phenylenediamine and mounted in Permafluor (Lipshaw-Immunon Inc.). Cells were observed using a standard fluorescence microscope (Axiophot photomicroscope; Zeiss).

**Flow cytometry.** Cells were prepared for flow cytometry as described elsewhere (23). Cells were resuspended in PBS and fixed in 70% ethanol. After being washed twice in 50 mM Tris (pH 8.0), cells were treated with RNase (1 mg/ml) for 2 h at 37°C and then incubated in proteinase K (40 μg/ml) for another 1 h at 50°C. Cells were pelleted and resuspended in propidum iodide (0.2 mg/ml in PBS). Before analysis, cells were sonicated for 5 s at the lowest setting. Fifty thousand cells from each sample were analyzed by FACScalibur (Becton Dickinson).

**Generation of polyclonal antibodies against scHec1p or Smc1p.** scHec1p was detected by mouse anti-Hec1p polyclonal antisera raised against a glutathione *S*-transferase–scHec1p fusion protein expressed in *E. coli*.

Two kinds of mouse anti-Smc1p polyclonal antisera, anti-Smc1B and anti-Smc1C, were raised against a glutathione *S*-transferase protein fused with aa 634 to 847 and aa 1021 to 1225, respectively, of Smc1p. Specificities of the antibodies compared with that of rabbit anti-Smc1p antisera (kindly provided by A. Strunnikov) (58), were checked. The antibodies recognized a 165-kDa cellular protein of the same size as described for Smc1p. The assays represented in Fig. 6 were repeated using rabbit anti-Smc1p antisera (data not shown).

**Immunoprecipitation and Western blotting.** Yeast cell lysates were prepared as described elsewhere (51), with the following modifications. Cell pellets were washed once and resuspended in 200 μl of yeast lysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS]) plus protease inhibitors. Two volumes of glass beads (0.5-mm diameter) were added, and the suspensions were vortexed at the highest speed for six periods totaling 3 min. The clarified lysates were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) followed by immunoblotting analysis as described previously (8). hsHec1p was detected by anti-hsHec1p MAb 9G3. For immunoprecipitation, the lysates were incubated with anti-scHec1p (diluted 1:500), 9G3 (1:2,000), anti-Smc1C (1:300), or anti-Gal4 transactivation domain (TAD; 1:100; Santa Cruz) at 4°C for 3 h; then protein A-Sepharose beads were added. After another 2 h of incubation, beads were washed with lysis 250 buffer (50 mM Tris [pH 7.5], 250 mM NaCl, 50 mM sodium fluoride, 0.5% Nonidet P-40) for immunoprecipitation or lysate 175 (175 mM NaCl) for coimmunoprecipitation. Finally, beads were boiled in 2× SDS sample buffer and subjected to SDS-PAGE followed by immunoblotting analysis with the antibodies indicated.

**Colony sectoring assay.** hs*HEC1* and hs*hec1-113* diploid strains for colony sectoring assay were mated from two haploid strains, YPH499 (*Mat***a**) and YPH1017 (*Matα*) (Table 1), in which sc*HEC1* was deleted and rescued by hs*HEC1* or hs*hec1-113* in the YCp-PA vector as described above. The resultant diploid strains contain a homozygous sc*hec1* null mutation marked by *URA3*, a homozygous *ade2-101* ochre color mutation, a chromosome fragment carrying a copy of *SUP11*, and a wild-type allele of hs*HEC1* or a ts allele hs*hec1-113* in a CEN-ARS1 vector marked by *TRP1*. The sc*HEC1* diploid strain used in this assay was mated from two wild-type haploid strains, CH2082 and CH2080 (29). Five single pink colonies of each diploid strain were picked and cultured in histidine-free SMM at 25°C for 3 days. Equal numbers of cells from each strain were inoculated at 37 or 25°C into fresh histidine-containing SMM and then cultured further for 3 h before plating on medium containing 6 mg of adenine per liter. The plates were incubated at 25°C for 6 days and 4°C overnight before observation.

## RESULTS

**hs*HEC1* can functionally substitute for yeast sc*HEC1*.** Using the hsHec1p sequence, we identified in a search of the current GenBank protein database a potential homologous protein, scHec1p, encoded by an ORF in the *S. cerevisiae* genome. Interestingly, this gene has been independently isolated twice, once as *TID3* in a screen for proteins interacting with *S. cerevisiae* Dmc1p (13) and again as *NDC80* in an attempt to identify spindle pole components. Ndc80p was previously proposed to be a potential homolog of hsHec1p, based on structural similarity (61). Consistently, we found that human and yeast Hec1p are 36% identical in their N-terminal regions, and their C-terminal regions constitute similar coiled-coil enriched structures. Such homologous sequences were also identified in fission yeast (61), *Caenorhebditis elegans*, and mouse genomes (data not shown).

Inactivation of *NDC80* has been shown to be lethal in budding yeast (61), which is consistent with our previous finding that hs*HEC1* is essential for cell viability since inactivation of hsHec1p in mammalian cells by specific antibodies leads to cell death. The structural and functional similarities between hsHec1p and scHec1p/Ndc80p indicated that scHec1p may be the hsHec1p homolog. Because of a marked overall structural divergence between the two proteins, however, it was necessary to test whether hs*HEC1* could functionally substitute for sc*HEC1* in yeast cells. To this end, the endogenous sc*HEC1* was inactivated by one-step gene disruption (53). The *URA3* marker gene, flanked by a 0.5 kb of 5′ sequence from the putative promoter region of sc*HEC1* and 1.6 kb of 3′ sequence of sc*HEC1* (Fig. 1A), was first used to replace one wild-type allele of sc*HEC1* in a diploid strain, YPH501. The mutated diploid strain exhibited segregation of two viable ($Ura^-$) and two lethal spores by tetrad analysis. Similar sc*HEC1* disruption was then performed with a haploid strain, YPH499. No yeast colonies survived after disruption of the sc*HEC1* allele; however, cotransformation of the sc*HEC1* disruption fragment with sc*HEC1* DNA in a *TRP1*-marked CEN-ARS1 vector rescued the lethal phenotype and generated strain WHL103 (Table 1; Fig. 1A). We therefore introduced the hs*HEC1* cDNA, in a *TRP1*-marked CEN-ARS1 vector, into selected haploid yeast cells in which the sc*HEC1* allele was disrupted (Fig. 1A). Expression of hs*HEC1* cDNA, under transcriptional control of the yeast sc*HEC1* promoter, was able to rescue the lethal phenotype resulting from sc*HEC1* disruption, thereby generating the strain WHL101 (Table 1; Fig. 1A). Similar results were obtained when hs*HEC1* was integrated into the sc*HEC1* locus to generate strain WHL102 (Table 1). Genotyping by PCR methods confirmed the replacements (Fig. 1B and C). Yeast cells in which sc*HEC1* was disrupted and reconstituted by hs*HEC1* expressed only hsHec1p; endogenous scHec1p was no longer detected (Fig. 1D).

To determine whether the hsHec1p can functionally substitute for scHec1p during yeast cell cycle progression, growth properties as well as cell morphology, spindle shape, and DNA content of the parental and rescued yeast strains were examined. Colony formation in solid plates and growth kinetics in liquid culture appeared to be indistinguishable in the strains with sc*HEC1* or with hs*HEC1* at 25°C (Fig. 1E). Fluorescence microscopy of cells stained with antitubulin antibodies and DAPI demonstrated similarities in morphology, spindle shape, and DNA content in the two strains; the distributions of cells in different phases of the cell cycle were also indistinguishable in these two strains (Fig. 1F). Neither the growth properties nor the morphologies of cells expressing hsHec1p were changed upon cultured at 37°C (data not shown). These results demonstrated that hsHec1p is indeed the functional homolog of scHec1p. Moreover, two yeast strains in which sc*HEC1* is replaced by the hs*HEC1* gene in either an episomal form (WHL101) or an integrated form (WHL102) were established for further studies.

**Hec1p localizes to the nucleus.** Our previous study showed that hsHec1p localized to the nucleus and that a portion of it moved to the centromere regions during mitosis (8). To determine whether hsHec1p localizes similarly in yeast cells, we performed immunostaining of Hec1p in yeast cells carrying

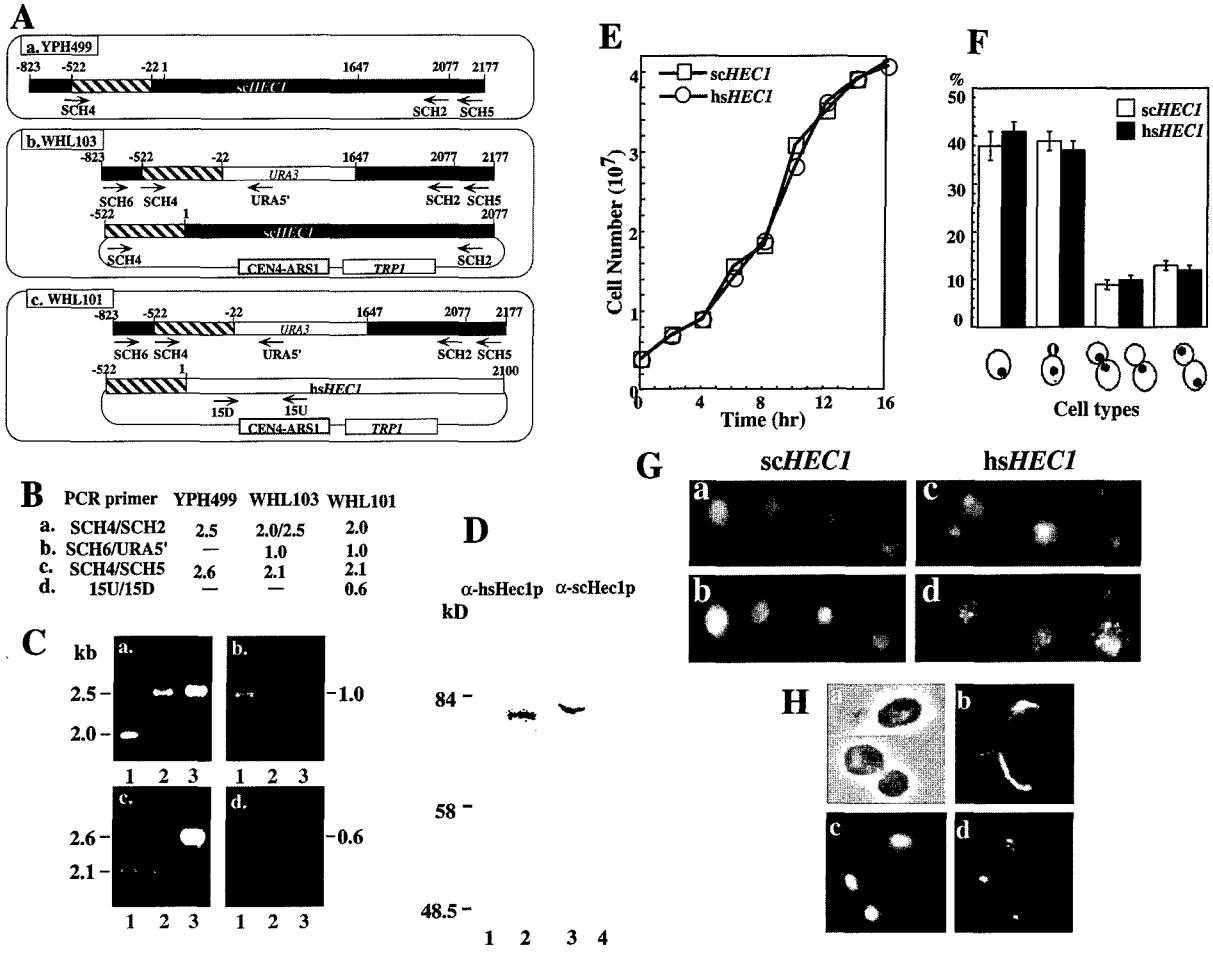


FIG. 1. Yeast cells with the sc*hec1* null mutation can be rescued by human hs*HEC1* (A) Schematic diagram of wild-type cell (a. YPH499) and yeast cells rescued by sc*HEC1* (b. WHL103) or hs*HEC1* (c. WHL101). The yeast sc*HEC1* promoter region is marked by hatched boxes; arrows indicate the primers used in PCR genotyping. (B) Predicted DNA fragment sizes determined by PCR genotyping using the primers shown in panel A. (C) DNA fragments of PCR genotyping analyzed by agarose gel electrophoresis. Lane 1, WHL101; lane 2, WHL 103; lane 3, YPH499. (D) Immunoblot analysis of total cell lysates from YPH499 (lanes 1 and 3) and WHL101 (lanes 2 and 4), using anti-hsHec1p MAb 9G3 and mouse anti-scHec1p sera, respectively. (E) Growth-density curve of the yeast strain carrying the sc*HEC1* allele (YPH499) and the isogenic yeast strain in which the sc*HEC1* allele is replaced by hs*HEC1* (WHL102) at 25°C. (F) The cell type distributions of yeast grown at 25°C for 4 h in (E) were observed with fluorescence microscopy. The percentages of cells at different phases of the cell cycle were determined according to budding morphologies, nuclear patterns, and spindle formation, and presented as histograms. (G) Immunostaining of Hec1p in yeast cells carrying the sc*HEC1* (a and b) or hs*HEC1* (c and d) allele. scHec1p was detected by mouse polyclonal anti-scHec1p antibodies (b); hsHec1p was detected by purified rabbit polyclonal anti-hsHec1p antibodies (d). Corresponding FITC-conjugated secondary antibodies were used. DNA was stained with DAPI (a and c). (H) Immunostaining of hsHec1p in yeast cells. a, phase-contrast; b, spindle stained by rat antitubulin antibodies and FITC-conjugated secondary antibodies; c, nuclear DNA strained by DAPI; d, hsHec1p stained by rabbit anti-hsHec1p antibodies and Texas red-conjugated secondary antibodies.

hs*HEC1* or sc*HEC1*. As shown in Fig. 1G, a nuclear staining pattern was observed in yeast cells carrying hs*HEC1*, and brighter speckles can be seen in nuclei. Costaining of spindles suggested that these brighter speckles were concentrated in the vicinity of the spindle pole body and confined to the periphery of the chromosomal DNA mass in unbudded or large budded anaphase cells (Fig. 1H). This pattern reflected the position of the centromeric DNA in these cells, as previously revealed by fluorescence in situ hybridization (20, 55), and was similar to the staining of centromere proteins, such as Mif2p (43), Cse4p (44), and Ctf19p (32). Consistently, the nuclear staining pattern and brighter speckles were also seen in the cells carrying sc*HEC1* (Fig. 1G).

**Isolation of ts mutants of hs*HEC1*.** The yeast strain in which the essential function is sustained by the hs*HEC1* gene has allowed us to explore the precise role of this human protein in mitotic division through isolating conditional alleles of hs*HEC1* in yeast. We performed random mutagenesis on an hs*HEC1* cDNA fragment, subcloned the resultant fragments in the CEN-ARS1 vector, and transformed the sc*HEC1*-disrupted strain for isolation of conditional lethal mutants. Eleven ts mutants which grew at 25°C but not 37°C were isolated, and their growth in liquid culture was examined. At 25°C, the growth pattern of the mutant yeast appears no different from that of the wild-type strain (WHL101). When the cultures were shifted to 37°C, however, the ts mutants stopped growing after the first or second division (Fig. 2A). Like mammalian cells in which hsHec1p is inactivated by specific antibodies, yeast cells without functional hsHec1p fail to arrest completely during mitosis and instead apparently proceed to cell division and cytokinesis. These mutant cells did not regain growth potential when shifted from the nonpermissive to the permissive temperature. Samples from these mutants cultured at 37°C for 3 h (approximately 1.5 generation times) were plated and incu-

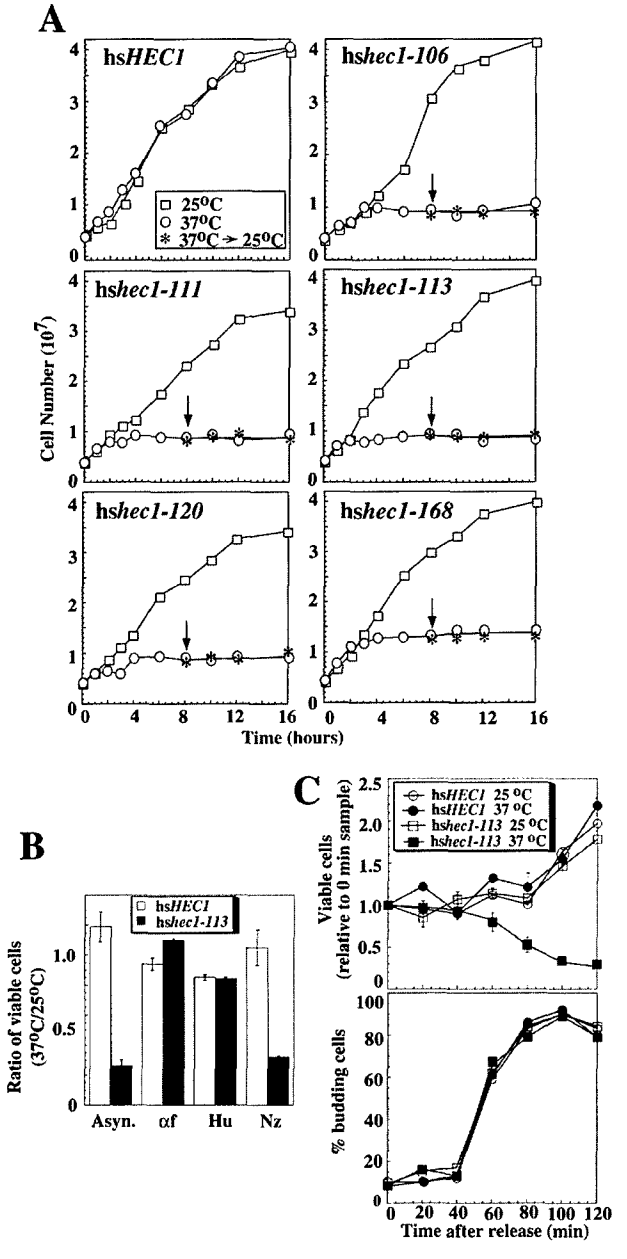


FIG. 2. Temperature sensitivity of mutated hs*hec1* alleles. (A) Growth-density curves of yeast strains carrying different mutant alleles or the wild-type allele of hs*HEC1* were measured by spectrophotometric methods. Log-phase cells originally growing at 25°C were inoculated and cultured at 25 or 37°C, respectively. After 8 h, half of the cultures grown at 37°C were shifted back to 25°C, as indicated by arrows. (B) Log-phase cells from hs*HEC1* or hs*hec1-113* strains were arrested with α-factor at 5 μg/ml, hydroxyurea at 0.1 M, or nocodazole at 20 μg/ml or were left untreated for 3 h at 25°C. The percentages of arrested cells, determined by DAPI staining and observation by fluorescence microscopy, were between 85 to 95%. While arrested, half of each culture was shifted to 37°C and the remaining was kept at 25°C. After another 3-h incubation, cells were released and plated at 25°C. The ratios between the numbers of colonies formed by the cells exposed to 37°C and those by the cells exposed to 25°C are shown by histograms. Asyn., asynchronous; αf, α-factor; Hu, hydroxyurea; Nz, nocodazole. (C) Log-phase cells from hs*HEC1* or hs*hec1-113* strains were arrested with α-factor at 5 μg/ml for 3 h at 25°C. Immediately after release, half of each culture was shifted to 37°C and the remaining half was kept at 25°C. The numbers of colonies formed by equal aliquots taken from these cultures every 20 min were counted from duplicated plates. Numbers of colonies formed for each sample relative to the 0-min sample were calculated to generate the curves in the upper panel. The lower panel shows the percentages of budding cells in each sample.

bated at 25°C. Only 30 to 40% of cells were able to form colonies on solid plates, whereas the wild-type strain showed no loss of viability at 37°C. These results suggested that mutations of hs*HEC1* lead to cell death at the nonpermissive temperature.

To determine whether the lethality at the nonpermissive temperature is specifically due to the failure of a cell cycle event, one of the ts mutants, hs*hec1-113*, was further examined. Treatment of exponentially growing cultures of hs*hec1-113* cells or cogenic wild-type hs*HEC1* cells with α-factor, hydroxyurea, or nocodazole at the permissive temperature resulted in cell cycle arrest at $G_1$, early S, or $G_2$/M phase, respectively. While arrested, these cells were shifted to 37°C for 3 h to inactivate hsHec1 proteins and then released by plating at 25°C. As controls, equivalent numbers of the arrested cells were cultured at 25°C for the same period of time. The ratio of viable cells at the nonpermissive temperature to those at the permissive temperature was used to determine viability. When the hsHec1p activity was depleted in $G_2$/M phase, this ratio dropped to 0.3, or approximately the same as the ratio in unsynchronized cells (Fig. 2B). In contrast, when the hsHec1p activity was depleted in either $G_1$ or S phase, the cell viability did not decrease. These results suggested that the function of hsHec1p is essential for cell viability during M phase. Increased lethality during M phase has also been observed in cells defective in proteins involved in sister chromatid cohesion or chromosome condensation, including Top2p (28), Smc1p (58), and Mcd1p (22). This resemblance is consistent with a relationship between Hec1p and these proteins suggested below.

The function of hsHec1p in M phase was further determined by following the viability of cells as they synchronously traversed a dynamic cell cycle (28). The hs*hec1-113* mutant and wild-type hs*HEC1* strains were synchronized in $G_1$ phase by α-factor at 25°C for 3 h and then released to fresh medium at 37 and 25°C as controls. At 20-min intervals, equal portions of the 37°C culture were shifted to 25°C and plated immediately to assess cell viability (Fig. 2C; upper panel). Cell cycle progression was monitored according to cell-budding morphologies (Fig. 2C; lower panel) and DNA content analysis (data not shown). During the initial 60 min, the viability of both strains at both temperatures remained relatively constant. After 60 min, however, the number of viable cells from the hs*hec1-113* strain grown at 37°C began to decrease. At 100 to 120 min after release from $G_1$ phase, the viability of the hs*hec-113* culture at 37°C continued to decrease; in contrast, the numbers of viable cells from all the other cultures doubled because their buds were released from the mother cells. Thus, death of the mutant cells could be prevented by a shift back to the permissive temperature during the initial 60 min, suggesting that the essential role of hsHec1p was not required during that period of time immediately following release from $G_1$. However, at 80 to 100 min after release (i.e., during M phase), cell death could no longer be prevented in cells containing inactivated hsHec1p. These results suggested that the essential function of hsHec1p is required when cells enter M phase.

**hs*hec1* mutants demonstrate mitotic delay and unequal chromosome segregation at the nonpermissive temperature.** Cytological analysis of the hs*hec1* mutant revealed accumulation of large-budded cells at the nonpermissive temperatures (Fig. 3A). The subpopulation of cells at anaphase consisted predominately of the cells in which DNA failed to separate into two discrete masses. Some cells with large buds contained DNA evident only at one pole (Fig. 3B). DNA content analysis showed that hs*hec1-113* mutant cells accumulated with a $G_2$ DNA content after shifting to 37°C; the shoulder of the $G_2$

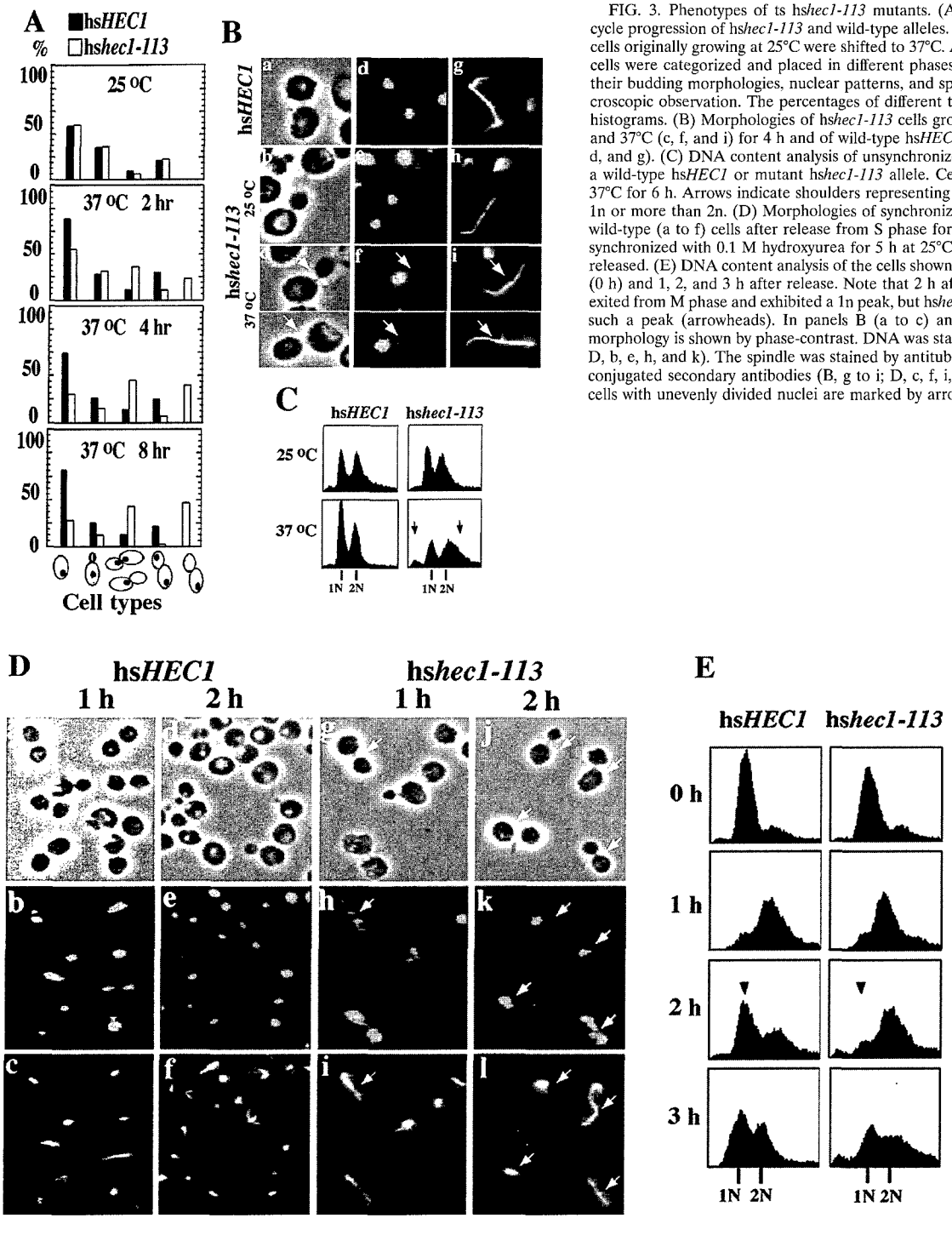


FIG. 3. Phenotypes of ts hs*hec1-113* mutants. (A) Characterization of cell cycle progression of hs*hec1-113* and wild-type alleles. Unsynchronized log-phase cells originally growing at 25°C were shifted to 37°C. At 2, 4, and 8 h, 300 to 500 cells were categorized and placed in different phases of cell cycle according to their budding morphologies, nuclear patterns, and spindle formation under microscopic observation. The percentages of different types of cells are shown by histograms. (B) Morphologies of hs*hec1-113* cells growing at 25°C (b, e, and h) and 37°C (c, f, and i) for 4 h and of wild-type hs*HEC1* cells growing at 37°C (a, d, and g). (C) DNA content analysis of unsynchronized log-phase cells carrying a wild-type hs*HEC1* or mutant hs*hec1-113* allele. Cells were cultured at 25 or 37°C for 6 h. Arrows indicate shoulders representing DNA content of less than 1n or more than 2n. (D) Morphologies of synchronized hs*hec1-113* (g to l) and wild-type (a to f) cells after release from S phase for 1 and 2 h. The cells were synchronized with 0.1 M hydroxyurea for 5 h at 25°C and shifted to 37°C when released. (E) DNA content analysis of the cells shown in panel D, before release (0 h) and 1, 2, and 3 h after release. Note that 2 h after release, wild-type cells exited from M phase and exhibited a 1n peak, but hs*hec1-113* cells did not exhibit such a peak (arrowheads). In panels B (a to c) and D (a, d, g, and j), cell morphology is shown by phase-contrast. DNA was stained with DAPI (B, d to f; D, b, e, h, and k). The spindle was stained by antitubulin antibodies and FITC-conjugated secondary antibodies (B, g to i; D, c, f, i, and l). The large-budded cells with unevenly divided nuclei are marked by arrows.

peak was extended to reflect a DNA content greater than 2n, and a small peak representing DNA content less than 1n appeared. This profile suggested the cells with the hs*hec1-113* allele become aneuploid at the nonpermissive temperature (Fig. 3C). Therefore, the phenotypes of hs*hec1* mutants are very similar to those observed in mutants of its yeast homolog (61), suggesting that human hsHec1p also plays a role in chromosome segregation.

Cell cycle progression of hs*hec1-113* mutant cells was monitored following synchronization with 0.1 M hydroxyurea at 25°C and release to fresh cultures at 37°C (Fig. 3D). Both wild-type and mutant cells entered M phase 1 h after release from hydroxyurea-induced S-phase arrest. After 2 h, however, only the wild-type cells exited from M phase. The hs*hec1-113* cells were still dominated by large-budded morphologies. Cells with unequal nuclear division and disperse DNA morphology were also observed. Results from DNA content analysis of the same cell samples were consistent with the cytological studies (Fig. 3E). A mitotic delay in hs*hec1-113* cells was thus suggested.

TABLE 2. Chromosome loss and nondisjunction in hs*hec1-113* diploids

| Genotype | Temp (°C) | No. of colonies[a] | Missegregation rate | |
|---|---|---|---|---|
| | | | % 1:0 events[b] | % 2:0 events[c] |
| sc*HEC1* | 25 | 10,021 | 0.02 | 0.01 |
| hs*HEC1* | 25 | 6,096 | 0.02 | 0.01 |
| | 37 | 6,827 | 0.03 | 0.01 |
| hs*hec1-113* | 25 | 4,299 | 1.35 | 0.37 |
| | 37 | 5,138 | 1.85 | 0.41 |

[a] Total number of pink colonies that carry one chromosome fragment.
[b] Number of half-red/half-pink colonies divided by the total number of pink colonies.
[c] Number of half-red/half-white colonies divided by the total number of pink colonies.

**hs*hec1* mutations increase the rate of chromosome missegregation.** Both cytological observations and DNA content analysis suggested that mutations of *HEC1/NDC80* cause a defect in chromosome segregation. To examine the rates at which this defect occurred in an hs*hec1* mutant, we used a colony sectoring assay in which diploid strains carried a homozygous *ade2* (ochre) color mutation and a single copy of ochre suppressor (*SUP11*) on a dispensable chromosome fragment (CF) (26). Exponentially growing cells from hs*HEC1* and hs*hec1-113* diploid strains were cultured at 25 or 37°C for 3 h and then plated at the permissive temperature. The total numbers of pink colonies representing 1:1 segregation, half-pink/half-red sectored colonies representing 1:0 segregation of CF, and half-white/half-red sectored colonies representing 2:0 segregation of CF were determined. The rates of chromosome loss and nondisjunction in the first division were determined by the frequencies of half-pink/half-red colonies and half-white/half-red colonies, respectively. Results suggested that the diploid strain carrying the wild-type hs*HEC1* allele exhibited very low rates of both chromosome loss and nondisjunction, which were indistinguishable from those for the strain carrying the wild-type sc*HEC1* allele (Table 2). The diploid strain carrying mutant hs*hec1-113* allele at 25°C, however, exhibited a 65-fold increase in the rate of chromosome loss and an 18-fold increase in nondisjunction compared to that carrying the wild-type alleles. After transient exposure to 37°C, the rate of chromosome missegregation was increased further. However, this increment was not dramatic, presumably because of excessive cell death at the nonpermissive temperature. These results showed that hs*hec1-113* mutant strain has missegregated chromosomes; the more severe phenotype represents chromosome loss and the moderate phenotype nondisjunction events.

Although defects in DNA metabolism may lead to large increases only in the rate of chromosome loss (47), the hs*hec1-113* mutant predominantly has a 2n DNA content at the nonpermissive temperature, making it unlikely that a failure of bulk DNA synthesis is responsible for the increasing rate of chromosome loss. Interestingly, the observed phenotype is consistent with roles of several key proteins in the process of chromosome segregation. For example, one member of a family of *ctf* (chromosome transmission fidelity) mutants, *spt4*, exhibits a similar increase in chromosome loss events, apparently due to a defect in kinetochore function (3). Another member, *ctf13-30*, has abnormal kinetochores and displays a comparable increase in both chromosome loss and nondisjunction events (11).

**hs*hec-113* contains a nonsense mutation and encodes a 45-kDa truncated protein.** To determine the mutations of hs*HEC1* leading to the ts phenotype, the plasmid DNA containing the hs*hec1-113* mutant allele was recovered from the yeast mutant strain WHL113 for sequence analysis. The hs*hec1-113* mutant allele was found to have a nonsense mutation, changing Trp395 to a stop codon in its translational sequence and resulting in the expression of a C-terminally truncated, 45-kDa protein (Fig. 4A). To further substantiate this finding, the hs*hec1-113* yeast lysates were analyzed by immunoprecipitation followed by Western blot analysis using an anti-hsHec1p MAb as probe. A 45-kDa protein, hshec1-113p, consistent with the predicted size, was detected in the mutant cells (Fig. 4B). The relative abundance of hshec1-113p did not appear to be significantly different at either 37 or 25°C. The ts phenotypes are, therefore, probably not the result of changes in protein expression at the nonpermissive temperature.

The staining pattern of hshec1-113p at 25°C was not distinguishable from that of wild-type Hec1p (Fig. 4C). The brighter speckles of hshec1-113p appeared to be restricted to the region proposed for the centromere localization. However, at 37°C, these brighter speckles were no longer confined to this region but randomly distributed. It remains to be explored whether

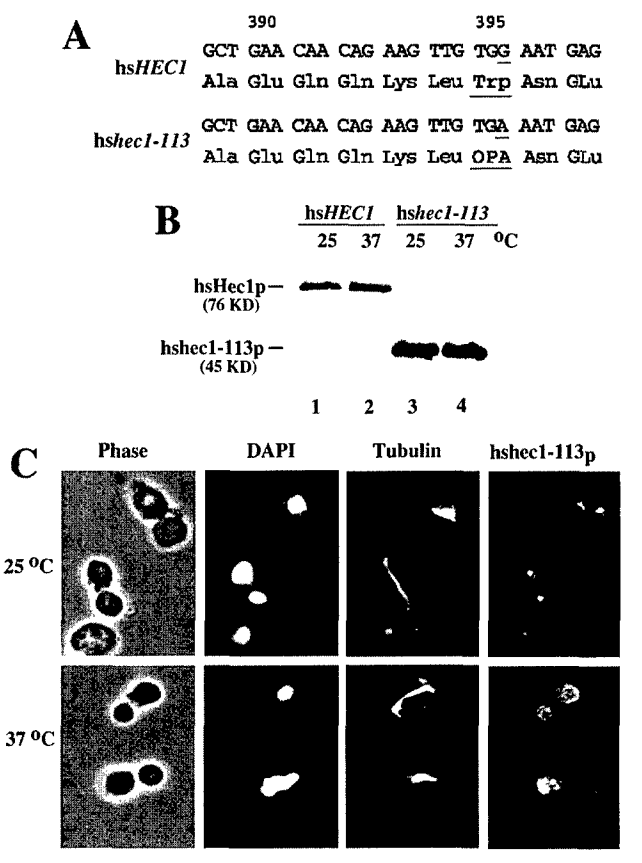


FIG. 4. Characterization of the mutant hshec1-113p. (A) Partial nucleotide and protein sequences of wild-type hs*HEC1* and mutant hs*hec1-113* alleles. The nucleotide substitution in *hshec1-113p* (G to A) and the corresponding amino acid change at position 395 (Trp to a stop codon OPA) are underlined. (B) Identification of hshec1-113p in mutant cells. Cell lysate prepared from wild-type (lanes 1 and 2) and hs*hec1-113* (lanes 3 and 4) cells growing at 25°C (lanes 1 and 3) or 37°C (lanes 2 and 4) for 4 h were immunoprecipitated by mouse anti-hsHec1p MAbs, followed by SDS-PAGE and Western blotting with the same antibodies. (C) Subcellular localization of the mutant hshec-113p. Log-phase cells of hs*hec1-113* were grown at 25°C or shifted to 37°C for 2 h. hshec-113p was stained by purified rabbit anti-hsHec1p antibodies and Texas red-conjugated secondary antibodies. Nuclear DNA of the same cells was stained by DAPI; spindle was stained by rat antitubulin antibodies and FITC-conjugated secondary antibodies.

TABLE 3. Interaction between scHec1p and hsHec1p-associated proteins

| hsHec1p-associated protein | Binding with hsHec1p[a] ($U^b$) | Binding with scHec1p | | Homolog |
|---|---|---|---|---|
| | | ($U^b$) | Color | |
| MSS1 | 371.5 ± 19.3 | | White | Cim5p |
| 15A2 | 468.9 ± 65.8 | 1,066 ± 40 | Blue | |
| sb1.8 | 273.3 ± 10.0 | 348 ± 20 | Blue | Smc1p |
| NEK2 | 239.4 ± 32.6 | 126 ± 2 | Blue | NIMA |
| 15A20 | 319.1 ± 16.6 | 309 ± 1 | Blue | |
| Subunit p45 of 26S proteasome | 1,105.2 ± 159.3 | 122 ± 2 | Blue | Sug1p/Cim3p |
| Rb | 8,345 ± 3,086 | | White | |

[a] Some data adapted from reference 9.
[b] β-Galactosidase activity units.

this abnormal pattern is reminiscent of the mislocalization of mutant hec1p or a rearrangement of centromeres in the mutant cells.

**hshec1-113p interacts with hsHec1p-associated proteins but not with SMC1 proteins at the nonpermissive temperature.** hsHec1p has been shown to interact with several proteins important for M-phase progression by yeast two-hybrid screening (9). To test whether scHec1p can bind to the hsHec1p-associated proteins, a yeast two-hybrid assay was used to score for β-galactosidase activity. The results showed that scHec1p can indeed interact with most of the hsHec1p-associated proteins, including sb1.8, p45, NEK2, and other novel proteins (Table 3). Interestingly, scHec1p does not interact with human Rb and MSS1; therefore, the interaction of Hec1 proteins with these proteins may be restricted to higher organisms.

Since most hsHec1p-associated proteins appear to have homologs in budding yeast, it is reasonable to postulate that hsHec1p may interact with the yeast homologs of these HEC-associated proteins. Mutant hshec1-113p contains the first complete coiled-coil domain but is missing most of the second domain. The first domain is sufficient for binding to many associated proteins (NEK2, sb1.8, and p45) but is insufficient for binding to MSS1 (9). The interaction between the truncated hshec1-113p and hsHec1p-associated proteins was tested at either 25 or 37°C. Mutant derivatives containing the first coiled-coil domain and a small part of the second domain (aa 251 to 394), which resembled the truncated C-terminal coiled-coil region of hshec1-113p, were able to interact with all of the associated proteins assayed at 25°C. However, the interaction with sb1.8 was specifically abolished at 37°C, while binding to other proteins was not changed (Fig. 5A). This result suggested that mutant hec1-113p may interact with yeast Smc1p in a ts manner similar to its binding to the human SMC1 protein in the yeast two-hybrid assay.

To determine which region(s) of Hec1p is necessary for interaction with the SMC1 protein, four hsHec1p deletion derivatives containing different coiled-coil regions fused to the Gal4 DNA-binding domain as previously described (9) were used in the yeast two-hybrid assay. The fragment of the human SMC1, sb1.8, originally cloned from yeast two-hybrid screening contains a 0.6-kb region (aa 621 to 836) encompassing the second coiled-coil domain. The putative homologous region of this 0.6-kb fragment (aa 634 to 847) of the yeast Smc1p, corresponding to the second coiled-coil domain, was also cloned. These two fragments, fused in frame to the Gal4 TAD, were used in the yeast two-hybrid assay. Both the first (aa 251 to 431) and the second (aa 361 to 547) coiled-coil regions of Hec1p are sufficient for interaction with human or yeast SMC1. The remaining region (aa 547 to 618), in which the coiled coil domain is poorly formed, although enriched in leucine heptad repeats, is not able to bind SMC1 (Fig. 5B). Similarly, the scHec1p appears to interact with both yeast and human SMC1.

The specific interaction between Hec1p and yeast Smc1p allows us to test whether the binding of the mutated hshec1-113p to Smc1p is temperature dependent in yeast cells. In assays using anti-hsHec1p MAbs, Smc1p was coimmunoprecipitated from the *hshec1-113* mutant cells cultured at the permissive temperature. Little Smc1p, however, was detectable in the immunocomplex precipitated by anti-hsHec1p antibodies from the cells cultured at the nonpermissive temperature (Fig. 5C). This result further confirmed that hshec113p fails to interact with Smc1p at nonpermissive temperatures, although the abundance of Smc1p is not changed.

**Hec1p also interacts with Smc2p.** The study of *Xenopus* condensin and cohesin complexes suggests that the SMC proteins of the SMC1/SMC3 subgroup and the SMC2/SMC4 subgroup have distinct functions in sister chromatid cohesion and chromosome condensation, respectively (41). Studies of *S. cerevisiae*, however, indicated that there are certain links between cohesion and condensation in certain organisms (21, 24, 56). Since Hec1p is associated with SMC1, it is possible that Hec1p is associated with other SMC proteins; in yeast, this association may also extend to condensation proteins such as Smc2p. To test this hypothesis, the same hsHec1p deletion derivatives were used to test their ability to bind to Smc2p. The full-length coding region of *SMC2* was cloned by PCR amplification from *S. cerevisiae* genomic DNA and fused to the Gal4 TAD (aa 768 to 881). As shown in Fig. 6A, the first coiled-coil-enriched region (aa 251 to 361) of hsHec1p was sufficient for binding to Smc2p; however, unlike the binding to Smc1p, the second coiled-coil enriched region (aa 361 to 547) was unable to bind Smc2p. These results suggested that Hec1p also interacts with Smc2p, albeit in a manner distinct from its interaction with Smc1p. Consistently, the binding ability of the first coiled-coil enriched region (aa 251 to 394) within the hshec1-113p mutant showed no differences at various temperatures (Fig. 6A). Therefore, the role of Hec1p in the modulation of SMC2 function may not be affected in *hec1* mutants at nonpermissive temperatures by the dissociation of these two proteins.

The interaction between scHec1p and Smc2p was further verified in vivo in yeast strain Y153 (*SMC2 gal4*Δ) (13). Smc2p was expressed in episomal form, tagged by TAD, and driven by the *ADH1* promoter as described above. Smc2p can be coimmunoprecipitated by anti-scHec1p antibodies and subsequently detected by immunoblot analysis using anti-TAD, an antibody that specifically recognize the TAD from cells expressing tagged Smc2p (Fig. 6B, lanes 4 to 6), but not from Y153 cells. On the other hand, anti-TAD can precipitate the TAD-Smc2p and coprecipitate scHec1p. These results suggested that Hec1p interacts with Smc2p in vivo.

**Genetic suppression of the *smc* mutants by overexpression of hs*HEC1* or sc*HEC1*.** The in vivo interaction between Hec1p and SMC1 or SMC2 may have biological significance. Both *smc1* and *smc2* mutations cause failure of chromosome segregation, leading to cell death at nonpermissive temperatures (57, 58). Such phenotypes are also observed with hs*hec1* mutations. If the interaction between Hec1p and Smc1p or Smc2p is critical for the Smc1p or Smc2p function in chromosomal segregation, the overexpression of *HEC1* may overcome the ts *smc1* or *smc2* phenotype. To test this possibility, we introduced an episomal plasmid containing hs*HEC1* or sc*HEC1* under control of the *GAL1* promoter into *smc1-2* and *smc2-6* ts mutants. After induction by galactose, the overexpression of hs*HEC1* or sc*HEC1* appeared to be able to suppress the lethal

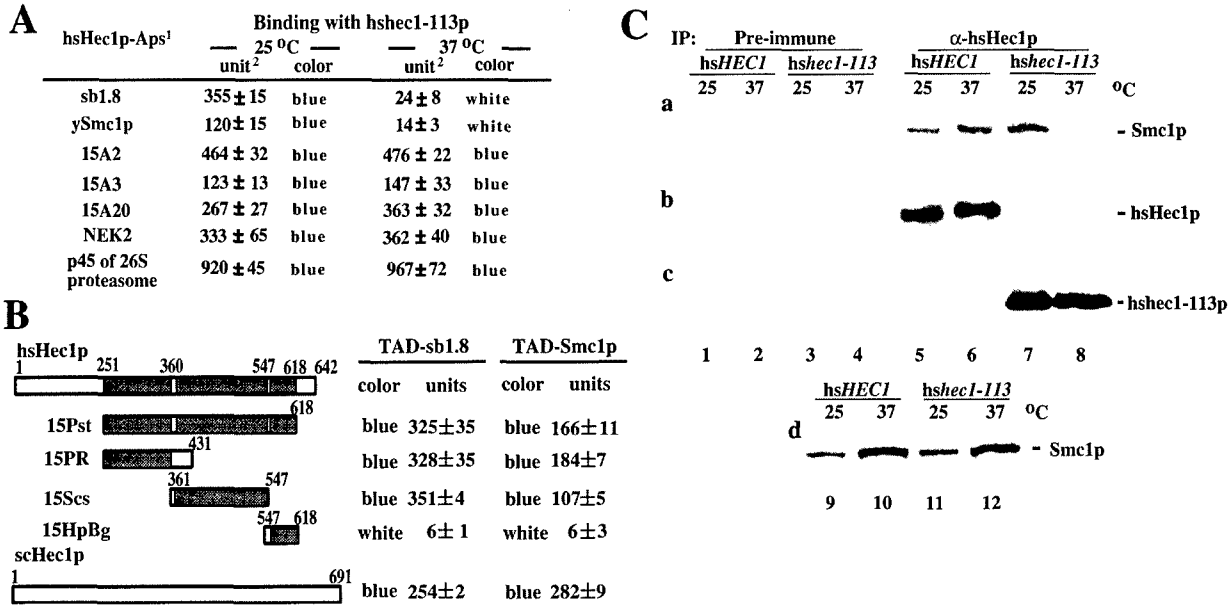


FIG. 5. The interaction between the mutated hshec1-113p and Smc1p is ts. (A) Binding between hsHec1-113p and hsHec1p-associated proteins (hsHec1p-Aps) at permissive and nonpermissive temperatures. 15ts113, the cDNA encoding the deletion mutant (aa 251 to 394) of hsHec1p that is truncated at the same position as the hshec1-113p mutant, was generated by in-frame fusion to the Gal4 DNA-binding domain. The same constructs in Table 3 were used to express hsHec1p-associated proteins that were fused with the Gal4 TAD. The interactions at 25 or 37°C were tested in yeast two-hybrid assays. Units represent β-galactosidase activity. (B) Domain mapping of the interaction between Hec1p and SMC1 proteins. The schematic structures of the regions in Hec1p that were fused with the Gal4 DNA-binding domain are shown. Shaded boxes are the three leucine heptad repeat-enriched regions. sb1.8/human SMC1 and Smc1p/yeast SMC1 were expressed as Gal4 TAD fusion proteins and used to test for interaction with Hec1 fusion proteins in yeast two-hybrid assays. (C) Log-phase yeast cells carrying an hs*hec1-113* or hs*HEC1* allele were transferred to fresh medium and grown at either 25 or 37°C. After 3 h, cells were collected and lysed, and equal amounts of protein extracts (about 15 mg) were immunoprecipitated (IP) with mouse preimmune serum (lane 1 to 4) or anti-hsHec1p MAb 9G3 (lane 5 to 8). The immunoprecipitates were then resolved by SDS-PAGE followed by immunoblotting with mouse anti-Smc1p antiserum for Smc1p (a), 9G3 for hsHec1p (b), or hshec1-113p (c). Equal amounts of the same lysates (about 2 mg) were separated by SDS-PAGE followed by immunoblotting with anti-Smc1p antiserum for detecting the expression of Smc1p (d, lanes 9 to 12). Lanes 1, 5, and 9, hs*HEC1* at 25°C; lanes 2, 6, and 10, hs*HEC1* at 37°C; lanes 3, 7, and 11, hs*hec1-113* at 25°C; lanes 4, 8, and 12, hs*hec1-113* at 37°C.

phenotype of both the *smc1-2* and *smc2-6* mutants at nonpermissive temperatures, while no suppression was observed with vector alone (Fig. 7). This result indicates that Hec1p proteins may modulate both sister chromatid cohesion and chromosome condensation, and that its interaction with Smc1p or Smc2p is crucial for chromosomal segregation.

## DISCUSSION

hsHec1p plays an essential role in mammalian cell mitosis. Cells injected with antibodies specific for hsHec1p exhibit several features of abnormal mitotic phenotypes, including the formation of multiple spindle poles and disordered metaphase chromosome alignment. These cells complete all aspects of division, including anaphase, cytokinesis, and re-formation of the nuclear envelope, but nonetheless missegregate chromosomes to daughter cells (8). To elucidate the molecular bases of hsHec1p in M-phase progression, an *S. cerevisiae* homolog of the hs*HEC1* gene was identified and characterized. Consistent with previous observations of Wigge et al. (61), we found that sc*HEC1/NDC80* encodes an 80-kDa cellular protein that is 36% identical to the human hsHec1p protein in its N-terminal one-third. Although the overall homology between the two proteins is not high, both proteins contain a long stretch of leucine heptad repeats that constitute two coiled-coil enriched domains in their otherwise divergent C-terminal regions. Genes with similar structure were also identified in fission yeast, *C. elegans*, and mouse genomes (data not shown). Moreover, the hs*HEC1* gene can complement the function of its yeast counterpart in cells deleted for sc*HEC1*. The result suggests that the essential functions of Hec1p proteins have been conserved throughout eukaryotic evolution.

The conservation of Hec1p function provided a useful tool to explore in a simple unicellular organism the molecular mechanisms by which hsHec1p functions. Our results suggest that hsHec1p function is essential for cell viability and M-phase progression. Its function is required during M phase, since conditional hs*hec1* mutations led to the mitotic delay, accumulation of large-budded cells, and increased lethality during M-phase arrest. Given the structural divergence that exists between hsHec1p and scHec1p, it was unexpected that the phenotypes of the mutants of these two proteins would be almost identical. This observation, however, strongly suggests that Hec1 proteins are functionally very conserved from yeast to human, and it is therefore feasible to study the in vivo properties of a human protein in an organism as simple as the budding yeast.

The chromosome missegregation resulting from mutated hs*hec1* may stem from a defect in the interaction between hsHec1p and Smc1p. The interaction between hsHec1p and the second coiled-coil domain of the human SMC1 protein was originally indicated by yeast two-hybrid screening. Later, the interaction of hsHec1p with yeast Smc1p was also found to be mediated by the corresponding region in this yeast homolog (Fig. 5). The coiled-coil domains of SMC proteins have been implicated in oligomerization or interaction with other proteins (40). The SMC1-type proteins, yeast Smc1p and *Xenopus* XSMC1, have been implicated in sister chromatid cohesion (21, 24, 41, 45, 56). The association of hsHec1p or scHec1p with Smc1p might therefore link one of the functions of Hec1p

A

| Gal4-DBD | TAD | —25 °C— color | units | —37 °C— color | units |
|---|---|---|---|---|---|
| hsHec1p | | | | | |
| 15Pst (251-618) | Smc2p | blue | 155 ± 10 | blue | 168 ± 9 |
| 15ts113 (251-394) | Smc2p | blue | 179 ± 16 | blue | 180 ± 4 |
| 15Scs (361-547) | Smc2p | white | 5 ± 1 | ND | |
| 15HpBg (547-681) | Smc2p | white | 4 ± 1 | ND | |
| 15ts113 (251-394) | Smc1p | blue | 258 ± 18 | white | 5 ± 1 |
| scHec1p (1-691) | Smc2p | blue | 177 ± 33 | ND | |

B

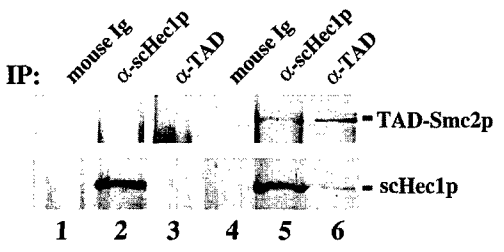


FIG. 6. Hec1p interacts with Smc2p. (A) Domain mapping and temperature sensitivity of the interaction between Hec1p and Smc2p. The same constructs of Hec1p as used for Fig. 5 were used in yeast two-hybrid assays to test their binding ability to yeast Smc2p expressed as a Gal4 TAD fusion protein. Binding ability was tested at 25 or 37°C. Gal4-DBD, Gal4 DNA-binding domain; ND, not determined. (B) In vivo interaction between Hec1p and Smc2p. Equal amounts of cell lysates from the log-phase culture of strain Y153 alone (lanes 1 to 3) or Y153 expressing TAD-Smc2p (lanes 4 to 6) were immunoprecipitated (IP) by the antibodies indicated. The resultant immunocomplexes were separated by SDS-PAGE followed by immunoblotting. The upper panel was blotted by polyclonal anti-TAD antibodies, and the lower panel was stained by anti-scHec1p. Ig, immunoglobulin.

to the regulatory machinery controlling the process of chromatin assembly. At nonpermissive temperatures, the mutant hshec1-113p fails to bind to Smc1p, and this may cause a defect in sister chromatid separation, leading to chromosome missegregation. Consistently, a defect in sister chromatid cohesion has been proposed as the cause leading to chromosome missegregation. Our observation that overexpression of both yeast and human Hec1p can suppress the temperature sensitivity of the *smc1* mutant suggests that increasing amounts of Hec1 proteins may augment their affinity for the mutant smc1p and help maintain the proper function of sister chromatid cohesion. Thus, the interaction between Hec1p and SMC1 protein is essential for the physiological function of Hec1p in chromosome segregation and cell viability.

Hec1p is also associated with Smc2p in yeast. Several lines of evidence have revealed the connection between sister chromatid cohesion and chromosome condensation (21, 24, 56). A subunit (Scc1p/Mcd1p) of the Smc1-containing cohesion complex is proposed to function as a linker molecule that connects these two different chromatin-structuring activities on yeast mitotic chromosomes (22). *S. cerevisiae* Trf4p, a protein required for rDNA condensation, interacts with both Smc1p and Smc2p (5), and a *trf4* mutant also exhibits a cohesion defect (24). Like these proteins, Hec1p may modulate both chromatin-structuring activities through association with Smc1p or Smc2p.

The biochemical basis of how Hec1p binds to Smc1p or Smc2p and participates in the activities of SMC proteins remains to be elucidated. It has been suggested by the in vitro activity of *Xenopus* condensins that SMC proteins may act as motors facilitating formation of chromosome loops in an energy-dependent fashion (22, 24, 37). The ATP-binding ability and ATPase activities of SMC protein complexes are proposed to provide them with motor energy and modulate their functions. Interestingly, other hsHec1p-associated proteins such as MSS1 and p45 of the 26S proteasome were also suggested to have ATPase activities (4), and hsHec1p is able to down-regulate the in vitro ATPase activity of MSS1, the human Cim5p homolog (9). Whether Hec1p can modulate ATPase activities of SMC proteins remains to be tested.

Besides a potential role in the modulation of sister chromatid cohesion or chromosome condensation, a kinetochore function can not be excluded for Hec1p in chromosome segregation since hsHec1p has been shown to localize to centromere regions in mammalian cells during M phase (8). The phenotypes of the mutant forms of both human Hec1p (Fig. 3) and the yeast Hec1p homolog (61) are similar to those of ndc10-1, a mutant form of a centromere-binding protein (18). The staining pattern observed for Hec1p/Ndc80p in this study and that previously reported by Wigge et al. (61) is reminiscent of the staining of the spindle pole body and is consistent with centromere localization. Moreover, the staining of Ndc80p on microtubules adjacent to the spindle pole body and along the short spindle, an important characteristic observed with centromere proteins including Ndc10p (18) and Cse4p (44), was also demonstrated in a previous report (50). A recent study suggested that Hec1p/Ndc80p interacts genetically with Ctf19p, which is a centromere protein (32). The yeast Hec1p/Ndc80p, however, was initially purified from the spindle pole (61), suggesting that Hec1p/Ndc80p may also localize to the spindle pole region. In addition, Hec1p may be distributed among other chromatin regions since cohesion and condensation occur at multiple places along the entire chromosome other than the centromere.

The interaction between Hec1p and SMC proteins may reflect a novel function for the SMC proteins. It is evident that SMC proteins are involved not only in sister chromatid cohesion or chromosome condensation but also in other activities such as DNA replication, recombination, and repair (24, 35, 54, 56, 59). The chromatin assembly activities involved in the cohesion and condensation processes that occur at the centromere have the potential to function in the structural remodeling of centromeric chromatin during mitosis. In higher eukaryotes, the assembly of highly ordered centromeric chromatin structure is suggested to be essential for kinetochore functions. In *S. cerevisiae*, specialized chromatin structures of the centromeres also appear to be important for kinetochore

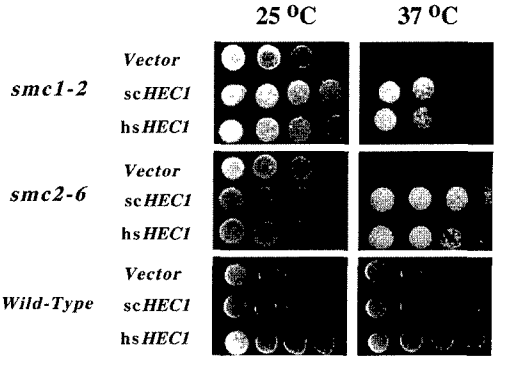


FIG. 7. Suppression of ts phenotype of *smc1* or *smc2* by overexpressing hs*HEC1* or sc*HEC1*. *smc1-2*, *smc2-6*, or wild-type cells were transformed by hs*HEC1* or sc*HEC1* in a *GAL1*-inducible vector or by the vector alone. Different dilutions of log-phase cells grown in 2% glucose at 25°C were inoculated on the plates with 2% galactose and incubated at 25 or 37°C.

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appropriately for other experiments, but, unless stated otherwise, the procedure for assembling the reaction mixtures was the same. In the analysis of rad51 K191R and rad51 K191A (Figure 5B), the reaction mixtures had a final volume of 50 μl and contained 3.3 μg (1.5 μM) of Rad51, rad51 K191R, or rad51 K191A and 2.4 μg Rad54 (440 nM).

### RecA-Mediated D Loop Formation

RecA (7.8 μM) was incubated with 500 ng of φX174 viral (+) strand (30 μM nucleotides) in 40 μl of buffer R for 5 min at 37°C, followed by the addition of 5.5 μg of *E. coli* SSB (6 μM) in 3 μl of storage buffer and a 5 min incubation at 37°C. The reaction mixture (50 μl final volume) was completed by adding 4 μl of 120 mM $MgCl_2$ and 600 ng φX174 replicative form I DNA (18.5 μM base pairs) in 3 μl. Samples were processed for agarose gel electrophoresis as described above for the Rad51/Rad54-mediated reaction.

### Topoisomerase I–Linked DNA Remodeling

The indicated concentrations of Rad54 were incubated with 120 ng of relaxed φX 174 DNA (18.5 μM base pairs) for 3 min at 23°C in 9.5 μl of buffer R with 50 mM KCl, followed by the addition of 4 units of calf thymus topoisomerase I (Gibco/BRL) or 100 ng of *E. coli* topoisomerase I in 0.5 μl. Reactions were incubated at 37°C for 10 min, deproteinized by treatment with SDS (0.5%) and proteinase K (1 mg/ml) for 15 min at 37°C. DNA species were resolved in 0.8% agarose gels run in TAE buffer at 23°C and stained with ethidium bromide. To examine the effect of Rad51, rad51 K191R, and rad51 K191A on DNA remodeling, these proteins were incubated with Rad54 and 120 ng of relaxed φX 174 DNA (18.5 μM base pairs) in 9.5 μl of buffer R for 3 min at 23°C, before the incorporation of *E. coli* topoisomerase I. For testing the effect of Rad51-RPA-ssDNA filament on DNA remodeling, the indicated concentration of Rad51 was incubated with pBluescript ssDNA (30 μM nucleotides) in 7.7 μl buffer R for 3 min at 37°C, and then 2.4 μg RPA (2 μM) was added in 0.5 μl storage buffer. After 3 min of incubation at 37°C, Rad54 in 0.5 μl storage buffer and 120 ng of relaxed φX 174 DNA (18.5 μM base pairs) in 0.8 μl TE were added, followed by a 3 min incubation at 23°C, before the incorporation of *E. coli* topoisomerase I. To analyze the effect of RecA or RecA-SSB-ssDNA, the same procedures were used to assemble the reaction. The completed reaction mixtures were incubated at 23°C for 10 min and then analyzed as described above.

### Two-Dimensional Gel Analysis of Form OW DNA

Kodak BioMax MP1015 agarose gel units were used for electrophoresis. The first dimension of the electrophoresis was carried out in 0.9% gels in TAE buffer at 110 mA and 23°C for 6 hr. The gels were soaked for 18 hr at 4°C in 30 gel volumes of TAE buffer containing 30 μM chloroquine diphosphate, and then subject to a second electrophoresis step in TAE containing 30 μM chloroquine diphosphate at 110 mA and 23°C for 6 hr. The DNA species were stained with ethidium bromide. The DNA samples in panels II and IV of Figure 3 were treated with 50 ng of *E. coli* topoisomerase I in buffer T for 10 min at 37°C prior to electrophoresis. The amount of negatively supercoiled DNA and Form OW DNA used in all the panels was 200 ng.

### P1 Sensitivity

Increasing concentrations of Rad54 were incubated with 120 ng of relaxed φX DNA (18.5 μM base pairs) in 9.5 μl of buffer R with 50 mM KCl for 2 min at 23°C, followed by the addition of 0.4 units of P1 nuclease (Roche) in 0.5 μl. Reaction mixtures were incubated at 23°C or 37°C for 10 min, quenched by 0.5% SDS, and treated with proteinase K (1 mg/ml) for 15 min at 37°C. Reactions products were resolved in agarose gels containing 10 μM ethidium bromide in TAE buffer at 23°C, followed by staining with ethidium bromide. For testing the effect of Rad51, Rad51-RPA-ssDNA complex, RecA, and RecA-SSB-ssDNA complex on P1 sensitivity, the procedures used in the topoisomerase I-linked DNA remodeling experiments described earlier were employed, except that 0.4 units of P1 nuclease replaced the topoisomerase.

### ATPase Assay

Rad54 (50 nM) was incubated with 120 ng of relaxed φX DNA (18.5 μM base pairs) in 10 μl of buffer A (30 mM Tris-HCl [pH 7.2], 5 mM $MgCl_2$, 1 mM DTT, 50 mM KCl, and 100 μg/ml BSA) and 1.5 mM [$\gamma$-$^{32}$P] ATP for the indicated times at 23°C, and the level of ATP hydrolysis determined by thin layer chromatography, as described (Petukhova et al., 1998). For testing the effect of Rad51 (360 nM) and Rad51-RPA-ssDNA (360 nM Rad51, 2 μM RPA, and 30 μM nucleotides of pBluescript ssDNA) complex on Rad54 ATPase, the procedures followed those described for the DNA supercoiling experiments earlier, except that Rad54 was preincubated with the other reaction components at 0°C instead of 23°C.

### DNA Mobility Shift

A mixture of φX174 viral (+) strand (30 μM nucleotides) and PstI-linearized φX replicative form (20 μM nucleotides) substrates were used with the indicated amounts of Rad51, rad51 K191R, or rad51 K191A in 10 μl buffer G (35 mM Tris-HCl [pH 7.2], 1 mM DTT, 3 mM $MgCl_2$, and 100 μg/ml BSA, with or without 2.5 mM ATP, as indicated). Following incubation at 23°C for 10 min, the reaction mixtures were mixed with 2 μl of loading buffer (0.1% orange G in 50% glycerol), subjected to electrophoresis in 0.9% agarose gels in TAE buffer at 23°C, and stained with ethidium bromide.

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Our work has also shown stimulation of the DNA remodeling ability of Rad54 by Rad51 and by the Rad51-RPA-ssDNA complex. Based on DNA binding results with the rad51 K191R and rad51 K191A mutants, we further suggest that the ability of Rad51 to stimulate Rad54 DNA remodeling activity requires DNA binding by Rad51. Thus, it seems likely that in the case of free Rad51 being added to the remodeling reaction, Rad51 stimulates DNA remodeling by first binding to the relaxed DNA used as substrate or to the supercoiled domains created by Rad54. We envision two different possibilities of how the Rad51-DNA complex stimulates DNA remodeling. First, it is possible that the complex of Rad51/Rad54 assembled on DNA has a higher intrinsic ability to remodel DNA. Alternatively, or in addition, the Rad51-dsDNA complex may facilitate the formation of closed DNA loops in the duplex via incorporation of the duplex molecule into the secondary binding site of the Rad51-ssDNA complex (Roca and Cox, 1997; Bianco et al., 1999; Sung et al., 2000). The establishment of such closed DNA loops is expected to further constrain the negative and positive supercoils formed by Rad54.

## Mechanism for D Loop Formation

Based on the findings from this study, we consider the following model for the observed cooperation between Rad51 and Rad54 in making D loop. In this model, the first step in the D loop reaction is the assembly of an active nucleoprotein complex on ssDNA that involves the binding of Rad51 protein and RPA to ssDNA and the incorporation of Rad54 protein via a specific interaction with Rad51. We propose this sequence of events because we have found that when Rad51 is first allowed to nucleate onto dsDNA, instead of the ssDNA, then D loop formation is suppressed in a Rad51 concentration-dependent manner. It should be noted that the role of RPA in the D loop reaction is less specific, as *E. coli* SSB can substitute for RPA at least partially (data not shown). In addition, we have found that the optimal amount of Rad51 protein (20 nucleotides per Rad51 monomer) for D loop formation is substantially lower than that required to saturate the ssDNA (3 nucleotides per Rad51 monomer), indicating that the most active form of the nucleoprotein filament contains short filaments of Rad51 with Rad54 protein bound to the filaments, with the remainder of the ssDNA substrate being covered with RPA. These biochemical results suggest that during recombination in vivo, efficient formation of D loop can in fact occur prior to the assembly of a contiguous Rad51 filament on the ssDNA tails generated as a result of the nucleolytic end-processing of the DNA break.

We envision that the DNA remodeling activity of Rad51-Rad54 could have two major consequences on the efficiency of the homologous DNA pairing reaction. First, an ability of Rad54 already associated with the Rad51-ssDNA nucleoprotein complex to thread the duplex through its fold (i.e., tracking) will enhance the rate at which the incoming duplex molecule is sampled for homology. Importantly, the transient DNA strand opening that occurs in the negatively supercoiled domain generated by Rad54 can be expected to facilitate the formation of a nascent DNA joint, when homology is located in the duplex. This latter suggestion is supported by our observation that Rad54 enables Rad51 to utilize even relaxed DNA substrate for efficient DNA joint formation. The nascent joint molecule formed will be paranemic in nature, if it is located within the interior of the initiating linear ssDNA molecule. However, if DNA joint formation occurs near one of the ends of the linear ssDNA molecule, then it has the potential of being converted into a plectonemic linkage or stabilizing a preexisting plectonemic joint.

## Experimental Procedures

### Recombination Proteins

Rad51, rad51 K191A, and rad51 K191R were purified from yeast strain tailored to overproduce these factors to near homogeneity, as described (Sung, 1994; Sung and Stratton, 1996). Rad54, rad54 K341, and rad54 K341R were purified to near homogeneity from overproducing yeast strains, as described (Petukhova et al., 1998, 1999). RPA was purified from *E. coli* cells transformed with a plasmid that simultaneously expresses all three subunits of this factor (He et al., 1996), using the purification procedure described in Sung (1997). RecA was a gift from Michael Cox, and SSB was purchased from Gibco/BRL.

### DNA Substrates

Replicative form I $\phi$X 174 DNA (Gibco/BRL) was relaxed with an excess of calf thymus topoisomerase I (Gibco/BRL) in buffer T (50 mM Tris-HCl [pH 7.5], 30 mM KCl, 0.5 mM DTT, and 10 mM $MgCl_2$) at 37°C. To make negatively supercoiled DNA, replicative form I DNA (120 $\mu$M base pairs) was incubated with 60 units of calf thymus topoisomerase I in 400 $\mu$l buffer T containing 5, 10, or 20 $\mu$M of ethidium bromide at 37°C for 2 hr. To make positively supercoiled DNA, topologically relaxed DNA (50 $\mu$M base pairs) was treated with Rad54 (400 nM) and 5 $\mu$g *E. coli* topoisomerase I in 500 $\mu$l of buffer R (in 35 mM Tris-HCl [pH 7.2], 2.5 mM ATP, 3 mM $MgCl_2$, 100 $\mu$g/ml bovine serum albumin, 1 mM DTT, 50 mM KCl, and an ATP regenerating system consisting of 30 mM creatine phosphate and 28 $\mu$g/ml phosphocreatine kinase) at 37°C for 15 min. To purify the DNA substrates, reaction mixtures were extracted with phenol and then run on 0.8% agarose gels without ethidium bromide (for positively supercoiled DNA and DNA negatively supercoiled with 10 and 20 $\mu$M ethidium bromide) or with 10 $\mu$M ethidium bromide (for relaxed DNA and DNA negatively supercoiled with 5 $\mu$M ethidium bromide). The DNA bands of interest were visualized by staining with ethidium bromide, excised, and purified using the Geneclean kit (Bio 101). DNA substrates were filter dialyzed in Centricon 30 microconcentrators into TE (10 mM Tris-HCl [pH 7.5] and 0.5 mM EDTA). The superhelical densities of the DNA substrates were determined by one-dimensional and two-dimensional gel analyses. The negatively supercoiled DNA species made with 5, 10, and 20 $\mu$M ethidium bromide had superhelical densities of −0.021, −0.039, and −0.076, respectively. The $\phi$X (+) strand DNA substrate used in the D loop assay was linearized by hybridizing a 26-mer oligonucleotide to create a PstI site, followed by treatment with an excess of this restriction enzyme. The linearized (+) strand was purified as described above.

### Rad51/Rad54-Mediated D Loop Formation

The reaction mixture (50 $\mu$l final volume) was assembled by mixing 3.3 $\mu$g Rad51 (1.5 $\mu$M) and 500 ng of linear $\phi$X viral (+) strand (30 $\mu$M nucleotides) in 40 $\mu$l of buffer R containing 50 mM KCl. After a 3 min incubation at 37°C, 12 $\mu$g RPA (2 $\mu$M) in 2 $\mu$l was added, followed by a 3 min incubation at 37°C, and then 1.2 $\mu$g Rad54 (220 nM) in 1 $\mu$l was incorporated, followed by a 3 min incubation at 23°C. To complete the reaction mixture, 600 ng $\phi$X174 DNA (18.5 $\mu$M base pairs) in 3 and 4 $\mu$l of 50 mM spermidine (4 mM) was incorporated. The reaction mixture was incubated at 23°C, and 5 $\mu$l portions were withdrawn at the indicated times and processed (Petukhova et al., 1998) for electrophoresis in 0.85% agarose gels containing 10 $\mu$M ethidium bromide in TAE buffer at 23°C. The gels were stained in ethidium bromide and then subjected to image analysis in a Nucleotech gel documentation station equipped with a CCD camera. The size of the reaction mixtures was scaled down

We next examined the ability of the two mutant rad51 proteins to promote D loop formation. The results, as shown in Figure 5B, indicated that while rad51 K191R cooperates with Rad54 in forming D loop, rad51 K191A mutant is defective in this reaction. Importantly, rad51 K191R stimulated the ability of Rad54 protein to remodel DNA structure, as measured by the formation of Form OW DNA in the *E. coli* topoisomerase I-linked reaction (Figure 5C) and by the assay that examines sensitivity of relaxed DNA to P1 nuclease (Figure 5D). The same results were obtained when rad51 K191R was preassembled into a nucleoprotein complex with RPA and ssDNA (data not shown). On the other hand, rad51 K191A (Figures 5C and 5D) and the mixture of rad51 K191A and RPA-ssDNA complex (data not shown) were incapable of stimulating the DNA remodeling ability of Rad54, as judged by both of the available criteria of DNA supercoiling (Figure 5C) and sensitivity to P1 nuclease (Figure 5D). Taken together, the results suggest that the DNA binding ability of Rad51 is indispensable for D loop formation and for stimulating the DNA remodeling activity of Rad54.

## Discussion

### Biochemical Requirements for D Loop Formation in Eukaryotic Recombination

Rad51 by itself has negligible D loop–forming ability (Petukhova et al., 1998; Mazin et al., 2000). In this work and other studies (Petukhova et al., 1998; Mazin et al., 2000), Rad54 has been shown to have a strong dsDNA-dependent ATPase activity and to promote robust D loop formation with Rad51 protein. Importantly, ATP hydrolysis by Rad54 is indispensable for the D loop reaction, in that rad54 K341A and rad54 K341R, which are, respectively, defective and greatly attenuated in ATPase activity (Petukhova et al., 1999), are inactive in the D loop reaction. Consistent with the in vitro results, genetic studies have revealed that the ATPase activity of Rad54 is required for DNA repair and recombination (Clever et al., 1997; Petukhova et al., 1999).

In the present work, we have also demonstrated that the ATP binding defective rad51 K191A mutant is inactive in D loop formation. On the other hand, rad51 K191R, which is greatly attenuated in ATPase activity but retains the ability to bind ATP and functions with the latter in making D loop. We have also provided evidence that rad51 K191R but not the rad51 K191A mutant binds DNA in an ATP-dependent manner. This result strongly suggests that the defect of rad51 K191A mutant in the D loop reaction is due to its inability to bind DNA. Accordingly, the results from genetic experiments have indicated that rad51 K191R is biologically active, whereas the rad51 K191A mutant is defective in recombination functions (Shinohara et al., 1992; Sung and Stratton, 1996).

### DNA Remodeling by Rad51 and Rad54

In previous studies, no DNA helicase activity was detected in yeast and human Rad54 proteins (Petukhova et al., 1998; Swagemakers et al., 1998). Yeast Rad54 also does not exhibit a DNA helicase activity in combination with yeast Rad51 (Petukhova et al., 1998). We have now shown that the free energy from ATP hydrolysis is used by Rad54 to remodel DNA structure in such a fashion that both negatively and positively supercoiled domains are generated in the DNA template. Within the concentration range of Rad54 employed for the D loop reaction, the negative and positive supercoils generated as a result of DNA remodeling by Rad54 are relatively unconstrained, as indicated by the fact that whereas treatment of *E. coli* topoisomerase I results in the formation of a positively supercoiled DNA species termed Form OW, similar incubation with calf thymus topoisomerase I did not result in a noticeable change in the DNA linking number. As shown in this work and in an earlier study (Petukhova et al., 1999), at much higher concentrations of Rad54, some of the negative supercoils become constrained, probably due to the preferential binding of Rad54 protein to the negatively supercoiled regions, resulting in the formation of Form U DNA upon treatment with calf thymus topoisomerase I.

We have also employed the single-strand-specific nuclease P1 to demonstrate DNA strand opening by Rad54. P1 does not act on topologically relaxed DNA, but negatively supercoiled DNA is susceptible to this nuclease, with the degree of sensitivity being proportional to the extent of negative supercoiling in the substrate. Incubation of topologically relaxed DNA with Rad54 renders the DNA sensitive to P1 nuclease in a manner that is completely dependent on ATP hydrolysis by Rad54, consistent with the suggestion that the negative supercoils induced by Rad54 lead to DNA strand opening.

Our results have shown a marked stimulation of the Rad54 DNA supercoiling ability by Rad51. Specifically, the formation of Form OW DNA in the *E. coli* topoisomerase I–linked reaction is enhanced by Rad51, and the sensitivity of the DNA substrate to P1 nuclease is similarly increased. The ability to stimulate the DNA supercoiling reaction is rather unique to Rad51, as RecA protein, either free or applied as a preassembled RecA-SSB-ssDNA complex, is devoid of the ability to enhance this Rad54-mediated DNA remodeling reaction. Taken together with our observation that the Rad54 has no stimulatory effect on the RecA-catalyzed D loop reaction, we conclude that the specific interaction noted between Rad51 and Rad54 (Jiang et al., 1996; Clever et al., 1997; Petukhova et al., 1998) is indispensable for the synergistic interactions between these two factors in DNA remodeling and D loop formation.

### Basis for Rad51/Rad54-Mediated DNA Supercoiling

A number of proteins, including type I restriction enzymes, DNA helicases, and RNA polymerase II, have been shown to be capable of simultaneously inducing positive and negative supercoils in duplex DNA. The supercoiling induced by these protein factors is thought to result from tracking of these factors along duplex DNA. In particular, the supercoiling produced by RNA polymerase II has been discussed in the context of the "twin-supercoiled-domain" model. In this model, RNA polymerase in the transcript elongation mode tracking along DNA produces a positively supercoiled domain ahead of the polymerase and a negatively supercoiled domain behind it (Wu et al., 1988). Based on these precedents, we speculate that the supercoiling function of Rad54 also stems from an ability of this protein to track along DNA, similarly producing positively and negatively supercoiled domains that are unconstrained, as revealed in the present study by treatment with eukaryotic and prokaryotic topoisomerases.

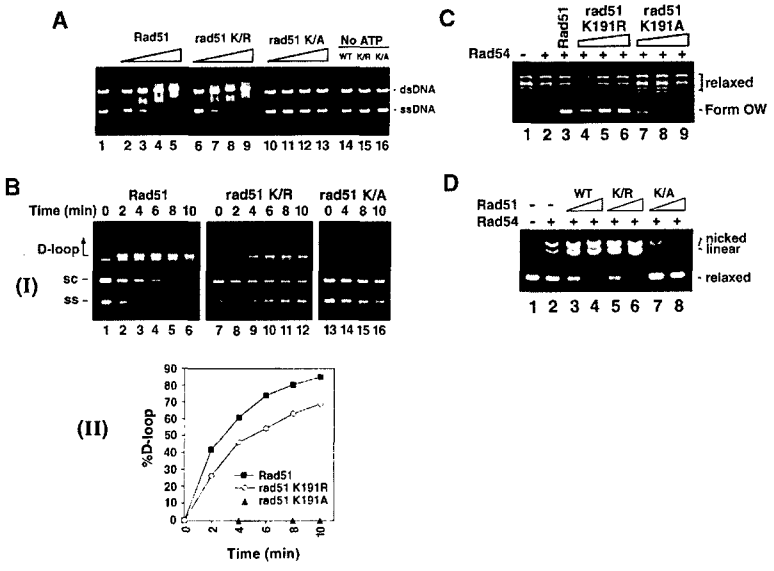


Figure 5. DNA Binding by Rad51 Is Required for DNA Supercoiling and D Loop Formation

(A) DNA binding ability of wild-type Rad51 and mutant rad51 proteins. Rad51 (1, 2, 4, and 6 μM in lanes 2 to 5, and 6 μM in lane 14), rad51 K191R (1, 2, 4, and 6 μM in lanes 6 to 9, and 6 μM in lane 15), and rad51 K191A (1, 2, 4, and 6 μM in lanes 10 to 13, and 6 μM in lane 16) were incubated with a mixture of $\phi$X viral (+) strand (30 μM nucleotides) and linear duplex (20 μM base pairs), either in the presence (lanes 1 to 13) or absence of ATP (14 to 16), for 10 min at 23°C. The reaction mixtures were run in a 0.9% agarose gel to detect the mobility shift of the DNA substrates.

(B) rad51 K191R but not rad51 K191A forms D loop with Rad54. Time courses of D loop formation (I) with Rad51 (1.5 μM), rad51 K191A (1.5 μM), and rad51 K191R (1.5 μM) using $\phi$X linear ssDNA (ss) and negatively supercoiled DNA (sc) with $\sigma = -0.06$ isolated from cells as substrates. The details are given in the Experimental Procedures. The datapoints from image analysis of the gels are plotted (II).

(C) rad51 K191R but not rad51 K191A cooperates with Rad54 in supercoiling DNA. The relaxed $\phi$X DNA substrate (18.5 μM) was incubated with Rad54 (90 nM in lanes 2 to 9), *E. coli* topoisomerase I (lanes 1 to 9), Rad51 (120 nM, lane 3), rad51 K191R (120, 240, and 360 nM in lanes 4, 5, and 6, respectively), and rad51 K191A (120, 240, and 360 nM in lanes 7, 8, and 9, respectively) for 10 min at 23°C, as indicated.

(D) Enhancement of DNA strand opening by rad51 K191R but not rad51 K191A. Relaxed $\phi$X DNA (18.5 μM base pairs) was incubated with P1 nuclease and the following components at 23°C for 10 min before gel analysis: Rad54 (90 nM in lanes 2 to 8), Rad51 (WT; 240 and 360 nM in lanes 3 and 4, respectively), rad51 K191R (K/R; 360 and 480 nM in lanes 5 and 6, respectively), and rad51 K191A (K/A; 360 and 480 nM in lanes 7 and 8, respectively), as indicated. The analyses in (D) were carried out in 0.85% agarose gels containing 10 μM ethidium bromide.

itself was, as expected, impervious to the action of P1 nuclease due to the lack of single-stranded nature in the DNA (Figure 4A, lane 2). Interestingly, treatment of the relaxed substrate with Rad54 rendered it sensitive to P1 nuclease, as revealed by the presence of nicked DNA in the gel analysis, with the level of P1-digested DNA increasing with the amount of Rad54 added (Figure 4A, lanes 4 to 6). The sensitivity of relaxed DNA to P1 nuclease is completely dependent on ATP hydrolysis by Rad54, as the omission of ATP or its substitution with ATP-$\gamma$-S abolished the sensitivity of the DNA substrate to P1 nuclease (Figure 4A, lanes 7 and 8), and even high levels of the ATPase defective rad54 K341A and rad54 K341R mutant proteins did not render the DNA substrate sensitive to P1 nuclease (data not shown).

Since in the topoisomerase-linked DNA remodeling reaction stimulation of the Rad54 DNA remodeling ability by either Rad51 or Rad51-RPA-ssDNA filament was seen (Figure 3), it became important to establish whether the P1 sensitivity of the relaxed DNA substrate undergoing remodeling is also similarly enhanced. To do this, we used exactly the same incubation conditions as those employed in the topoisomerase-linked DNA remodeling experiments (Figure 3), with the exception that P1 nuclease was used instead of topoisomerase. The results from these experiments indicated that either the addition of free Rad51 or a preassembled complex of Rad51-RPA-ssDNA results in a marked increase of the DNA sensitivity to P1 nuclease (Figure 4B, panel I). Control experiments showed that neither free Rad51 nor Rad51-RPA-ssDNA complex alone has any ability to promote scission of DNA by P1 nuclease (Figure 4B, panel II). In addition, RecA and the RecA-SSB-ssDNA complex are also devoid of the ability to promote P1 scission of DNA (Figure 4C, lanes 2 to 5), nor do they significantly enhance the ability of Rad54 to make DNA sensitive to P1 (Figure 4C, lanes 6 to 9). The synergistic action of Rad51/Rad54 and Rad51-RPA-ssDNA/Rad54 in the P1 sensitivity assay is completely dependent on the ability of Rad54 to hydrolyze ATP, as verified by substitution of Rad54 with the rad54 K341A and rad54 K341R mutant proteins (data not shown). Taken together, the results from the analyses conducted with P1 nuclease provide direct evidence that ATP hydrolysis-driven negative supercoiling of DNA by the combination of Rad51 and Rad54 leads to transient DNA strand opening.

### D Loop Formation and DNA Remodeling Require DNA Binding by Rad51

Rad51 protein, like RecA, assembles onto ssDNA to form a nucleoprotein filament in which the search for DNA homology in the incoming homologous duplex and formation of DNA joints with the duplex occur. The assembly of the Rad51-ssDNA nucleoprotein filament requires ATP binding (reviewed in Bianco et al., 1998; Roca and Cox, 1997; Sung et al., 2000). We have previously described mutant variants of Rad51 altered in their ability to interact with ATP: rad51 K191A, which does not bind ATP, and rad51 K191R, which retains the ability to bind ATP but has greatly attenuated ATPase activity (Sung and Stratton, 1996). As shown in previous studies (Zaitseva et al., 1999) and reiterated here, wild-type Rad51 binds DNA in a mobility shift assay in an ATP-dependent manner (Figure 5A). While the rad51 K191A mutant is defective in DNA binding, the rad51 K191R mutant, as expected, retains the ability to bind DNA with a dependence on ATP (Figure 5A).

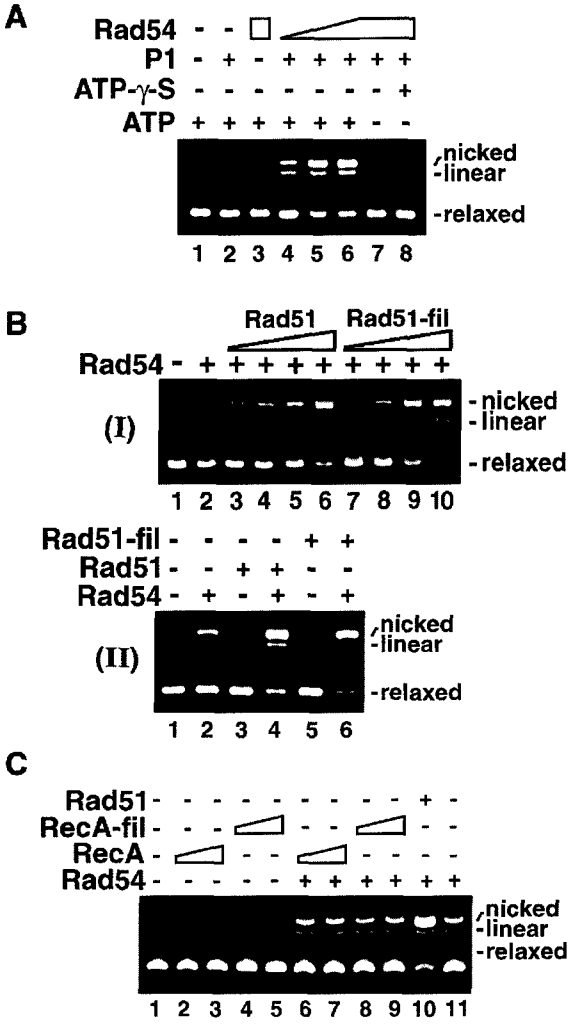


Figure 4. DNA Supercoiling Leads to DNA Strand Opening

(A) Rad54 renders topologically relaxed DNA sensitive to P1 nuclease. Relaxed DNA (18.5 μM base pairs, lanes 1 to 8) was incubated at 23°C for 10 min with P1 nuclease and increasing concentrations of Rad54 protein (90, 250, and 500 nM in lanes 4 to 6, respectively) in the presence of ATP. In lane 3, Rad54 (500 nM) was incubated with relaxed DNA in the presence of ATP but without P1, and in lanes 7 and 8, Rad54 (500 nM), relaxed DNA, P1 nuclease were incubated in the absence of ATP or with ATP-γ-S, as indicated.

(B) Rad51 enhances P1 sensitivity. In (I), relaxed ϕX DNA (18.5 μM base pairs in lanes 1 to 10) was incubated with P1 nuclease (lanes 1 to 10), Rad54, (50 nM; lanes 2 to 10), and increasing amounts of Rad51 (60, 120, 240, and 360 nM in lanes 3 to 6, respectively) or nucleoprotein complexes containing pBluescript ssDNA (30 μM nucleotides), RPA (2 μM), and increasing amounts of Rad51 (60, 120, 240 and 360 nM in lanes 7 to 10, respectively) at 23°C for 10 min. In lane 1, relaxed DNA and P1 nuclease were incubated in the absence of Rad51 and Rad54. In (II), relaxed ϕX DNA (18.5 μM base pairs in lanes 1 to 6) was incubated with P1 nuclease (lanes 1 to 6), Rad54 (50 nM in lanes 2, 4, and 6), and Rad51 (360 nM in lanes 3 and 4) or a nucleoprotein complex of Rad51-RPA-ssDNA (Rad51-fil, consisting of 360 nM Rad51, 2 μM RPA, and 30 μM pBluescript ssDNA; lanes 5 and 6), as described in (I).

(C) RecA does not promote DNA strand opening. Relaxed ϕX DNA (18.5 μM base pairs; lanes 1 to 11), P1 nuclease (lanes 1 to 11), Rad54 (50 nM in lanes 6 to 11), and Rad51 (360 nM, lane 10), RecA (525 nM in lanes 2 and 6, and 2.6 μM in lanes 3 and 7), or nucleoprotein complexes of RecA-SSB-ssDNA (RecA-fil; lanes 4, 5, 8, and 9) consisting of a fixed amount of SSB (6 μM), pBluescript ssDNA (30 μM nucleotides), but an increasing level of RecA (525 nM in lanes 4 and 8, and 2.6 μM in lanes 5 and 9). The analyses in (A) through (C) were carried out in 0.85% agarose gels containing 10 μM ethidium bromide.

DNA remodeling ability of Rad54 is influenced by Rad51. For this purpose, we examined Form OW DNA formation by incubating increasing amounts of Rad51 or nucleoprotein complexes preassembled with pBlueScript ssDNA, RPA, and increasing amounts of Rad51 with a quantity of Rad54 (22.5 nM, corresponding to 822 base pairs/Rad54 monomer) that by itself gave only a trace of Form OW DNA. Both Rad51 alone or Rad51/RPA prebound to ssDNA markedly stimulated the ability of Rad54 to produce Form OW DNA (Figure 3A). Control experiments showed that Rad51 or the complex of Rad51-RPA-ssDNA alone was devoid of DNA remodeling ability (Figure 3B, lanes 3 and 8), and the complex of RPA on ssDNA without Rad51 did not stimulate the DNA remodeling ability of Rad54 (Figure 3B, lane 10). Interestingly, ssDNA alone inhibited the ability of Rad54 to remodel duplex DNA (Figure 3B, compare lane 7 to lane 2), and other experiments have suggested that this is due to preferential binding of Rad54 to ssDNA and its sequestration from the DNA remodeling reaction (data not shown). In the presence of Rad51 or Rad51-RPA-ssDNA complex, DNA remodeling is also dependent on ATP hydrolysis by Rad54, as revealed by the substitution of Rad54 with either rad54 K341A or rad54 K341R mutant protein (Figure 3C). Consistent with the results from DNA remodeling experiments, Rad51 and Rad51-RPA-ssDNA both stimulated the dsDNA-dependent ATPase activity of Rad54 significantly (Figure 3D). As expected, RPA-ssDNA complex alone has no stimulatory effect on the Rad54 ATPase activity. A similar degree of stimulation of the Rad54 supercoiling and ATPase activities was observed when we used ϕX ssDNA instead of pBluescript ssDNA for assembling the Rad51-RPA-ssDNA complex (data not shown), indicating that DNA homology is not required for stimulation.

We have also examined whether a nucleoprotein complex of RecA-SSB-ssDNA has the ability to remodel DNA and to stimulate the Rad54 DNA remodeling and ATPase activities. The results indicated that RecA and the RecA-SSB-ssDNA nucleoprotein complex are devoid of the ability to remodel DNA (Figure 3B, lanes 5 and 11), and in addition that RecA and the RecA-SSB-ssDNA complex do not stimulate the DNA remodeling capability of Rad54 (Figure 3B, lanes 6 and 12) or its ATPase activity (data not shown). Taken together, the above observations indicate a specific cooperation between Rad51 and Rad54 in DNA remodeling, consistent with the physical interaction noted between these two proteins (Jiang et al., 1996; Clever et al., 1997; Petukhova et al., 1998).

### Nuclease P1 Sensitivity Reveals DNA Strand Separation by Rad51/Rad54-Mediated Supercoiling

Since formation of a heteroduplex DNA joint involves the melting of the target duplex to facilitate invasion by the initiating ssDNA substrate (Gupta et al., 1999), we wished to determine whether the negative supercoiling induced by Rad54 leads to DNA strand separation. We addressed this point by asking if treatment of a relaxed duplex with Rad54 would render the DNA sensitive to the single-strand-specific nuclease P1. Relaxed DNA by

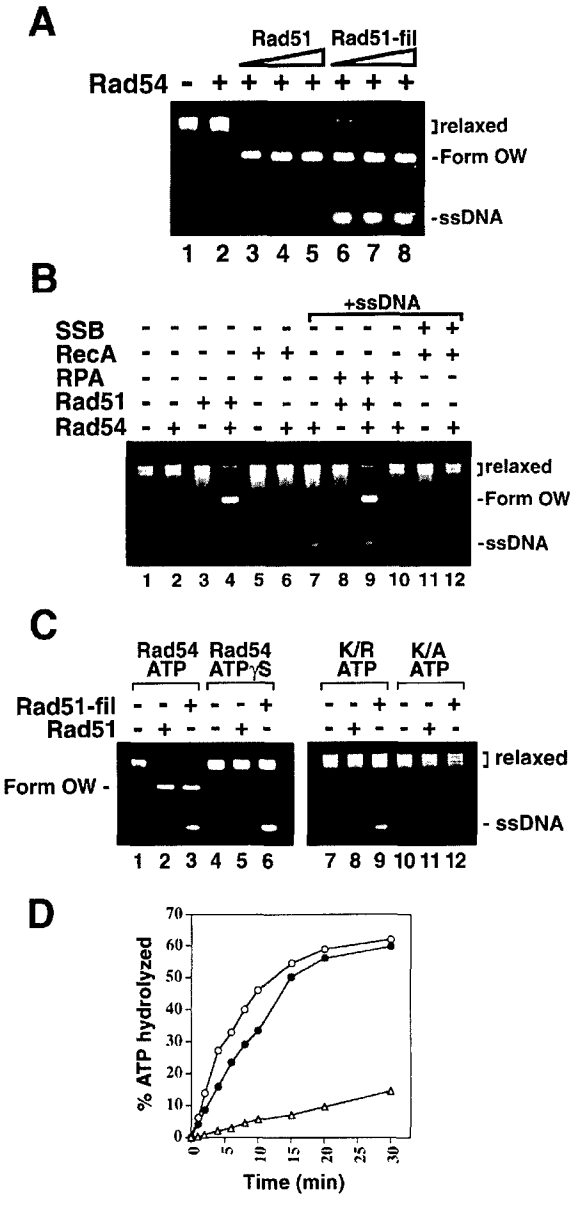


Figure 3. Effect of Rad51 on the Rad54 DNA Supercoiling and ATPase Activities

(A) Stimulation of DNA supercoiling by Rad51 and Rad51-RPA-ssDNA complex. Rad54, at 22.5 nM (lanes 2 to 8), was incubated with relaxed $\phi$X DNA (18.5 $\mu$M base pairs, lanes 1 to 8), *E. coli* topoisomerase I (lanes 1 to 8), and increasing amounts of Rad51 (120, 240, and 360 nM in lanes 3 to 5) or nucleoprotein complexes of Rad51-RPA-ssDNA (Rad51-fil) containing a constant amount of RPA (2 $\mu$M) and pBluescript ssDNA (30 $\mu$M nucleotides) but an increasing level of Rad51 (60, 120, and 240 nM in lanes 6 to 8) at 23°C for 10 min.

(B) RecA does not stimulate DNA supercoiling. Relaxed DNA (lanes 1 to 12) was incubated with *E. coli* topoisomerase I (lanes 1 to 12), Rad54 (lanes 2, 4, 6, 7, 9, 10, and 12), Rad51 (lanes 3 and 4), Rad51-RPA-ssDNA complex (lanes 8 and 9), RecA (lanes 5 and 6), and RecA-SSB-ssDNA complex (lanes 11 and 12), all in the presence of ATP. The concentrations of the reaction components were Rad54, 90 nM; Rad51, 120 nM; RPA, 2 $\mu$M; pBluescript ssDNA, 30 $\mu$M nucleotides; relaxed $\phi$X DNA, 18.5 $\mu$M base pairs; RecA, 1 $\mu$M; and SSB, 6 $\mu$M. The reaction mixtures were incubated at 23°C for 10 min.

(C) Dependence of DNA supercoiling on ATP hydrolysis. Relaxed DNA (lanes 1 to 6) was incubated with *E. coli* topoisomerase I (lanes 1 to 6), Rad54 (lanes 1 to 6), and Rad51 (lanes 2 and 5) or a nucleoprotein complex of Rad51, RPA, and ssDNA (Rad51-fil; lanes 3 and 6) in the presence of ATP (lanes 1 to 3) or ATP-$\gamma$-S (lanes 4 to 6), as indicated. Likewise, relaxed DNA (lanes 7 to 12) was incubated with *E. coli* topoisomerase I (lanes 7 to 12), rad54 K341R (K/R; lanes 7 to 9), and rad54 K341A (K/A; lanes 10 to 12) and either Rad51 (lanes 8 and 11) or the Rad51-RPA-ssDNA complex (Rad51-fil; lanes 9 and 12), all in the presence of ATP. The concentrations of the reaction components were Rad54, rad54 K341A, and rad54 K341R proteins, 90 nM; relaxed $\phi$X DNA, 18.5 $\mu$M base pairs; Rad51, 120 nM; RPA, 2 $\mu$M; and pBluescript ssDNA, 30 $\mu$M nucleotides. The reaction mixtures were incubated at 23°C for 10 min.

(D) Rad51 and Rad51-RPA-ssDNA complex stimulate Rad54 ATPase activity. Rad54, at 50 nM, was incubated with radiolabeled ATP and relaxed $\phi$X DNA without other protein (open triangles), with Rad51 (closed circles), or with Rad51-RPA-ssDNA complex (open circles) at 23°C.

be fully relaxed by calf thymus topoisomerase I (data not shown) it is refractory to *E. coli* topoisomerase I (Figure 2C, panel IV).

It is important to note that at relatively low Rad54 concentrations where no significant DNA linking number change was observed with calf thymus topoisomerase I (Figure 2A, lanes 2 to 4), Rad54 was very much adept at generating Form OW DNA with *E. coli* topoisomerase I (Figure 2A, lanes 9 to 11). For instance, while 90 nM of Rad54 (corresponding to 205 base pairs per Rad54 monomer) produced a substantial amount of Form OW DNA (Figure 2A, lane 10), this concentration of Rad54 did not cause any linking number change in DNA when calf thymus topoisomerase I was used (Figure 2A, lane 3). At 225 nM of Rad54 (corresponding to 82 base pairs per Rad54 monomer), while the majority of the input substrate was converted to Form OW DNA (Figure 2A, lane 11), only a trace of Form U DNA was seen (Figure 2A, lane 4). These results strongly suggest that equivalent levels of both unconstrained negative and positive supercoils are generated during DNA remodeling by relatively low levels of Rad54 (up to 225 nM), and treatment with *E. coli* topoisomerase I removes only the negative supercoils, thus resulting in the accumulation of the positive supercoils and the formation of Form OW DNA. At these relatively low concentrations of Rad54, little or no linking number change in the DNA is seen with calf thymus topoisomerase I, because both the unconstrained negative and positive supercoils are removed by the eukaryotic topoisomerase. Results from these analyses have also allowed us to deduce that at concentrations of 225 nM of Rad54 and above, some of the negative supercoils produced as a result of DNA remodeling become constrained, as revealed by the formation of Form U DNA in the reaction that uses calf thymus topoisomerase I (Figure 2A, lanes 4 to 7). This deduction is also supported by the observation that at high concentrations of Rad54, the formation of Form OW DNA with *E. coli* topoisomerase I in fact becomes progressively inhibited (Figure 2A, lanes 13 and 14). The constraining of negative supercoils seen at high concentrations of Rad54 (Figure 2A, lanes 5 to 7) is likely due to secondary binding of Rad54 to the regions that contained these supercoils.

### DNA Remodeling Is Stimulated by Rad51

Since Rad54 physically interacts with Rad51 and cooperates with the latter in the formation of DNA joints, it was of considerable interest to examine whether the

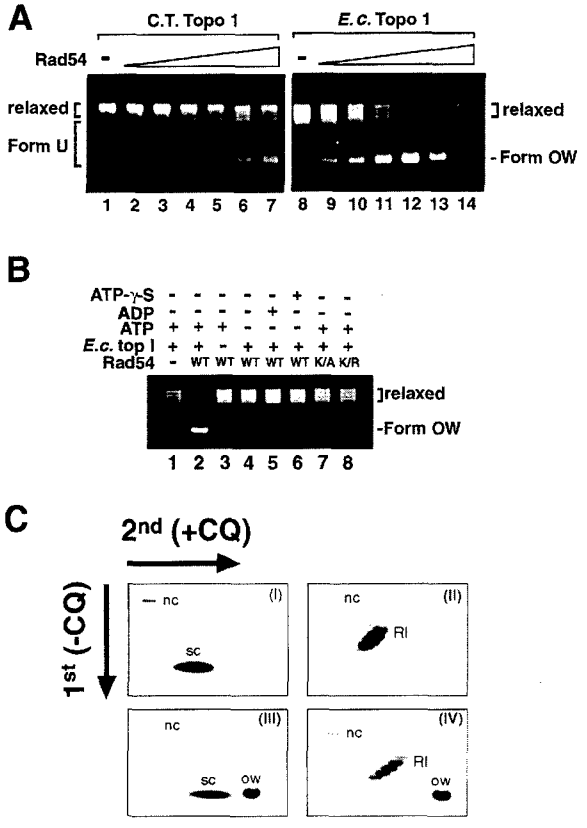


Figure 2. DNA Remodeling by Rad54 as Revealed by Topoisomerase Treatment

(A) Increasing concentrations of Rad54 protein (0.045, 0.09, 0.23, 0.45, 0.9, and 1.8 μM in lanes 2 to 7 and lanes 9 to 14, respectively) were incubated with relaxed DNA and with either calf thymus topoisomerase I (lanes 2 to 7) or *E. coli* topoisomerase I (lanes 9 to 14), as indicated. In lanes 1 and 8, DNA was incubated in buffer with topoisomerase but no Rad54 protein. The novel DNA species generated by the combination of Rad54 and topoisomerase I is designated as Form OW, and the heterogeneous novel DNA species generated by the combination of Rad54 and calf thymus topoisomerase I are collectively designated as Form U. The DNA concentration was 18.5 μM base pairs, and all the reactions were incubated at 37°C for 10 min.

(B) Formation of Form OW DNA requires ATP hydrolysis. Rad54 (0.3 μM) was incubated with relaxed DNA and *E. coli* topoisomerase I in the presence of ATP (lane 2), ADP (lane 5), ATP-γ-S (lane 6), or in the absence of a nucleotide (lane 4), as indicated. The reaction in lane 1 contained relaxed DNA without Rad54, and in lane 3, relaxed DNA was incubated with Rad54 and ATP without topoisomerase. In lanes 7 and 8, rad54 K341A (K/A) and rad54 K341R (K/R) mutant proteins, 0.3 μM each, were incubated with relaxed DNA, *E. coli* topoisomerase I, and ATP, as indicated. The DNA concentration was 18.5 μM base pairs, and the reactions were carried out at 37°C for 10 min.

(C) Two-dimensional gel analysis of Form OW DNA. In (I) and (II), negatively supercoiled ϕX DNA isolated from cells ($\sigma = -0.06$) without (I) or with (II) prior treatment with *E. coli* topoisomerase I was subject to two-dimensional gel analysis. In (III) and (IV), a mixture of negatively supercoiled ϕX DNA and purified Form OW DNA without (III) or with (IV) prior treatment with *E. coli* topoisomerase I was subject to two-dimensional gel analysis. Note in (IV) that the negatively supercoiled DNA, but not Form OW DNA, was relaxed by *E. coli* topoisomerase I. OW, Form OW DNA; SC, negatively supercoiled DNA; RI, relaxed DNA; and nc, nicked circular DNA. In these gel analyses, the first dimension was conducted in the absence of chloroquine (−CQ) and the second dimension in the presence of chloroquine (+CQ).

Importantly, no D loop was formed by RecA with topologically relaxed DNA or positively supercoiled DNA. Even with the supercoiled substrate with $\sigma = -0.021$, only a trace of D loop was seen. These results demonstrate that topological constraints in the relaxed and less negatively supercoiled DNA molecules prevent the formation of a stable joint with the linear ssDNA molecule by RecA protein. Importantly, addition of Rad54 protein did not enhance the ability of RecA to make D loop with the negatively supercoiled DNA, nor did it enable RecA to promote D loop formation with relaxed or positively supercoiled substrate (data not shown). These results suggest that the physical interaction noted between Rad51 and Rad54 (Jiang et al., 1996; Clever et al., 1997; Petukhova et al., 1998) is likely to be functionally important in the D loop reaction.

In summary, unlike RecA, negative supercoiling alone does not appear to compensate for a deficit in the ability in Rad51 to form D loop. Addition of Rad54 overcomes this deficit and in fact renders D loop formation efficient even with relaxed DNA, indicating a specific role of Rad54 in overcoming the topological constraint inherent in DNA joint formation. The experiments described below begin to address the mechanism by which Rad54 promotes DNA joint formation with Rad51.

### Rad54 Generates Both Negatively and Positively Supercoiled Domains in DNA

We have shown that incubation of yeast Rad54 with topologically relaxed DNA and calf thymus topoisomerase I results in a change in the DNA linking number (Petukhova et al., 1999; Figure 2A, lanes 1 to 7), in a manner that is completely dependent on ATP hydrolysis by Rad54 (Petukhova et al., 1999). The product of the DNA remodeling reaction that uses calf thymus topoisomerase I, which removes both negative and positive supercoils efficiently, is negatively supercoiled and was designated Form U (underwound) (Petukhova et al., 1999). In this reaction, a relatively high concentration of Rad54 protein (≤ 50 base pairs per Rad54 monomer) is required to see any linking number change (Petukhova et al., 1999; Figure 2A, lanes 1 to 7), suggesting that either Rad54 protein produces only limited unwinding of the DNA double-helix or that in fact both negative and positive supercoils are produced by low concentrations of Rad54, but that both types of supercoils are removed by calf thymus topoisomerase I. To distinguish between these possibilities, we have used *E. coli* topoisomerase I, which removes negative supercoils but is completely inactive toward positive supercoils in DNA, together with Rad54 and relaxed DNA substrate in the DNA remodeling reaction.

Incubation of the relaxed ϕX DNA substrate, Rad54, and *E. coli* topoisomerase I resulted in the formation of a novel DNA species that migrated ahead of the relaxed DNA (Figure 2A, lanes 8 to 14). Since subsequent analyses showed that this DNA species was positively supercoiled, we have designated it Form OW (overwound) DNA. Formation of Form OW DNA was completely dependent on ATP hydrolysis by Rad54, as revealed by either the omission of ATP or its substitution by ADP and the nonhydrolyzable analog ATP-γ-S (Figure 2B). Consistent with this result, neither rad54 K341A nor rad54 K341R was active in the DNA remodeling reaction (Figure 2B). That Form OW DNA was positively supercoiled was revealed by two-dimensional gel analysis (Figure 2C, panel III) and by the finding that while it can

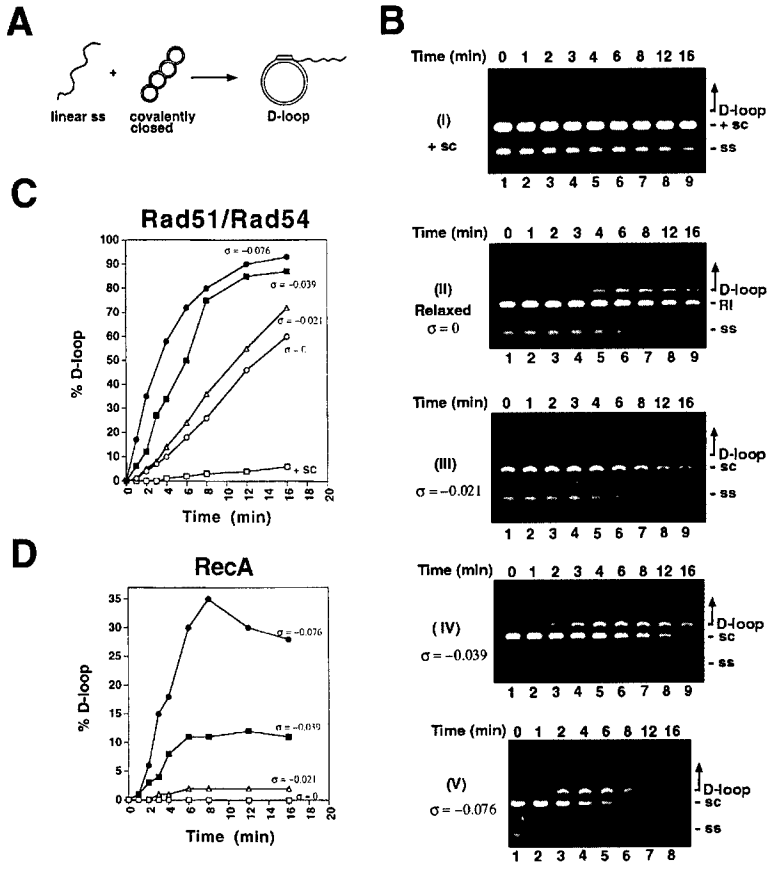


Figure 1. Effect of Superhelicity on D Loop Formation

(A) Schematic of the D loop reaction. Linear ssDNA is paired with a covalently closed duplex, such as a negatively supercoiled species as shown, to yield the D loop. A variety of covalently closed duplex substrates differing in their topological state were employed in the present study (see below).

(B) Superhelicity and D loop formation. In (I) through (V), a preassembled nucleoprotein complex of Rad51-RPA-ssDNA was mixed with Rad54 and duplex DNA substrates that were positively supercoiled (+SC in [I]), relaxed (RI in [II]), and with $\sigma$ values of −0.021 (III), −0.039 (IV), and −0.076 (V), as indicated. The reaction mixtures were analyzed in agarose gels containing 10 μM ethidium bromide to effect the separation of D loop species from other DNA species (nicked circular duplex and linear duplex, which are not marked). At later time points, more complex D loop species appeared, which likely corresponded to multiple molecules of ssDNA and dsDNA paired together. The linear single-strand is designated as ss.

(C) The results in (B) are plotted.

(D) Results from D loop reactions in which RecA-SSB-ssDNA complex was reacted with duplex substrates of the indicated topological state are plotted.

topological states (see Experimental Procedures for technical details), ranging from positively supercoiled, relaxed (average $\sigma = 0$), to negatively supercoiled (average $\sigma$ values of −0.021, −0.039, and −0.076). In addition to yeast Rad51/Rad54/RPA, *E. coli* RecA was also used in conjunction with *E. coli* SSB. The reaction temperature used for examining D loop formation was 23°C, as this relatively low temperature enabled us to more easily follow the reaction kinetics.

Rad51, at 3 nucleotides/monomer and 23°C, was unable to form D loop with the entire range of DNA substrates used, including the most highly negatively supercoiled DNA with $\sigma = -0.076$. This conclusion was validated with a wide range of Rad51 concentrations from 60 nucleotides/monomer to 1 nucleotide/monomer and at the higher reaction temperatures of 30°C and 37°C (data not shown). We next characterized the effect of adding Rad54 on D loop formation with the same DNA substrates, using an amount of Rad51 protein corresponding to 20 nucleotides per protein monomer. This ratio of Rad51 to ssDNA was used because even although 3 nucleotides/Rad51 monomer was optimal for DNA joint formation in the model reaction that employs circular ssDNA and linear duplex (Sung and Robberson, 1995), increasing Rad51 to this level in fact resulted in about 2- to 3-fold reduction in D loop formation (S. V. K. and P. S., unpublished data). As shown in Figure 1B, addition of Rad54 protein rendered the formation of D loop by Rad51 robust, as even with relaxed DNA as substrate, about half of the input DNA had been converted into D loop species at the reaction endpoint of 16 min. Compared to the relaxed DNA substrate, a significantly higher level of D loop was seen when the $\sigma$ value of the substrates decreased to −0.039 and −0.076, but only a small enhancement in the rate and extent of D loop formation was detected with the slightly negatively supercoiled substrate with $\sigma = -0.021$ (Figures 1B and 1C). The result with the relaxed substrate (Figures 1C and 1D) was rather surprising, as RecA protein requires a high level of negative supercoiling in the DNA substrate for D loop formation (Kowalczykowski et al., 1994; see later).

D loop formation by the combination of Rad51 and Rad54 requires that ATP is hydrolyzed by Rad54, as the substitution of Rad54 with mutant variants (Petukhova et al., 1999) of Rad54 either defective in ATP hydrolysis (rad54 K341A) or greatly attenuated for its ATPase activity (rad54 K341R) abolished D loop formation with the entire range of DNA substrates that differed in their topological state (data not shown). RPA is needed for optimal D loop formation (Petukhova et al., 1998), and other experiments have shown that Rad54 does not replace RPA in DNA joint formation, nor does it promote D loop formation without Rad51 (Petukhova et al., 1998; S. V. K. and P. S., unpublished data). In addition, no D loop was formed if we preincubated Rad51 with the duplex, indicating that DNA joint formation requires that Rad51 is bound to the ssDNA substrate (data not shown).

Figure 1D presents the results obtained with the combination of *E. coli* RecA and SSB proteins. As reported before, RecA makes D loop, in a manner that is strongly dependent on the topological state of DNA, such that a dramatic increase in the yield of D loop occurred with decreasing $\sigma$ (reviewed in Kowalczykowski et al., 1994).

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# Superhelicity-Driven Homologous DNA Pairing by Yeast Recombination Factors Rad51 and Rad54

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## Summary

**Yeast Rad51 recombinase has only minimal ability to form D loop. Addition of Rad54 renders D loop formation by Rad51 efficient, even when topologically relaxed DNA is used as substrate. Treatment of the nucleoprotein complex of Rad54 and relaxed DNA with topoisomerases reveals dynamic DNA remodeling to generate unconstrained negative and positive supercoils. DNA remodeling requires ATP hydrolysis by Rad54 and is stimulated by Rad51-DNA nucleoprotein complex. A marked sensitivity of DNA undergoing remodeling to P1 nuclease indicates that the negative supercoils produced lead to transient DNA strand separation. Thus, a specific interaction of Rad54 with the Rad51-ssDNA complex enhances the ability of the former to remodel DNA and allows the latter to harvest the negative supercoils generated for DNA joint formation.**

## Introduction

*Saccharomyces cerevisiae* genes of the *RAD52* epistasis group, consisting of *RAD50, RAD51, RAD52, RAD54, RAD55, RAD57, RAD59, MRE11, XRS2,* and *RDH54/TID1*, are indispensable for homologous recombination and the repair of DNA double-strand breaks induced by ionizing radiation and other DNA damaging agents. Because of a requirement for meiotic recombination to effect the proper disjunction of homologous chromosomes in meiosis I, mutations in the *RAD52* group genes often lead to meiotic abnormalities, including lethality (reviewed in Petes et al., 1991; Paques and Haber, 1999; Sung et al., 2000).

Much of our current understanding of homologous recombination in eukaryotes has been garnered from studies on mating type switching and meiotic recombination in *S. cerevisiae*. In both types of recombination, DNA double-strand breaks are generated in a developmentally programmed fashion. Once formed, the ends of these breaks are processed nucleolytically to yield long ssDNA overhangs. These ssDNA tails are then utilized by the recombination machinery to invade an intact DNA homolog, either the sister chromatid or homologous chromosome, to form a heteroduplex DNA joint called D loop. The recombination event is completed by DNA synthesis primed from the D loop and the resolution of DNA intermediates. The repair of DNA double-strand breaks induced by ionizing radiation very likely follows the same mechanistic route, as it too shows a dependence on the same *RAD52* epistasis group genes (Petes et al., 1991; Paques and Haber, 1999; Sung et al., 2000). Cloning studies have revealed a high degree of functional and structural conservation of the components of the recombination machinery, from yeast to humans. Emerging evidence has implicated the homologous recombination machinery in cancer prevention in mammals, underscoring the importance of dissecting the functional and mechanistic intricacies of this machinery (reviewed in Dasika et al., 1999).

The enzymatic process that leads to the formation of heteroduplex DNA during recombination is referred to as "homologous DNA pairing and strand exchange" (reviewed in Kowalczykowski et al., 1994; Roca and Cox, 1997; Sung et al., 2000). *RAD51*, a key member of the *RAD52* group, encodes a protein homologous to the *Escherichia coli* homologous DNA pairing and strand exchange enzyme RecA. Even though Rad51 catalyzes homologous DNA pairing and strand exchange in model systems that involve oligonucleotides or plasmid length circular ssDNA and linear duplex molecules, it has little ability to pair a linear ssDNA and a covalently closed duplex molecule to form D loop (Petukhova et al., 1998; Mazin et al., 2000). Previous studies have indicated that the *RAD54* encoded product, a member of the *RAD52* group and a Swi2/Snf2-like factor (see Eisen et al., 1995, for a discussion), promotes efficient D loop formation by Rad51 (Petukhova et al., 1998; Mazin et al., 2000).

Rad54 interacts with Rad51 in two-hybrid analysis and in vitro (Jiang et al., 1996; Clever et al., 1997; Petukhova et al., 1998). Rad54 also possesses a robust ATPase function that is completely dependent on DNA, dsDNA in particular, for activation (Petukhova et al., 1998; Swagemakers et al., 1998). Upon ATP hydrolysis, Rad54 induces a linking number change in the DNA that can be trapped by treatment of relaxed DNA with calf thymus topoisomerase I or nicked circular duplex with DNA ligase, indicating that Rad54 remodels DNA (Petukhova et al., 1999; Tan et al., 1999). However, the nature of DNA remodeling and the manner in which Rad51 and Rad54 functionally cooperate to make D loop have remained mysterious. Here, we show that Rad54 supercoils DNA and report on the synergistic cross-talk between Rad51 and Rad54 in DNA supercoiling and D loop formation. Our results highlight the main distinctions between the prokaryotic and eukaryotic recombination machineries in effecting DNA strand invasion to form DNA joints, and they also suggest a novel general principle for a protein machinery to promote DNA strand opening via supercoiling rather than unwinding.

## Results

### Rad51/Rad54-Mediated D Loop Formation

For examining D loop formation (Figure 1A, schematic), we made covalently closed duplex DNAs with different

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prone compensation for defective homologous recombination-based mechanisms. On the contrary, it has recently been demonstrated that inactivation of individual NHEJ DNA repair pathway components, such as XRCC4 and Ku, also leads to severe GCR (Difilippantonio *et al.*, 2000; Gao *et al.*, 2000). Moreover, a severe defect in NHEJ has recently been observed in Brca1-deficient MEF cells (Zhong *et al.*, 2000). Thus, both homologous recombination and NHEJ are likely to be required for maintaining genome integrity in the face of chromosome breakage. Defects in either of these repair pathways could result in inefficient and improper repair of damaged chromosomes. These latter studies therefore imply that gross chromosomal rearrangements may not be a consequence of non-homology-dependent DNA repair which is proposed to be error-prone, but due instead to an unknown mechanism that remains to be defined.

Clearly, a more precise understanding of BRCA1/2 function at the mechanistic level will not only provide insight into the pathogenesis of breast cancer, but also enhance our knowledge of the molecular basis by which a cell maintains its genomic integrity. We can also expect that novel therapeutic tools designed to specifically interfere with these molecular events will be developed and tested for tumor suppression in a growing company of genetically defined mammary tumor models.

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BRCA1/2 apparently accelerates the rate of mutation and increases the incidence of tumor formation, since predisposed individuals who inherit one mutant copy of BRCA1 or BRCA2 experience a 10-fold greater chance of developing breast cancer than non-predisposed individuals (Hall *et al.*, 1990; Wooster *et al.*, 1994). BRCA1/2 function in sequence-specific transcriptional regulation, and it has been demonstrated that BRCA1 can regulate a diverse array of genes involved in cell proliferation, cell cycle control and differentiation. Moreover, BRCA1/2 are unique to metazoans, a fact that distinguishes them from other member genes in the caretaker class. These distinctive features of BRCA1/2 render it possible that their functional inactivation may, apart from their participation in the maintenance of global genome stability, contribute to a critical step in malignant transformation during breast tumorigenesis. Accordingly, it will be important to examine whether reintroduction of BRCA1/2 at levels approaching those occurring physiologically can rescue a transformed cellular phenotype.

In sporadic breast cancers and other cancers, p53 mutations must arise through selection during clonal evolution. This implies that p53 gene mutations are more frequently selected because they either confer growth advantages to tumor cells or, alternatively, that the p53 gene locus is more susceptible to mutation. On the other hand, the fact that somatic mutations in BRCA1/2 are rare events may imply that no growth advantage accompanies the loss of BRCA1/2 function in the adult breast tumor cells, and perhaps even normal adult mammary gland cells. This has raised speculation that BRCA1/2 have distinct functions at different stages of mammary gland development. Consistent with this notion is the observation that expression of BRCA1 and BRCA2 is regulated during this process (Lane *et al.*, 1995; Marquis *et al.*, 1995; Rajan *et al.*, 1996). In this regard, understanding how BRCA1/2 contribute to mammary gland development might weigh equally as important as understanding how their mutational inactivation leads to breast cancer formation.

The rarity of BRCA1/2 gene mutations in sporadic breast and ovarian cancer cases implies that aberrant expression of the BRCA1/2 genes or, alternatively, improperly regulated activity of their encoded products could contribute to non-familial breast cancer formation. Consequently, detailed knowledge of their normal biological function and regulation will be required for a thorough appreciation of how direct or indirect functional inactivation of BRCA1/2 ultimately leads to breast tumorigenesis. Specifically, functional analyses of BRCA1/2-associated proteins and the operative mechanisms underlying their respective roles in transcription regulation and DNA damage repair could contribute to a more complete understanding of the etiological bases of the remaining 90% of breast cancer cases that do not involve germline BRCA1/2 mutations.

Another unsolved question concerns the tissue-specific nature of tumor suppressive properties of BRCA1/2. BRCA1/2 are expressed ubiquitously and participate in universal cellular pathways; however, functional inactivation of BRCA1/2 leads specifically to breast or ovarian cancer formation. One possibility to account for this apparent paradox is that BRCA1/2, apart from their universal activities, exert distinct functions in breast or ovarian tissues. For example, BRCA1/2 may transcriptionally regulate specific genes that are critical for growth control of mammary gland cells. Consequently, a more complete understanding of how BRCA1/2 target specific genes for transcriptional regulation may illuminate unique pathways leading to breast tumorigenesis. Furthermore, the fact that BRCA1 has been implicated in the modulation of estrogen signaling suggests a potential mechanism through which its functional inactivation may impact cells in a specific hormonal environment. It remains to be established whether and how BRCA1 modulates the estrogen response *in vivo* and how its loss affects this cellular response. An alternative possibility to account for the breast- and ovarian-specific nature of BRCA1/2 tumor suppression is that the activities of BRCA1/2 are more important for tumor suppression in these as opposed to other tissues. BRCA1/2 function ubiquitously in the maintenance of genomic integrity, and it is probable that the DNA damage-induced cellular signaling pathways that converge on them are conserved in most cell types. Indeed, the DNA damage-induced target genes controlled transcriptionally by BRCA1, as well as the components of the DNA double-strand break repair machinery with which it functionally interacts exhibit broad cell type expression profiles. It thus appears likely that BRCA1 occupy a fundamental and universally conserved role in the DNA damage response through their control of transcription and DNA repair. The tissue-specificity of their tumor suppressive properties may therefore lie not within the tissue-specific regulation of BRCA1/2 activity but, rather, in the tissue-specific nature of the DNA damage to which it responds than the others. In this regard, it is significant that specific metabolic byproducts of estrogen itself have been documented to be genotoxic in nature (reviewed by Liehr, 2000). These collective observations raise the intriguing possibility that BRCA1/2 may play a role in protecting breast and ovarian tissue from estrogen-induced DNA damage. Clearly, a precise understanding of the molecular basis underlying the tissue-specificity of the tumor suppressive properties of BRCA1/2 will be essential for the design and implementation of strategies to delay, and ultimately to prevent, breast tumor formation.

Recent advances in the studies of BRCA1/2 have also provided insight into the mechanistic role of DSB repair in the suppression of tumorigenic mutations. It is well-established that cancer-causing mutations such as large deletion mutations, chromosomal translocations, and partial or complete chromosome loss often arise from gross chromosomal rearrangement and lead to loss of heterozygosity which is implicated in the inactivation of tumor suppressor genes (Lasko *et al.*, 1991). Hence, it has been proposed that one role for BRCA1/2 in homologous recombination-based DSB repair is to suppress gross chromosomal rearrangements (GCR) including genetic exchange between nonhomologous chromosomes after chromosome breakage (Moynahan *et al.*, 1999; Yu *et al.*, 2000). However, it is not presently clear whether GCR that are observed in BRCA1/2 mutant cells represent resolution of chromosome breaks by non-homology-based repair pathways including NHEJ as an error-

are also hypersensitive to MMC (Liu *et al.*, 1998). Moreover, gross chromosomal rearrangements and genetic exchange between nonhomologous chromsomes that are observed in BRCA2-deficient cells are proposed to result from a defect in homologous recombination. Taken together, these observations present further evidence for a role of BRCA2 in homologous recombination. Like BRCA1, BRCA2 is not evolutionarily conserved, implicating that BRCA2 is not essential for the basic machinery of DSB repair, but is necessary for an efficient DNA repair process that is required by more complicated organisms to ensure the integrity of a more complicated genome.

*BRCA2 in DNA-damage induced checkpoint control*
Although the enforcement of checkpoint activated by DNA damage is largely preserved in cells from mice homozygous for Brca2 truncations (Patel *et al.*, 1998), tumors formed by these mice display loss of mitotic spindle checkpoint presumably due to acquired mutations in BUB1, MAD3L and p53, three genes involved in mitotic checkpoint control (Lee *et al.*, 1999). It is possible that Brca2 truncation at exon 11, retaining the first three BRC repeats, possesses a partial function that is able to rescue the embryonic lethality and preserve an intact DNA damage-induced checkpoint (Chen *et al.*, 1998a). The specific disruption of the interaction between RAD51 and BRCA2 by expressing a peptide containing BRC repeats results in loss of G2/M checkpoint control, thereby, suggesting that the role of BRCA2 in G2/M checkpoint may be associated with its function in DNA repair (Chen *et al.*, 1998a).

## Perspectives

Both BRCA1 and BRCA2 have been proposed to be caretakers that function in the maintenance of global genome stability. Although functional inactivation of a caretaker is not sufficient to convert a normal cell to a cancer cell, it destabilizes the genome such that mutations in other genes can accumulate more frequently (Kinzler and Vogelstein, 1997). The subsequent mutational inactivation or deletion of a gatekeeper gene represents the precipitating event that initiates transformation to the neoplastic state. Gatekeepers are exemplified by genes that encode activities that directly regulate cell growth, and loss of gatekeeper activity leads to aberrant control of cell growth or cell death. While this hypothesis has provided a framework for understanding the contribution of inactivation of tumor suppressor genes to the genesis of cancer, its arbitrary distinction between caretakers and gatekeepers may obscure what in actuality represents the contribution of a potentially overlapping set of functional activities. More specifically, it seems evident that classically-defined gatekeepers could reasonably function as caretakers and vice versa. The cases of BRCA1 BRCA2, and p53 provide examples to illustrate this point.

In a variety of model systems, functional inactivation of BRCA1/2 leads to overt global genome instability; BRCA1/2 therefore clearly conform to the class of caretakers. However, while p53 fulfils the definition of a gatekeeper in many instances, other models exist in which it functions more akin to a caretaker. For example, while p53 is likely to play a role in restraining breast cancer formation in BRCA1/2 mutation carriers, the loss of its function does not appear to represent the threshold event that is proposed to accelerate the rate of mutation in cancer development. Rather, in these instances, functional loss of p53 appears only to permit cells with accumulated genetic abnormalities by bypass checkpoint control and DNA-damage induced apoptosis. A similar effect is observed in a Ku80 deficient mouse tumor model. In this system, the absence of Ku80 precipitates genomic instability, which is not augmented by the additional loss of p53 (Difilippantonio *et al.*, 2000). Under such circumstances, it appears that inactivation of BRCA1/2 (or Ku80) and inactivation of p53 represent two distinct but coordinate events; the first destabilizes the genome, while the second assists the first in evading the custodial effects of cellular surveillance systems such as DNA-damage induced checkpoint control or apoptosis. BRCA1/2 and p53 thus appear to function as a coordinate caretaker; inactivation of both genes will permit cells with unstable genomes to proliferate freely and ultimately become tumorigenic.

Consistent with the possibility that p53 may function as a caretaker, germline inactivation of p53 itself will lead to genome instability and an increased frequency in tumor formation (reviewed by Lane, 1992). Hence, p53 appears to conform to the class of caretakers which are proposed to function as guardians of the genome (Lane, 1992). Genome instability need not necessarily arise through germline inactivation of specific genes such as BRCA1 and BRCA2 that function in the maintenance of chromosomal integrity, but conceivably could arise by spontaneous insult from intrinsic or extrinsic agents. For example, environment, age, diet, or other etiological factors could represent causative agents in the precipitation of chromosomal damage in sporadic cancers, as could sporadic mutations in genes involved in the maintenance of chromosomal integrity. As it represents the most direct means to effect bypass of cellular surveillance systems, p53 mutation is most frequently observed in breast cancers or in familial cancers with BRCA1/2 mutations. However, mutational inactivation of genes that function in p53-mediated pathways, such as p19ARF (de Stanchina *et al.*, 1998; Pomerantz *et al.*, 1998; Zindy *et al.*, 1998), or genes that function in other checkpoint control pathways, such bub1, have also documented in human cancers (Cahill *et al.*, 1998).

Thus while p53 can evidently fulfill, at least in part, the duties of a caretaker, it must be emphasized that it can also function as a classical gatekeeper. Its dual character likely derives from the fact that p53 exerts its influence over cellular functions other than DNA-damage induced checkpoint conrol and apoptosis. As a sequence-specific transcription factor, p53 regulates multiple genes involved in many cellular pathways (el-Deiry, 1998; Prives and Hall, 1999; Tokino and Nakamura, 2000). Inactivation of p53 could lead to uncontrolled proliferation and/or invasive growth and, in so doing, contribute to a distinct step in malignant transformation. By similar reasoning, it seems plausible that BRCA1/2, which clearly function as caretakers in the sense that their inactivation is invariably accompanied by widespread genome instability, could also fulfill the role of gatekeepers. Indeed, inactivation of

*Other potential functions of BRCA1*

BRCA1 also associates with BARD1 (Wu *et al.*, 1996), which functionally interacts with polyadenylation factor CstE-50 (Kleiman and Manley, 1999), and with BAP1 (Jensen *et al.*, 1998), which is a de-ubiquitinating enzyme, suggesting two additional roles of BRCA1 in RNA polyadenylation and in ubiquitin-dependent protein degradation. The potential role of BRCA1 in post-transcriptional RNA processing may coordinate with its role in transcriptional regulation. The N-terminal RING domain of BRCA1 has been suggested to function with or as a ubiquitin–protein ligase (Lorick *et al.*, 1999), which is particularly interesting as protein degradation may influence the processes of transcription and DNA repair. In sum, it remains to be elucidated how BRCA1 participate in these pathways and the biological significance of these functions.

*BRCA2 in transcriptional regulation*

Contrary to BRCA1, the role of BRCA2 in transcriptional regulation is less certain. However, several lines of evidence support such a role for BRCA2. First, the product of BRCA2 exon 3 (amino acids 23–105), when fused to DNA-binding domains, activates transcription in yeast and mammalian cells (Milner *et al.*, 1997). Consistently, the amino acid sequence of BRCA2 within this region shares some similarity with the transactivation domain present in c-Jun (Milner *et al.*, 1997). Intriguingly, in a breast cancer case of a Swedish patient, a deletion of exon 3 at the BRCA2 allele was found (Nordling *et al.*, 1998). Moreover, a missense mutation (tyrosine 42 to cysteine), identified in a familial breast cancer case, within the primary activating region severely compromised the transactivation activity of BRCA2 (Milner *et al.*, 1997) suggesting that the abrogation of the BRCA2 putative transcription activity may be a predisposition to tumor development. Second, overexpression of exogenous BRCA2 inhibited p53-mediated transcription (Marmorstein *et al.*, 1998). Third, BRCA2 was demonstrated to have histone acetyltransferase activity and consistently, it was found to interact with the transcription co-activator P/CAF (p300/CBP-associated factors), suggesting a role of BRCA2 as a coactivator (Fuks *et al.*, 1998). To date, it remains unclear what the exact role of BRCA2 is in the regulation of transcription. More importantly, it will be critical to understand how the loss of this putative transcriptional activity of BRCA2 contributes to tumorigenesis.

*BRCA2 in DNA repair*

*The interaction between BRCA2 and RAD51* The potential role of BRCA2 in DNA repair was first revealed by identification of its interaction with human or mouse RAD51 in yeast two-hybrid screens (Chen *et al.*, 1998b; Sharan *et al.*, 1997; Wong *et al.*, 1997). Consistently, mouse embryos lacking BRCA2 exhibit similar radiation hypersensitivity to mouse embryos lacking RAD51 (Sharan *et al.*, 1997). Human and mouse RAD51 are mammalian homologs of the *Escherichia coli* protein RecA (Clark, 1996) and of yeast ScRad51 (Shinohara *et al.*, 1992), a membrane of the RAD52 epistasis group in *Saccharonmyces cerevisiae* (McKee and Lawrence, 1980). All of the proteins in the RAD52 epistasis group are required for the repair of DSB and for mitotic and meiotic recombination in yeast. Eukaryotic RAD51 protein, similar to RecA, has ATP-dependent DNA binding activity and multimerizes to form a nucleoprotein filament on single-stranded DNA. Furthermore, RAD51 can catalyze homologous DNA pairing and DNA strand exchange in an *in vitro* recombination reaction (Baumann *et al.*, 1996; Baumann and West, 1997; Sung, 1994; Sung and Robberson, 1995).

Apparently, multiple discrete regions in BRCA2, including six BRC repeats, mediate its interaction with RAD51 (Chen *et al.*, 1998b; Sharan *et al.*, 1997; Wong *et al.*, 1997). It is, therefore, tempting to hypothesize that BRCA2 may increase the efficiency for RAD51-nucleoprotein filament formation by binding multiple RAD51 subunits. It would be interesting to determine the precise stoichiometry of the BRCA2-RAD51 complex, and examine how BRCA2 influences homologous DNA paring and strand exchange activity of mammalian RAD51. It has been shown that other protein components, such as a single-strand DNA binding protein, replication protein A, RAD52, and RAD55/RAD57 heterodimer are necessary for the formation of RAD51-filament by enhancing the activity of RAD51 (reviewed by Shinohara and Ogawa, 1999). It is conceivable that BRCA2 is also required for the formation of RAD51-filament and the proper function of RAD51 (Figure 3). Consistent with this notion, IR-induced RAD51 foci formation is diminished in BRCA2-deficient cells (Yu *et al.*, 2000; Yuan *et al.*, 1999) or in cells in which the interaction between BRCA2 and RAD51 is specifically disrupted (Chen *et al.*, 1998a). Therefore, BRCA2 does not appear to be necessary for the assembly of RAD51 complexes upon DNA damage.

*Interaction between BRCA1 and BRCA2* It has been suggested that BRCA1 and BRCA2 interact *in vivo* (Chen *et al.*, 1998a). A region adjacent to, but not at the extreme C-terminus of BRCA1 was shown to mediate this interaction. BRCA2 also localizes in nuclear dots in mitotic cells at S or G2 phase and, colocalizes with BRCA1 on synaptonemal complexes of meiotic chromosomes (Chen *et al.*, 1998a). As discussed before, BRCA1 colocalizes with RAD51 in a specific period after DNA damage, and this association is distinct from the colocalization of IR-induced BRCA1/RAD50 foci. In short, the functional consequence of the interaction between BRCA1 and BRCA2 remains elusive, although it is possible that this interaction may be involved in coupling the functions of RAD50 and RAD51.

*BRCA2 in DSB repair* By revealing the interaction between BRCA2 and RAD51, the potential role of BRCA2 in DSB repair through homologous recombination is supported, but direct evidence is still desired. Brca2-truncated mouse lymphocytes are hypersensitive to mitomycin C (MMC) (Yu *et al.*, 2000), which causes DNA interstrand cross-links that are repaired primarily by recombination between homologous sequences (Li *et al.*, 1999a). Other mutant cells lacking DNA recombination genes such as XRCC2 and XRCC3

both serines at residues 1423 and 1524 to alanines failed to rescue radiation hypersensitivity in BRCA1-deficient cells (Cortez *et al.*, 1999). These data suggested that ATM-dependent phosphorylation of BRCA1 on S1423 and S1524 is necessary for BRCA1-mediated DNA damage response.

However, as detected by its mobility shift in SDS gels, hyperphosphorylated forms of BRCA1 still exist in ATM-deficient cells upon DNA damage (Scully *et al.*, 1997c), suggesting that multiple kinase activities are responsible for DNA damage-induced hyperphosphorylation of BRCA1. Consistently, hCds1/ChK2 has been shown to phosphorylate BRCA1 on serine 988 upon IR (Lee *et al.*, 2000). The BRCA1 mutant carrying the S988A mutation also fail to rescue radiation hypersensitivity of BRCA1-deficient HCC1937 cells. It is therefore possible that ATM-dependent and Chk2-dependent phosphorylation of BRCA1 are involved in cellular responses to different levels of DNA damages. Alternatively, two kinase pathways may, through modulating different functions of BRCA1, regulate multiple downstream effectors.

In addition to ATM and hCds1/Chk2, ATR and DNA-PK were shown to phosphorylate BRCA1 *in vitro* (Lim *et al.*, 2000). It remains to be determined whether these kinases are involved in phosphorylation of BRCA1 *in vivo*. Moreover, additional kinases responsible for phosphorylation of BRCA1 upon treatments of other DNA damaging agents such as UV and MMS remains to be explored.

*Functional links of ATM and BRCA1 in DNA damage signaling* Phosphorylation of BRCA1 is apparently important for DNA damage response. However, it remains unclear how phosphorylation modulates the activities of BRCA1. Since BRCA1 forms IRIFs that colocalize with RAD50/MRE11/NBS1 and DNA-damage sites following IR-treatment, it is obvious to speculate that phosphorylation of BRCA1 is involved in this process. Mutation of ATM or hCds1/Chk2 phosphorylation sites on BRCA1, however, did not affect BRCA1 foci formation IR (Cortez *et al.*, 1999; Lee *et al.*, 2000). Therefore, it is likely that ATM or Chk2-mediated phosphorylation of BRCA1 regulates the activities of BRCA1 in DNA repair processes, rather than targeting the BRCA1-RAD50 complexes to damaged sites. Alternatively, phosphorylation of BRCA1 may influence the activities of BRCA1 in transcription regulation, thereby, alter the transcription of DNA damage responsive genes.

Nevertheless, the kinase activity of ATM has been functionally linked to BRCA1 through the BRCA1-associated protein, CtIP. Recent studies indicated that ATM phosphorylates CtIP *in vitro* and *in vivo* following IR-treatment (Li *et al.*, 2000). This ATM-dependent phosphorylation of CtIP is required for dissociation of the CtIP/CtBP corepressor complex from BRCA1 and, subsequently relieving BRCA1-mediated repression of GADD45 transcription (Figure 4) (Li *et al.*, 2000). Therefore, ATM can modulate the activities of BRCA1, not only through a direct modification on BRCA1, but also through phosphorylation of its binding partner, CtIP. These studies suggest that CtIP mediates one of the functional links between ATM and BRCA1 in DNA-damage signaling pathways.

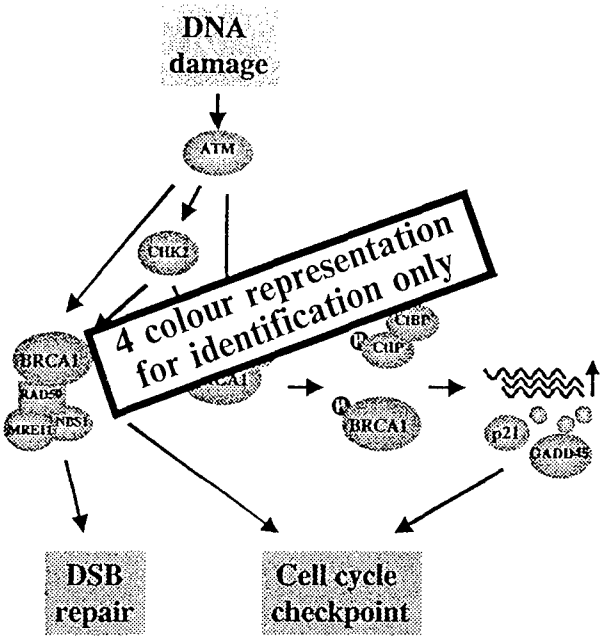


**Figure 4** BRCA1 is involved in cellular response to DNA damage. BRCA1 participates in DNA damage repair and cell cycle checkpoint control, and coordinates both cellular processes in response to DNA damage. BRCA1 may directly participate in the DSB repair process through interacting with the RAD50/MRE11/NBS1 complex. BRCA1 may control cell cycle checkpoint by induction of p21 or GADD45 transcription. Upon IR, CtIP is phosphorylated by ATM. Phosphorylated CtIP dissociates from BRCA1, leading to the relief of repression activity of BRCA1 and, thereby, the induction of GADD45 or p21. Induction of p21 or GADD45 results in cell cycle arrest at G1/S or G2/M transition. The BRCA1/RAD50/MRE11/NBS1 complex may be also involved in the cell cycle checkpoint control

*An integrated signaling network in BRCA1-mediated DNA-damage response* BRCA1 and CtIP are apparently not the only proteins that are phosphorylated by ATM upon IR-treatment. It has been demonstrated that ATM phosphorylates multiple components in BRCA1-containing complexes, including p53 and NBS1. ATM-dependent phosphorylation of p53 is required for G1/S checkpoint and p53-mediated apoptosis (Canman *et al.*, 1998). Phosphorylation of NBS1 by ATM is necessary for the formation of IRIF, suggesting that one function of NBS1 activated by ATM is to relocalize Rad50/Mre11/NBS1 to sites of DNA damage (Zhao *et al.*, 2000). Derivatives of NBS1 carrying mutations in the ATM-phosphorylation sites fail to correct the RDS defect in NBS1-deficient cells, suggesting that another function of NBS1 activated by ATM is in S phase checkpoint control (Gatei *et al.*, 2000; Lim *et al.*, 2000; Wu *et al.*, 2000; Zhao *et al.*, 2000). These BRCA1-associated proteins are also likely to be regulated by other kinases such as ATR and Chk2. Interestingly, MRE11 is also phosphorylated upon DNA damage in an NBS1-dependent manner, although the consequences of this phosphorylation event remains unknown (Dong *et al.*, 1999). It is apparent that a complicated but integrated network is utilized to transmit DNA-damage signals to BRCA1 and its associated proteins, and regulate their functions at different levels.

ment of an *in vivo* DSB repair assay system (Moynahan *et al.*, 1999) in which the frequency of repair at a defined DSB may be measured has proven instrumental in elucidating the contribution of BRCA1 to both homologous and nonhomologous recombinational repair pathways (Figure 3). Using this system, Brca1-deficient embryonic stem cells were found to exhibit a significant defect in homologous recombination (Moynahan *et al.*, 1999). A recent study using a similar approach has shown that NHEJ is also severely reduced in Brca1-deficient mouse embryonic fibroblast cells (Zhong *et al.*, 2000) although such a defect was not observed previously in embryonic stem cells (Moynahan *et al.*, 1999). Moreover, retroviral integration, which relies on the NHEJ-dependent repair pathway, is inefficient in Brca1-deficient embryonic fibroblast cells (Zhong *et al.*, 2000). This same study also demonstrated that Brca1-deficient cell extract as well as normal cell extract blocked by BRCA1-specific antibodies both exhibit reduced activity in catalyzing DNA end-joining *in vitro*, suggesting that BRCA1 participates directly in the NHEJ repair process (Zhong *et al.*, 2000).

The involvement of BRCA1 is both homologous recombination and NHEJ is consistent with its interaction with the RAD50/MRE11/NBS1 complex, which is required for both DNA recombination processes. It has been demonstrated that cells with component defects in either of these recombinational repair pathways are prone to radiation hypersensitivity, genetic instability, and increased tumor susceptibility (Difilippantonio *et al.*, 2000; Gao *et al.*, 2000; Varon *et al.*, 1998a).

It is worth noting that both BRCA1 and BRCA2 genes are not evolutionarily conserved, while the basic DSB repair machinery itself is (reviewed by Featherstone and Jackson, 1999; Karran, 2000). BRCA1 is therefore dispensable for normal frequencies of DNA recombination and DSB repair in lower eukaryotes such as *Saccharomyces Cerevisiae*. Thus, BRCA1 is likely to increase the repair efficiency of damaged DNA in the context of a much larger and more complicated chromatin environment such as that of the mammalian genome. Moreover, BRCA1 could additionally function to effect the efficient coordination of DNA repair with other cellular processes that are critical to support metazoan existence.

*BRCA1 in transcription-coupled repair* Brca1-deficient mouse embryonic stem cells have been shown to be defective in the ability to carry out transcription-coupled repair (TCR), a process in which DNA damage is repaired more rapidly in transcriptionally active DNA than in the genome as a whole (Gowen *et al.*, 1998). DNA damage induced by UV as well as oxidative DNA damage caused by IR or $H_2O_2$ can be repaired by TCR. It has been demonstrated that Brca1-deficient cells are defective in TCR of oxidative DNA damage. but not in TCR of UV-induced DNA damage. Consistent with this observation, these Brca1-deficient cells are hypersensitive to oxidative DNA damage. Presently, it is not clear whether BRCA1 itself participates directly in TCR or, alternatively, whether it functions as a transcription factor essential for the expression of genes whose products are required for TCR of oxidative damage (Gowen *et al.*, 1998).

The hypersensitivity of BRCA1-deficient cells to IR and $H_2O_2$ cannot be explained exclusively by a defect in TCR because DSB are also generated following treatments with these two agents. This observation is consistent with the aforementioned role of BRCA1 in recombinational repair of DSBs through both homologous and nonhomologous pathways. Therefore, BRCA1 is involved in multiple DNA repair pathways that ensure global genome stability.

*BRCA1 in DNA-damage checkpoint controls* DNA repair process must be coordinated with cell cycle control mechanism to ensure that damaged chromosomal DNA is fixed before it is replicated or segregated. BRCA1 appears to play such a role through its dual participation in the repair process of damaged DNA and in the control of cell cycle checkpoint. Although the underlying mechanism for BRCA1 functioning in checkpoint control has not been clarified, the observations showing the effects of BRCA1 on the transcription of genes involved in cell cycle controls, as aforementioned, may evoke a model for it. Alternatively, BRCA1 may regulate DNA damage-induced checkpoint through its associated DSB repair complexes. Recently, a potential role of Rad50/Mre11 in G2/M checkpoint was suggested by showing that the rad50 or mre11 mutant can suppress the adaptation of hdf1, a yeast Ku70 mutant, to G2/M arrest after DNA damage (Lee *et al.*, 1998). Whether the interaction between BRCA1 and the RAD50/MRE11/NBS1 complex is involved in G2/M checkpoint control remains interesting to be examined.

*BRCA1 in DNA-damage signaling* An important step in the cellular response to DNA damaged is to transduce damaged signals to downstream effectors involved in the arrest of cell cycle and repair of damaged DNA. Many kinases have been implicated to be responsible for the transduction of DNA damaged signals. In mammalian cells, several kinases, such as ATM, ATR, DNA-PK, Chk1 and hCds1/Chk2 are activated in response to DNA damage (for review see Dasika *et al.*, 1999; and references therein). Consistently, ATM-deficient cells are defective in DNA damage-induced checkpoint control as well as DNA repair (reviewed by Shiloh, 1997). Chk2, which itself is phosphorylated and regulated by ATM, is essential for G2/M checkpoint control (Matsuoka *et al.*, 1998).

BRCA1 becomes hyperphosphorylated in response to treatment of a variety of DNA damaging agents, including UV, hydroxyurea, mitomycin C, MMS, IR, $H_2O_2$ and adriamycin (Chen *et al.*, 1996c; Li *et al.*, 1999b; Scully *et al.*, 1997b). Recently, multiple phosphorylation sites at serine (S) residues, including S1330, S1423, S1466, S1524 and S1542, have been detected by mass spectrometry analysis of recombinant BRCA1 peptides phosphorylated *in vitro* by ATM (Cortez *et al.*, 1999). Moreover, S1387 was shown to be phosphorylated by ATM *in vitro* based on a screening of peptides containing potential ATM-phosphorylation sites (Lim *et al.*, 2000). Among these serine residues, S1457, S1524 and S1542 were shown to be phosphorylated *in vivo* by mass spectrometry analysis of transfected BRCA1 in irradiated human 293T cells (Cortez *et al.*, 1999). Furthermore, the phosphorylation-defective mutant of BRCA1 carrying changes of

chromatin structural reconfiguration (Alani *et al.*, 1989). NBS1, the human equivalent of yeast Xrs2, was identified based on its copurification with RAD50 and MRE11, and simultaneously as the product of the gene mutated in Nijmegen break syndrome (Carney *et al.*, 1998; Varon *et al.*, 1998b). The RAD50/MRE11/NBS1 complex exhibits several activities that are not observed in the absence of NBS1, including partial DNA duplex unwinding and efficient cleavage of fully paired hairpins (Paull and Gellert, 1999). Apart from its role in double-strand break repair, this complex, or individual complex components, may function in other aspects of the DNA damage response. Radio-resistant DNA synthesis (RDS), a hallmark of ataxia telangiectasia (A-T) cells (Painter and Young, 1980), has also been observed in NBS1-deficient cells, implying a role for NBS1 in the S phase DNA damage checkpoint control, which could represent a potential mechanism for downregulation of DNA synthesis when DNA is damaged during the early stages of S phase (Painter and Young, 1987).

BRCA1 appears to interact with the RAD50/MRE11/NBS1 complex directly through RAD50 (Zhong *et al.*, 1999). Similar to the formation of RAD50/MRE11/NBS1 complex, the association of BRCA1 with this complex does not change in response to DNA damage. Rather, the nuclear partitioning of this BRCA1-containing complex changes and BRCA1 forms ionizing irradiation-induced foci (IRIF), which is also a characteristic of RAD50, MRE11 and NBS1 (Maser *et al.*, 1997). Upon IR treatment, BRCA1 nuclear dots originally observed in untreated cells are disrupted, and later gradually reassemble into bright foci, which also colocalize with RAD50 foci in the portion of cells that display both RAD50 and BRCA1 foci (Zhong *et al.*, 1999). It has been suggested that RAD50/MRE11 complexes localize to the sites of DSB upon IR (Nelms *et al.*, 1998); therefore, the colocalization of BRCA1 with this complex implies that BRCA1 is relocated to the sites of DSB upon IR. This dynamic redistribution of BRCA1 could reflect an aspect of the cellular response to DNA damage in a manner analogous to the translocation of RAD50 complexes to the sites of DSBs. Consistently, BRCA1 has been found to be important for efficient formation of IRIF (Zhong *et al.*, 1999). Its association with the RAD50/MRE11/NBS1 complex suggests that BRCA1 could participate directly in the RAD50-mediated DNA repair process. Alternatively, BRCA1 could facilitate the repair of DSBs on chromatin templates through its direct recruitment of chromatin remodeling activities (Hu *et al.*, 1999). The precise role that BRCA1 plays in complex with RAD50/MRE11/NBS1, however, remains to be definitively established.

Besides RAD50/MRE11/NBS1, other components involved in DNA damage repair, such as MSH2, MSH6, MLH1, ATM, BLM, have been found to reside in a large BRCA1-containing DNA repair complex (Wang *et al.*, 2000). In addition, DNA replication factor C and PCNA were also found in this complex (Wang *et al.*, 2000). This complex has been proposed to represent a BRCA1-associated genome surveillance complex (BASC) since many of its constituent proteins individually recognize distinctly abnormal DNA structures, such as double-strand breaks, base-pair mismatches, and stalled replication forks. Many of these proteins are involved in replication or repair of damage that can occur at replication forks. Therefore, the association of BRCA1 with these proteins suggests that BRCA1 may also participate in the resolution of aberrant DNA structures that occur during DNA replication or when DNA replication is stalled (Wang *et al.*, 2000). Consistent with this notion is the previous observation that BRCA1 foci at S phase disperse in response to DNA damage or replication blocks, and relocalize to PCNA-containing structures (Scully *et al.*, 1997b), suggestive of a role for BRCA1 in replicational DNA repair.

BRCA1 has also been proposed to associate, through a region encoded by the 3′ end of exon 11, with RAD51 although it is still not clear whether this association is mediated by direct or indirect interaction (Scully *et al.*, 1997c). This association is supported primarily by the observation that BRCA1 foci partially colocalize with RAD51 foci during S phase, and relocalize to PCNA-containing structures in response to UV-treatment or replication block by hydroxyurea (Scully *et al.*, 1997b). BRCA1 has also been observed in IR-induced RAD51 foci; however, such foci are distinct from those comprising BRCA1 and the RAD50/MRE11/NBS1 complex (Zhong *et al.*, 1999). While the RAD50/MRE11/NBS1 complex has been proposed to function in end-processing, an early step in both homologous recombination and non-homologous end-joining based repair of DNA double-strand breaks, RAD51 is involved in strand-exchange, a later step in homologous recombination (reviewed by Baumann and West, 1998). The biological implications underlying the dual participation of BRCA1 in two distinct steps of homology-based recombinational DSB repair remain to be resolved.

*BRCA1 in DNA double-strand break repair* Cumulative evidence is consistent with the direct participation of BRCA1 in DNA DSB repair through both homologous recombination and NHEJ. The develop-

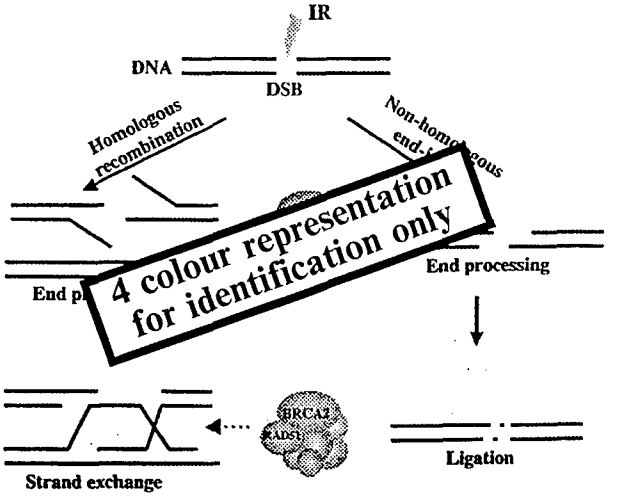


**Figure 3** BRCA1 and BRCA2 participate in DSB repair. BRCA1 forms a complex with RAD50, MRE11, NBS1 and other proteins. This complex is involved in both homologous recombination and non-homologous end-joining processes of DSB repair. BRCA2 forms a complex with RAD51, which is involved in DNA strand exchange during homologous recombination

SAP30, and additional unidentified factors (for review, see Pazin and Kadonaga, 1997; and references therein). Sin3 and nuclear receptor corepressors, N-CoR/SMRT, are also components of the histone deacetylase complex and appear to function in establishing the protein–protein links between DNA-bound repressors and the histone deacetylases. The association of N-CoR/SMRT with HDAC1/2 is mediated by mSin3 (Pazin and Kadonaga, 1997). Besides HDAC1 and HDAC2, which comprise class I histone deacetylases, other HDAC family proteins have been identified, including class I HDAC3, and class II HDAC4, HDAC5, HDAC6 and HDAC7. Recently, N-CoR/SMRT have been found to interact directly with class II histone deacetylases, suggesting that these corepressors can recruit histone deacetylases in a mSIN3-independent manner (for review, see Glass and Rosenfeld, 2000; and references therein). BRAC1 has been shown to associate with at least four components of histone, deacetylase complexes, including HDAC1/2 and RbAp46/RbAp48 (Yarden and Brody, 1999). This observation suggests that BRCA1-mediated transcriptional repression may derive, at least in part, from active recruitment of HDACs.

While the precise mechanism by which BRCA1 mediates gene-specific transcriptional repression remains to be established, current experimental observation is consistent with several alternative possibilities (Figure 2). First, BRCA1 corepression could involve targeted chromatin remodeling. For example, BRCA1 could, by virtue of its direct recruitment of HDAC complexes, alter the chromatin structure of its target genes into a repression-favored status. Alternatively, BRCA1 could affect remodeling via its interaction with the CtIP-CtBP corepressor complex. CtBP itself interacts directly with HPC2, the human homolog of *Drosophila* polycomb (Sewalt *et al.*, 1999). HPC2 is part of a polycomb group (PcG) protein complex that functions in repression of homeotic gene expression during vertebrate development. The PcG proteins have been proposed to confer heritable and stable transcriptional repression by packaging target genes into heterochromatin-like configurations or, alternatively, by relocalizing target genes into heterchromatic compartments (Sewalt *et al.*, 1999). Recently, a histone deacetylase-independent CtBP repression mechanism has been described (Koipally and Georgopoulos, 2000), suggesting the possibility of multiple independent paths to achieve repression through a putative BRCA1-CtIP-CtBP complex. Alternatively, the association of BRCA1 with the RNA polymerase II holoenzyme may evince a distinct mechanism for corepression by which BRCA1 directly targets either the general transcription machinery or interacting coactivators.

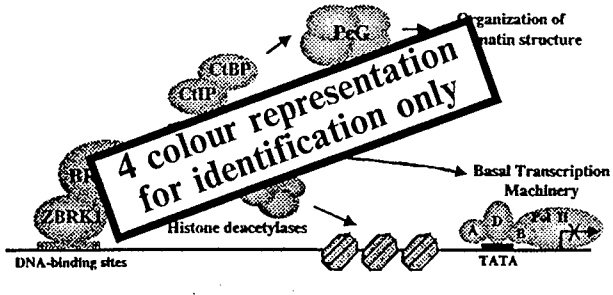


**Figure 2** Sequence-specific transcription repression mediated by BRCA1. ZBRK1 represents the sequence-specific transcription repressor that recruits BRCA1 to the specific DNA-binding sites in its target genes. BRCA1 mediates transcription repression through three potential mechanisms. First, BRCA1 may recruit CtIP/CtBP to mediate reorganization of higher order chromatin structure. Second, BRCA1 may recruit the histone deacetylase complex to mediate local gene silencing. Third, BRCA1 may repress transcription by regulating the basal transcription machinery

*BRCA1 in DNA repair*

BRCA1 appears to participate in the cellular DNA damage response at multiple stages. In normal cells, responses to DNA damage include sensing damaged DNA, transducing DNA damage signals, relocating repair machinery to damage sites, completing a repair process, and coordinating cell cycle progression with the DNA repair process. Accumulating evidence suggests that BRCA1 functions not only in association with the DNA repair machinery, but also in DNA-damage induced cell cycle checkpoint control. Additionally, BRCA1 may regulate the expression of genes involved in DNA damage repair and, significantly, it directly participates in the repair process itself. Finally, phosphorylation of BRCA1 upon DNA damage implies a role for BRCA1 in DNA damage-induced signal relay.

*The interaction between BRCA1 and the DNA repair machinery* BRCA1 has been shown to associate directly with the RAD50/MRE11/NBS1 complex (Zhong *et al.*, 1999). Its equivalent complex in yeast, the Rad50/Mre11/Xrs2 complex, functions in both non-homologous end-joining (NHEJ) and homologous recombinational repair of DNA double-strand breaks. Yeast strains deficient in MRE11, RAD50, or XRS2 exhibit a 50- to 100-fold decrease in NHEJ in the absence of the RAD52-dependent homologous recombination pathway (Ivanov *et al.*, 1992; Johzuka and Ogawa, 1995). A role in facilitating homologous recombination in mitotic cells has also been established for this complex (Bressan *et al.*, 1999). Meanwhile, Mre11, Rad50, and Xrs2 are necessary for introduction of chromosomal double-strand breaks (DSB) that lead to homologous recombination during meiosis in yeast (Ivanov *et al.*, 1992; Johzuka and Ogawa, 1995; Ohta *et al.*, 1998). In addition, they are involved in other cellular processes including chromatin configuration and telomere maintenance (Boulton and Jackson, 1998; Chamankhah *et al.*, 2000; Chamankhah and Xiao, 1999; Gerecke and Zolan, 2000; Nugent *et al.*, 1998; Ohta *et al.*, 1998). It has been proposed that RAD50/MRE11/XRS2 is responsible for end-processing of double-strand breaks (Tsubouchi and Ogawa, 1998). In support for this idea, recombinant MRE11 proteins and purified human RAD50/MRE11/NBS1 complexes exhibit exonuclease and endonuclease activities (Paull and Gellert, 1998; Trujillo *et al.*, 1998). The mechanistic role of RAD50 in this complex is not clear, although it has been shown that the exonuclease activity of MRE11 in complex with RAD50 is moderately increased (Paull and Gellert, 1998). Based on the structural similarity between RAD50 and SMC family proteins, it has been proposed that RAD50 may be a chromatin-associated protein and participate in

within GADD45 through the sequence-specific DNA-binding transcription repressor ZBRK1 (Zheng *et al.*, 2000). This study also reveals that relief of ZBRK1-directed GADD45 repression may be achieved by ectopic overexpression of BRCA1, most likely by altering the balance of repression components specifically recruited to DNA-bound ZBRK1. This unexpected observation raises the possibility that activation of GADD45 transcription in the natural setting may reflect the concerted effects of both derepression and true activation.

While derepression by BRCA1 overexpression may accurately reflect some aspect of its function *in vivo*, more physiologically relevant mechanisms for derepression are likely to involve alterations in the phosphorylation and/or protein interaction status of BRCA1. In this regard, DNA damage-induced dissociation of a CtIP-CtBP corepressor complex from BRCA1 could relieve ZBRK1 repression of *GADD45* transcription, thereby leading to *GADD45* induction (Li *et al.*, 2000) in response to DNA damage-induced signaling. A similar mechanism may also underlie p21 induction.

BRCA1-mediated control of p21 and GADD45 gene transcription may contribute to its role in cell cycle checkpoint control, since p21 and GADD45 have been implicated in DNA-damage induced G1/S and G2/M checkpoint control, respectively (el-Deiry *et al.*, 1993; Deng *et al.*, 1995; Brugarolas *et al.*, 1995; Wang *et al.*, 1999). Consistent with this possibility, GADD45 induction in response to MMS or UV has been shown to be dependent on BRCA1 (Harkin *et al.*, 1999). Thus, mutational inactivation of BRCA1 or its associated factors could lead to alterations in the normal induction profile of GADD45 and a resultant failure of cells to achieve an appropriate G2/M arrest. While GADD45 induction triggered by IR has been shown to require ATM and p53 (Kastan *et al.*, 1992), the involvement of BRCA1 is not clear. However, several recent studies have implicated BRCA1 in the IR pathway possibly leading to GADD45 induction. First, it has been shown that BRCA1 can potentiate transcription from p53-response elements within the GADD45 gene in a p53-dependent manner (Harkin *et al.*, 1999). Second, BRCA1 has been identified as a downstream target of ATM in a DNA damage induced signaling pathway following IR (Cortez *et al.*, 1999). Nonetheless, direct supporting evidence of a regulatory role for BRCA1 in GADD45-mediated G2/M checkpoint control is currently lacking.

Gene expression profiling studies have established a number of genes to be downregulated in response to BRCA1 overexpression, thus identifying potential targets of BRCA1-mediated transcriptional repression. Included among these are Cyclin B1 and PIN1 (MacLachlan *et al.*, 2000). Cyclin B1, the activating subunit of cdc2 kinase (reviewed by Nurse, 1994), and PIN1, a peptidyl-prolyl isomerase (Lu *et al.*, 1996), are both involved in mediating cellular progression into and through mitosis. BRCA1-mediated repression of these genes could be expected to arrest cells at the G2/M cell cycle transition phase, thereby providing an additional potential mechanism through which BRCA1 could achieve G2/M cell cycle checkpoint control (MacLachlan *et al.*, 2000). Precisely how DNA damage-induced signaling might activate BRCA1 to effect transcriptional repression of Cyclin B1 or PIN1, however, remains to be elucidated. Alternatively, it is possible that BRCA1 could regulate G2/M phase traversal during the normal cell cycle through regulation of Cyclin B1 and PIN1.

A recent study documenting BRCA1-mediated repression of estrogen receptor transcriptional activity invokes a potential role of BRCA1 in the estrogen-signaling pathway (Fan *et al.*, 1999). This pathway controls multiple aspects of breast and ovarian cell growth, differentiation and homeostasis. Furthermore, estrogen itself is a distinct etiological factor in breast and ovarian cancer. By affecting hormone response pathways, BRCA1 may regulate growth or a differentiation in a cell-type specific manner.

*Mechanistic basis for BRCA1-mediated transcriptional repression* Insight into the potential mechanism(s) by which BRCA1 mediates the repression of specific target genes has come from protein interaction studies that link BRCA1 to established transcriptional repression activities, including the CtIP/CtBP corepressor complex and histone deacetylases. Human CtBP was initially identified as an adenovirus E1A C-terminal interacting protein capable of attenuating E1A-mediated transcriptional activation and tumorigenesis (Schaeper *et al.*, 1995). A corepressor function for CtBP was subsequently revealed through the interaction of *Drosophila* CtBP with three transcriptional repressors, Knirps, Snail, and Hairy (Nibu *et al.*, 1998; Poortinga *et al.*, 1998). Mammalian homologs of CtBP have also been found to serve as corepressors for a variety of DNA-binding transcriptional repressors (Furusawa *et al.*, 1999; Postigo and Dean, 1999; Turner and Crossley, 1998). Each of these CtBP-interacting transcriptional repressors harbors a conserved amino acid sequence motif, PLDLS, that was originally identified in E1A and that specifies the association of each with CtBP (Sollerbrandt *et al.*, 1996).

The same sequence motif present in ctBP-interacting transcriptional repressors also specifies the interaction of CtBP with CtIP, a protein initially identified by virtue of this interaction (Schaeper *et al.*, 1998). Remarkably, an interaction between CtIP and BRCA1 was simultaneously identified by several groups (Li *et al.*, 1999; Wong *et al.*, 1998; Yu *et al.*, 1998), and CtIP was found to link the corepressor CtBP to BRCA1 (Li *et al.*, 1999). Interestingly, a second tumor suppressor, the retinoblastoma (Rb) protein, as well as one of its associated family members, p130, also interact with CtIP. This observation led to the hypothesis that the transcriptional repression activity of Rb and p130 might be mediated by recruitment of CtBP through CtIP (Meloni *et al.*, 1999). Its specific interaction with two distinct tumor suppressor proteins, BRCA1 and Rb, imply a fundamental role for CtIP in tumor suppression.

Another distinct corepressor complex with which BRCA1 interacts is the histone deacetylase complex (Yarden and Brody, 1999). Initially identified based on its copurification with mSin3, histone deacetylase complex components include two proteins initially identified as Rb-associated proteins, RbAp48 and RbAp46, two histone deacetylases, HDAC1 and HDAC2, two additional polypeptides, SAP18 and

First, BRCA1 could play a role in the recruitment of chromatin remodeling activities. BRCA1 has been demonstrated to interact directly or indirectly with chromatin modifying activities including p300 (Pao *et al.*, 2000), hBRG1 (Neish *et al.*, 1998), and BRCA2 (Chen *et al.*, 1998a) which itself is associated with histone acetyltransferase activity (Fuks *et al.*, 1998; Siddique *et al.*, 1998). In addition, it has recently been demonstrated that the BRCA1 carboxyl-terminal transactivation domain, when targeted to chromatin via a heterologous DNA-binding domain, can alter local chromatin structure (Hu *et al.*, 1999). Significantly, the same cancer-predisposing mutations that abolish its transcriptional activation function also abrogate the ability of this domain to effect chromatin remodeling (Hu *et al.*, 1999), perhaps implicating direct recruitment of chromatin modifying activities as a mechanistic basis for the disruptive influence of this region.

A second step at which BRCA1 is likely to function in transcriptional activation involves RNA polymerase II holoenzyme recruitment. This possibility is supported by several studies that link BRCA1 to the RNA polymerase II holoenzyme through demonstrated interactions with constituent holoenzyme components, including RNA helicase A (RHA) (Anderson *et al.*, 1998; Scully *et al.*, 1997a), CBP/p300 (Pao *et al.*, 2000), and RNA polymerase II itself (Schlegel *et al.*, 2000). The ability of BRCA1 to bind to RNA helicase A has been shown to be essential for the transactivation activity of BRCA1, suggesting a direct functional link between BRCA1 and the holoenzyme. CBP/p300 has been found to interact with BRCA1 *in vitro* and *in vivo*, and to stimulate BRCA1-directed transcription activation (Pao *et al.*, 2000). Recently, BRCA1 has been shown to bind specifically to RNA polymerase II subunits hRPB2 and hRPB10a, but not other polymerase subunits, were found to be capable of blocking activated transcription *in vitro* by the BRCA1 carboxyl-terminal transactivation domain, suggesting that direct interactions between BRCA1 and core RNA polymerase II could potentially mediate BRCA1-dependent transcriptional activation. Taken together, these observations implicate multiple components within the RNA polymerase II holoenzyme for potential contact by BRCA1. Accordingly, a plausible model to account for transcriptional activation by BRCA1 is that gene-specific activators, by virtue of their interaction with BRCA1, recruit the RNA polymerase II holoenzyme onto target promoters in order to effect an increase in the transcription rate of genes under their control.

Yet another mechanism by which BRCA1 could potentially function to stimulate transcription invokes targeting of post-initiation processes, including transcription elongation. The observation that Brca1-deficient ES cells are defective in transcription-coupled repair of oxidative DNA damage (Gowen *et al.*, 1998) suggests a link between BRCA1 and RNA polymerase II actively engaged in transcript synthesis. Future studies will be required to more precisely define the potential regulatory role that BRCA1 plays in steps subsequent to RNA polymerase II transcription initiation.

*BRCA1-mediated transcriptional repression* Somewhat paradoxically, the carboxyl-terminus of BRCA1 that both binds transcriptional coactivators and encodes a potent transactivation domain also mediates the interaction of BRCA1 with transcriptional corepressors including the CtIP/CtBP complex and histone deacetylases (HDACs) (Li *et al.*, 1999; Yarden and Brody, 1999). Interestingly, familial breast cancer-derived mutations that compromise its transactivation activity also abolish the binding of BRCA1 to CtIP and HDACs. These observations have prompted the speculation that BRCA1 may function analogously to nuclear receptors, which function both as activators and repressors depending on their associated cofactors (Pao *et al.*, 2000). These observations also suggest the intriguing possibility that apparent BRCA1-mediated transcriptional induction may derive, at least in part, from derepression by BRCA1-mediated corepressor titration.

BRCA1 has been shown to repress c-Myc-mediated transcriptional activation (Wang *et al.*, 1998) and also to inhibit the transactivation activity of estrogen receptor (Fan *et al.*, 1999). The negative effect of BRCA1 on c-Myc-mediated transactivation could derive from transrepression involving inhibition of either Myc-Max heterodimer formation or DNA binding by Myc-Max heterodimers (Wang *et al.*, 1998). BRCA1-mediated inhibition of estrogen receptor-mediated transactivation could reflect either direct repressive effects at the promoter or, alternatively, indirect repression through disruption of signaling events that activate the estrogen receptor (Fan *et al.*, 1999).

The association of BRCA1 with CtIP/CtBP or HDACs suggests a more direct role for BRCA1 in active repression and, thus provides an alternative explanation for the negative effect of BRCA1 on transcription. A direct role for BRCA1 in transcriptional repression is supported by a recent study showing that BRCA1 can mediate sequence-specific transcriptional repression through its selective recruitment by a novel DNA-binding transcription factor, ZBRK1 (zinc-finger and BRCA1-interacting protein with a KRAB domain). In this study, BRCA1 is shown to mediate ZBRK1-directed repression through a ZBRK1 binding site identified in intron 3 of the GADD45 gene, thus providing a potential mechanistic link between the activities of BRCA1 in gene-specific transcription control, the cellular DNA damage response, and the maintenance of genome integrity (Zheng *et al.*, 2000).

*Biological implications of BRCA1-mediated transcriptional repression* BRCA1 has been reported to repress c-Myc-mediated transcriptional activation from synthetic promoters carrying c-Myc response elements as well as from the natural c-Myc-responsive CDC25A promoter (Wang *et al.*, 1998). Furthermore, this BRCA1-mediated transcriptional repression can be correlated with its inhibition of Myc-mediated cellular transformation, thus providing one potential mechanism for BRCA1-mediated tumor suppression (Wang *et al.*, 1998).

BRCA1-mediated transcriptional repression has also been implicated in silencing the DNA-damage inducible p21 and GADD45 genes in their uninduced states. Support for this model has been provided by a recent study showing that BRCA1 may be physically tethered and functionally linked to a specific regulatory locus

sequence motif termed the BRC repeat. Each BRC repeat, designated BRC1 to BRC8, is approximately 30 amino acids in length. Among the eight BRC repeats within BRCA2, the sequences of six are highly conserved among the repeats and can mediate the interaction of BRCA2 with RAD51. The two remaining repeats, BRC5 and BRC6, are less conserved and do not bind to RAD51 (Chen *et al.*, 1998b; Wong *et al.*, 1997). A third notable region within the BRCA2 primary amino acid sequence, spanning residues 2472 to 2957, represents a region of higher sequence conservation between human and mouse BRCA2 than the coding sequence as a whole. An evolutionarily conserved protein, DSS1 (deleted in split hand/split foot), has been found to interact with BRCA2 in this region, although the significance of this interaction is not clear (Marston *et al.*, 1999).

Protein interaction studies thus provide independent support for a potential role of BRCA1 and BRCA2 in DNA damage repair by revealing their interactions with RAD50 and RAD51, respectively. Correspondingly, a role for BRCA1 and BRCA2 in transcription regulation is supported by both the identification in each of an autonomous transactivation function, and protein interaction profiling that reveals the interaction of each with a variety of transcriptional activators and repressor proteins.

## Molecular basis of BRCA1 and BRCA2 function

### *BRCA1 in transcription regulation*

*BRCA1-mediated transcriptional activation* A role for BRCA1 in transcriptional regulation was initially indicated by the identification of an acidic domain near the carboxyl-terminus of BRCA1 with an inherent transactivation function that is sensitive to cancer-predisposing mutations (Chapman and Verma, 1996; Chen *et al.*, 1996a; Monteiro *et al.*, 1996). When fused to a heterologous DNA-binding domain, a carboxy-terminal fragment of BRCA1 (amino acids 1560–1863) was observed to exhibit strong transcriptional activity in mammalian cells, and this activity was completely abolished by familial breast cancer-derived BRCA1 mutations (Chapman and Verma, 1996; Monteiro *et al.*, 1996). This region, as well as a second, partially overlapping region (amino acids 1142–1643) was also found to confer similar transactivation activity in yeast cells (Chen *et al.*, 1996a). An inherent transactivation function within this region of BRCA1 is further supported by a recent study showing that this region, when expressed recombinantly with a heterologous DNA binding domain, can activate transcription *in vitro* in a highly purified reconstituted transcription system (Haile and Parvin, 1999). The presence of an autonomous transactivation function within BRCA1, coupled with the absence of demonstrable sequence-specific DNA-binding activity, had led to the hypothesis that BRCA1 functions as a co-activator of transcription.

*Biological implications of BRCA1-mediated transcriptional activation* Recent studies utilizing gene expression profiling methodologies have revealed that ectopic overexpression of BRCA1 can induce a diverse array of genes implicated in cell growth control, cell cycle regulation, and DNA replication and repair. Included among these are the genes encoding p21, GADD45, EGR1, PCNA, CDC34, Ku70, K80, and GADD153 (Harkin *et al.*, 1999; MacLachlan *et al.*, 2000). As BRCA1 protein levels increase between mid-S and G2 phases of the cell cycle (Chen *et al.*, 1996c), it is possible that BRCA1 overexpression strategies may simulate the status of BRCA1 during these physiological periods and thereby provide at least a limited window onto the spectrum of target genes under its transcriptional control.

While the transactivation function of BRCA1 is likely to contribute to its role in the regulation of gene expression, it is presently not clear how BRCA1 mediates gene-specific transcription control. Since BRCA1 does not appear to bind specific DNA sequences, it seems likely that it must interact with sequence-specific DNA-binding transcription factors in order to target unique genetic loci. As a sequence-specific DNA-binding transcription factor, the universal tumor suppressor p53 may represent an important link between BRCA1 and gene-specific transcription control. p53 lies at the heart of a cell-signaling pathway that is triggered by genotoxic stresses, including DNA damage. Stress-induced p53-initiated cell cycle arrest and/or apoptosis ensures the timely repair or elimination of potentially deleterious genetic lesions. Significantly, p53 and BRCA1 appear to regulate transcription from an overlapping set of DNA damage-inducible target genes, including p21 and GADD45. This observation initially implied a functional interaction between these two important tumor suppressors, a prediction that has since been borne out experimentally. BRCA1 and p53 have been demonstrated to interact physically and synergize functionally to activate transcription through p53 binding sites located in both the *p21* promoter and *GADD45* intron 3 sequences (Chai *et al.*, 1999; Ouchi *et al.*, 1998; Zhang *et al.*, 1998). The ability of BRCA1 to potentiate p53-dependent transcription absent DNA binding has led to the hypothesis that BRCA1 functions as a p53-specific co-activator, possibly linking the biochemical activities of these two proteins to a common pathway of tumor suppression. Nevertheless, BRCA1 can also regulate promoter activity and induce gene expression in a p53-independent manner (Somasundaram *et al.*, 1997; Harkin *et al.*, 1999). Therefore, additional unidentified DNA-binding transcription factors must function to recruit BRCA1 to specific target genes.

*Mechanistic basis for BRCA1-mediated transcriptional activation* The initiation of RNA polymerase II transcription represents a principal step targeted for regulation within the cell. Gene-specific activators function to stimulate the rate of transcription initiation largely through the recruitment of either chromatin remodeling activities and/or the general transcription machinery in order to override nucleosome-mediated promoter repression and assemble transcription-competent pre-initiation complexes, respectively. While the underlying mechanism by which BRCA1 mediates gene-specific transcriptional activation remains to be established, current experimental observation is consistent with a role for BRCA1 in both of these recruitment steps.

following IR-treatment, suggesting that Brca2 is also required for efficient DNA repair (Connor *et al.*, 1997).

## Analysis of functional domains recognized in BRCA1 and BRCA2

Genetic studies have revealed *BRCA1* and *BRCA2* to be essential for cell growth and survival, critical for an appropriate cellular response to DNA damage, and important etiological factors in the development of cancer. In parallel, functional analyses of their corresponding gene products have been carried out in order to understand how BRCA1 and BRCA2 execute these functions.

The *BRCA1* gene encodes a nuclear phosphoprotein of 1863 amino acids (Chen *et al.*, 1996b,c; Miki *et al.*, 1994) characterized by the presence of two outstanding structural motifs at each of its flanking termini (Figure 1a). At its amino terminus, BRCA1 harbors a structurally conserved RING finger domain (amino acids 24–64). The RING fingers is a zinc-binding motif characterized by a set of spatially conserved cysteine and histidine residues that follow the linear order C3HC4 within the primary amino acid sequence. The RING finger motif of BRCA1 does not appear to represent a DNA-binding domain, but is apparently involved in protein–protein interactions (Saurin *et al.*, 1996). Two proteins, BARD1 and BAP1, have been identified based on their ability to bind to the BRCA1 RING finger domain (Jensen *et al.*, 1998; Wu *et al.*, 1996).

The C-terminal region of BRCA1 was first characterized as a transactivation domain (Chapman and Verma, 1996; Chen *et al.*, 1996a; Monteiro *et al.*, 1996). This region also contains two tandem BRCT (BRCA1 C-terminal) domains (amino acids 1640–1863). An autonomous folding unit defined by conserved clusters of hydrophobic amino acids, the BRCT domain is found in a diverse group of proteins implicated in DNA repair and cell cycle check-point control (Bork *et al.*, 1997; Callebaut and Mornon, 1997; Koonin *et al.*, 1996). While no specific cellular function has been ascribed to the BRCT domain, this motif is likely to represent a protein interaction surface (Saka *et al.*, 1997). The BRCT domain in BRCA1 mediates its interaction with proteins such as RNA helicase A, CtIP, and histone deacetylases (Table 1) (Anderson *et al.*, 1998; Li *et al.*, 1999; Wong *et al.*, 1998; Yarden and Brody, 1999; Yu *et al.*, 1998).

Yet another region in BRCA1, which is encoded by the 5′-region of exon 11 appears to have an emerging role as a functionally relevant protein–protein interaction surface (Chen *et al.*, 1999). Although the structure of this region has not yet been defined, it nonetheless mediates the interaction of BCA1 with many proteins including BRAP2, p53, c-Myc, and RAD50 (Table 1) (Li *et al.*, 1998; Wang *et al.*, 1998; Zhang *et al.*, 1998; Zhong *et al.*, 1999). This region also includes two putative nuclear localization signals, which have been shown to interact with importin α (Chen *et al.*, 1996a).

The BRCA2 gene encodes a nuclear phosphoprotein of 3418 amino acids (Figure 1b) (Bertwistle *et al.*, 1997; Wooster *et al.*, 1995). Sequence analysis has revealed that its exon 3-encoded region shares some sequence similarity with the transactivation domain present in c-Jun, and functional analysis has confirmed the presence of an inherent transactivation function within this region (Milner *et al.*, 1997). A prominent architectural feature resident within the BRCA2 primary amino acid sequence comprises eight tandem copies of a repetitive

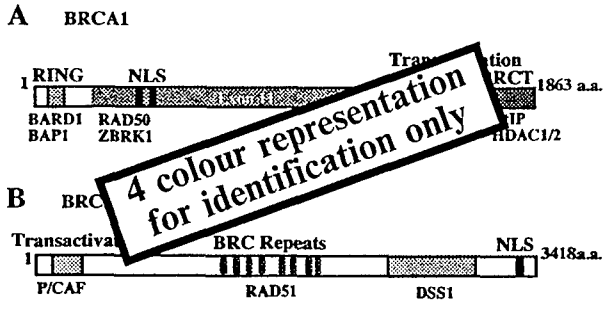


**Figure 1** Structural and functional motifs recognized in BRCA1 and BRCA2. (**a**) Schematic structure of BRCA1. RING domain, transactivation domain, BRCT domain, nuclear localization signals (NLS), as well as the exon 11-coding regions are indicated. Representative proteins that interact with BRCA1 in three different regions are indicated. (**b**) Schematic structure of BRCA2. Transactivation domain, BRC repeats, and NLS are indicated. Representative BRCA2-interacting proteins are indicated

**Table 1** Putative BRCA1-interacting proteins

| *BRCA1-interacting protein* | *Function* | *Interaction domain* | *Reference* |
|---|---|---|---|
| BARD1 | Interacting protein of polyadenylation factor CstF-50 | N-terminal RING domain | Wu *et al.* |
| BAP1 | De-ubiquinating enzyme | N-terminal RING domain | Jensen *et al.* |
| RAD50 | DSB repair protein | Exon 11 5′-coding region | Zhong *et al.* |
| ZBRK1 | Sequence-specific transcription repressor | Exon 11 5′-coding region | Zheng *et al.* |
| BRAP2 | Cytoplasmic retention protein | Exon 11 5′-coding region | Li *et al.* |
| p53 | Transcription factor tumor suppressor | Exon 11 5′-coding region and 2nd BRCT domain | Zhang *et al.*; Ouchi *et al.*; Chai *et al.* |
| c-Myc | Transcription factor, oncogene | Exon 11 5′-coding region | Wang *et al.* |
| Rb | Cell cycle regulator, Tumor suppressor | Exon 11 5′-coding region | Aprelikova *et al.* |
| STAT1 | Signal transducer and activator of transcription | Exon 11 5′-coding region | Ouchi *et al.* |
| Importin α | Nuclear transportation | Nuclear localization signals | Chen *et al.* |
| RAD51 | DSB repair protein | Exon 11 3′-coding region | Scully *et al.* |
| RNA Helicase A | Component of RNA polymerase II holoenzyme | BRCT domains | Anderson *et al.* |
| CtIP | Transcription co-repressor, CtBP-interacting protein | BRCT domains | Li *et al.*; Yu *et al.*; Wong *et al.* |
| HDAC1/2 | Histone deacetylases | BRCT domains | Yarden and Brody |
| CBP/p300 | Transcription co-activator | BRCT domains | Pao *et al.* |

out of 23 MMTV-Cre or WAP-Cre female mice developed diverse mammary tumors by 10–13 months of age (Xu *et al.*, 1999a). Most of the tumors analysed were found to carry p53 mutations, an observation consistent with previous reports from studies of human BRCA1 familial breast tumors (Crook *et al.*, 1997; Eisinger *et al.*, 1997). These observations imply a link between p53 mutation and Brca1-associated mammary tumor development, a notion further supported by a documented acceleration in both the frequency and age of onset of breast tumor formation accompanying inactivation of one germline copy of p53 in these conditional Brca1 knockout mice (Xu *et al.*, 1999a). Collectively, these observations support a role for BRCA1 as a breast cancer suppressor gene.

Evidence to support a role for BRCA2 as a tumor suppressor includes the observation of tumorigenesis in mice homozygous for a Brca2 truncation mutation at the 3′ end of exon 11 (Connor *et al.*, 1997; Friedman *et al.*, 1998). Inactivating mutations in mitotic checkpoint genes such as Bub1, Mad3L and p53, whose products are pressed into action as a consequence of chromosomal damage, are believed to relieve growth arrest caused by BRCA2 deficiency and precipitate neoplastic transformation (Lee *et al.*, 1999). Nevertheless, a suitable breast cancer model for studying the role of BRCA2 in breast cancer development remains to be established.

Both BRCA1 and BRCA2-deficient cells are characterized by cumulative chromosome abnormalities, including chromosomal breaks, aberrant mitotic exchanges and aneuploidy (Lee *et al.*, 1999; Patel *et al.*, 1998; Xu *et al.*, 1999b). Chromosomal instability has been proposed as the pathogenic basis for mammary tumor formation caused by BRCA1 and BRCA2 deficiency. Paradoxically, chromosomal instability is invariably accompanied by growth arrest or increased cell death, and the early embryonic lethality associated with BRCA1 or BRCA2 deficiency has been attributed to these cellular responses. How then might BRCA1 or BRCA2 mutation lead ultimately to uncontrolled cell growth and tumor formation? One answer to this question may lie in the observation that tumors found in Brca1 and Brca2-deficient mice harbor additional inactivating mutations in p53 and mitotic checkpoint genes (Lee *et al.*, 1999; Xu *et al.*, 1999b). Thus, mutational inactivation of p53, which governs the G1/S cell cycle checkpoint, may circumvent the growth arrest that is normally induced upon DNA damage, and also inhibit p53-mediated apoptosis, thereby permitting the survival of cells with severe chromosomal damage. Consistently, the embryonic lethality associated with brca1-null mutations can be partially rescued by targeted deletion of p53 or p21 (Hakem *et al.*, 1997). On the other hand, inactivation of mitotic checkpoint genes could bypass mitotic arrest and permit aberrant chromosomes to segregate into progeny cells. Hence, the cumulative evidence suggests that the genetic instability arising in Brca1- or Brca2-deficient cells plays a pivotal role in tumorigenesis, leading first to compensatory gene mutations that override chromosomal damage-induced cell cycle arrest and apoptosis and, subsequently, to the accrual of functionally inactivating mutations at genetic loci involved in breast tumorigenesis.

## Analysis of BRCA1- and BRCA2-deficient cells

The establishment of culture cell lines deficient in either BRCA1 or BRCA2 has facilitated studies designed to define and characterize their corresponding biological activities. Cell lines established from clinical tumor specimens include the BRCA1-deficient human breast adenocarcinoma HCC1937 cell line and the BRCA2-deficient human pancreatic carcinoma CAPAN-1 cell line. Brca1 and Brca2-deficient mouse embryos derived from gene targeting events have also served as an invaluable source of Brca1 and Brca2-deficient stem (ES) and fibroblast (MEF) cell lines for fundamental research purposes.

BRCA1-deficient HCC1937 cells, BRCA1-null ES cells, and Brca1-exon 11 deletion MEF cells are all characterized by radiation hypersensitivity. Increased sensitivity to the radiomimetic agent methyl methanesulfonate (MMS) and ionizing radiation (IR), but not to ultraviolet (UV) radiation, has also been observed in BRCA1-deficient cells (Gowen *et al.*, 1998; Scully *et al.*, 1999; Xu *et al.*, 1999b; Zhong *et al.*, 1999). Reintroduction of a wild-type BRCA1 allele, but not clinically validated BRCA1 missense mutant alleles, can complement the MMS and IR sensitivity of BRCA1-deficient cells, suggesting that the cellular response to DNA damage is compromised in breast cancer patients carrying BRCA1 mutations (Scully *et al.*, 1999; Zhong *et al.*, 1999). BRCA1-null ES cells are defective in the repair of both oxidative DNA damage by transcription-coupled processes (Gowen *et al.*, 1998) and chromosomal double-strand breaks by homologous recombination (Moynahan *et al.*, 1999). Defective control of the DNA-damage induced G2/M checkpoint has also been observed in BRCA1-exon 11 deletion MEFs, thereby implicating BRCA1 in cell cycle checkpoint control (Xu *et al.*, 1999b). Improper centrosome duplication is another prominent characteristic of these cells leading to multipolar spindle formation and consequent unequal chromosomal segregation and micronuclei formation (Xu *et al.*, 1999b). Although a function in centrosome duplication is consistent with the reported localization of the BRCA1 protein centrosomes (Hsu and White, 1998), multiple centrosomes could be formed as a consequence of accumulated DNA damage in Brca1-deficient cells, as it has been shown that DNA damage can trigger improper centrosome activity followed by micronuclei formation (Sibon *et al.*, 2000; Su and Vidwans, 2000). Nonetheless, improper centrosome duplication is likely to exacerbate pre-existing genomic instability that has arisen from defects in the surveillance and repair of damaged DNA. Taken together, the phenotypic characteristics of BRCA1-deficient cells suggest that BRCA1 occupies a central role in the cellular DNA damage response by virtue of its dual participation in DNA damage repair and cell cycle checkpoint control.

Hypersensitivity to genotoxic agents including UV, MMS, and IR has been reported to be characteristic of BRCA2-deficient cells, including tumor-derived CAPAN-1 cells, Brca2-deficient mouse blastocysts and MEFs (Chen *et al.*, 1998b; Connor *et al.*, 1997; Patel *et al.*, 1998; Sharan *et al.*, 1997). It has also been observed that the level of unrepaired DNA double-strand breaks is abnormally elevated in Brca2-deficient MEFs

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# Lessons learned from BRCA1 and BRCA2

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**BRCA1 and BRCA2 are breast cancer susceptibility genes. Mutations within BRCA1 and BRCA1 are responsible for most familial breast cancer cases. Targeted deletion of Brca1 or Brca2 in mice has revealed an essential function for their encoded products, BRCA1 and BRCA2, in cell proliferation during embryogenesis. Mouse models established from conditional expression of mutant Brca1 alleles develop mammary gland tumors, providing compelling evidence that BRCA1 functions as a breast cancer suppressor. Human cancer cells and mouse cells deficient in BRCA1 or BRCA2 exhibit radiation hypersensitivity and chromosomal abnormalities, thus revealing a potential role for both BRCA1 and BRCA2 in the maintenance of genetic stability through participation in the cellular response to DNA damage. Functional analyses of the BRCA1 and BRCA2 gene products have established their dual participation in transcription regulation and DNA damage repair. Potential insight into the molecular basis for these functions of BRCA1 and BRCA2 has been provided by studies that implicate these two tumor suppressors in both the maintenance of genetic stability and the regulation of cell growth and differentiation.** *Oncogene* (2000) **19**, 000–000.

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## BRCA1 and BRCA2, two genetic models of breast cancer

Breast cancer is one of the most frequent malignancies affecting women. The cumulative lifetime risk of a female for the development of this disease is about 10% (Claus *et al.*, 1991). For this reason, breast cancer has been the subject of intense study; however, the mechanism underlying breast cancer formation is still largely unknown. In the last decade of the 20th century, two breast cancer susceptibility genes, *BRCA1* and *BRCA2*, which together are responsible for most of the hereditary breast cancer cases, were identified (Hall *et al.*, 1990; Miki *et al.*, 1994; Wooster *et al.*, 1994, 1995). Mutations in *BRCA1* account for almost all of the hereditary breast and ovarian cancer cases and up to 40–50% of families with hereditary breast cancer only (Easton *et al.*, 1993). Mutations in *BRCA2* are linked to the other half of inherited breast cancer families and also to male breast cancer (Wooster *et al.*, 1994, 1995). The identification of familial breast cancer susceptibility genes has provided two human genetic models for studies of breast cancer.

To understand how the loss of BRCA1 or BRCA2 function leads to breast cancer formation, mouse genetic models for *BRCA1* or *BRCA2* mutations have been established. This work has revealed that Brca1 homozygous deletions are lethal at early embryonic days (E)5.5–13.5 (Gowen *et al.*, 1996; Hakem *et al.*, 1996; Liu *et al.*, 1996; Ludwig *et al.*, 1997). Three independent groups generated distinct mutations within brca1, yet nonetheless observed similar embryonic phenotypes, including defects in both gastrulation and cellular proliferation, and death at E6.5 (Hakem *et al.*, 1996; Liu *et al.*, 1996; Ludwig *et al.*, 1997). A fourth group that generated a distinct brca1 mutation observed embryos that survived until E13.5 and exhibited defects in neural development, including anencephaly and spina bifida to varying degrees (Gowen *et al.*, 1996). A fifth group generated a mouse model with a targeted deletion of Brca1 exon 11. The resultant mutant embryos expressed an exon 11-deletion variant of Brca1 and died at E12–18.5 (Xu *et al.*, 1999b). Collectively, these findings imply a role for the Brca1 gene product in growth and/or differentiation during mouse embryogenesis.

Similarly, three separate groups have demonstrated that mice homozygous for a Brca2 truncation mutation at the 5′ end of exon 11 die at E8.5–9.5 of gestation (Ludwig *et al.*, 1997; Sharan *et al.*, 1997; Suzuki *et al.*, 1997). Prior to growth arrest, the mutant embryos appear to have been differentiating and to be forming mesoderm, suggesting that the influence of Brca2 during mouse embryogenesis is manifest more on proliferation than differentiation. Mice homozygous for a Brca2 truncation mutation at the 3′ end of exon 11 also exhibit progressive proliferative impairment and die prenatally or perinatally; those animals that do survive to adulthood, however, develop lethal thymic lymphomas (Connor *et al.*, 1997; Friedman *et al.*, 1998).

Although these mouse models have revealed a fundamental role for BRCA1 and BRCA2 in embryogenesis, mice carrying heterozygous Brca1 and Brca2 mutations develop normally and are no more susceptible to tumors than their normal littermates. The lack of tumor formation in mice heterozygous for Brca1 or Brca2 has rendered it difficult to study the pathogenesis of breast cancer. However, the recent establishment of a conditional knockout animal model has provided a useful system with which to study early events in breast tumor formation (Deng and Scott, 2000).

By exploiting the mammary epithelium-specific expression of a MMTV-Cre or WAP-Cre transgene to induce a Cre-LoxP mediated deletion of brca1 exon 11 in mammary epithelium, it was reported that five

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assembly (60). Therefore, it is, possible that the interaction between Hec1p and SMC proteins is involved in the process of centromeric chromatin assembly and modulation of kinetochore function.

Hec1p may play multiple roles in M-phase progression since it has been shown to associate with other proteins such as the human homologs of Sug1p/Cim3p, Cim5p, and NIMA. It has been shown that Sug1p/Cim3p, Cim5p, and NIMA are important M-phase players because mutants of these proteins lead to $G_2$/M arrest (16, 46). Interestingly, the interactions between Hec1 proteins and their associated proteins appear to have been conserved (Table 3). These interactions are likely to be common modes for regulation of M-phase progression in all eukaryotes.

Although hsHec1p may serve as a regulator of multiple mitotic pathways, it is itself regulated by higher-level modulators. The retinoblastoma protein Rb, as an hsHec1p-associated protein, is likely to be one of these modulators. hsHec1p is not the first protein linking Rb to M-phase progression. The association of Rb with mitosin/CENP-F (62), H-nuc/CDC27 (7), and protein phosphatase 1α (13) has provided circumstantial evidence that Rb has an important role in M-phase progression. The higher-level regulatory function that we propose for Rb is less conserved in lower eukaryotes. First, no gene with sequence similarity to Rb exists in the entire *S. cerevisiae* genome. Second, the specific I-C-E motifs found in hsHec1p sequences could well serve as Rb-binding domains (6). The protein sequence of either the budding or fission yeast homolog, however, contains no I-C-E motif, consistent with the lack of interaction between budding yeast Hec1p and human Rb in the yeast two-hybrid system (Table 3). The lack of Rb in yeast will allow us to address Rb function by using yeast machinery as a powerful assay tool, without interference from endogenous Rb. The strain in which the sc*HEC1* gene has been replaced by the hs*HEC1* gene will be available for future studies of the in vivo interaction between Rb and hsHec1p and the biological consequences of such an interaction.

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The first two authors contributed equally to this report.

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# Retinoblastoma Protein Enhances the Fidelity of Chromosome Segregation Mediated by hsHec1p

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**Retinoblastoma protein (Rb) plays important roles in cell cycle progression and cellular differentiation. It may also participate in M phase events, although heretofore only circumstantial evidence has suggested such involvement. Here we show that Rb interacts, through an IxCxE motif and specifically during $G_2$/M phase, with hsHec1p, a protein essential for proper chromosome segregation. The interaction between Rb and hsHec1p was reconstituted in a yeast strain in which human hs*HEC1* rescues the null mutation of sc*HEC1*. Expression of Rb reduced chromosome segregation errors fivefold in yeast cells sustained by a temperature-sensitive (ts) hs*hec1-113* allele and enhanced the ability of wild-type hsHec1p to suppress lethality caused by a ts *smc1* mutation. The interaction between Hec1p and Smc1p was important for the specific DNA-binding activity of Smc1p. Expression of Rb restored part of the inactivated function of hshec1-113p and thereby increased the DNA-binding activity of Smc1p. Rb thus increased the fidelity of chromosome segregation mediated by hsHec1p in a heterologous yeast system.**

Genetic instability is one of the most important hallmarks of cancer. It occurs at two different levels. On one level, increased mutation rates result from defective repair of damaged DNA or replication errors, which leads to missense, nonsense, or other small but functionally important mutations in several types of cancer. On another level, improper segregation of whole chromosomes or pieces of chromosomes during mitosis leads to aneuploidy or translocations, traits commonly observed in cancers (35). Chromosome segregation is controlled by a large group of proteins that together coordinate M phase progression (43, 44, 48, 58). Loss of function of key proteins important for the structure and dynamics of mitotic chromosomes would be expected to lead to cell death and thus to prevent passage of mutations of such fundamental proteins to daughter cells. Loss of function of proteins that play subtler regulatory roles in mitosis, however, may not be immediately lethal but instead may lead to high frequencies of chromosome abnormalities and to neoplasia.

Associations of oncoproteins or tumor suppressors with the process of chromosome segregation provide possible links between carcinogenesis and chromosomal instability. Recent studies suggest that both p53 and retinoblastoma protein (Rb) play important roles in the prevention of aneuploidy in human and rodent cells (12, 28, 34, 56). When treated with microtubule-destabilizing agents, cells lacking functional Rb or p53 do not finish mitosis properly but nonetheless enter a new cell cycle, leading to hyperploidy (28, 34). p53 has been found to be associated with centrosome duplication activity (15) and mitotic or postmitotic checkpoint control (18, 34), loss of these functions would result in aberrant mitosis and contribute to the observed increase in ploidy. Similarly, the propensity of Rb-deficient cells to become hyperploid is most likely due to the loss of a novel function of Rb in M phase of the cell cycle, although supportive evidence remains scarce.

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Study of the function of Rb has been centered on the progression of $G_1$ phase (17, 23, 52). However, accumulating evidence has suggested potential functional roles for Rb during other phases of the cell cycle (27, 31, 46), especially during M phase. First, the functional, hypophosphorylated form of Rb is present at this phase of the cell cycle (8, 38). Second, hypophosphorylated Rb is associated with at least three cellular proteins that have crucial functions in M phase progression (50). One example is the human H-nuc2 (also called hCDC27) protein (9), a subunit of the anaphase-promoting complex that controls the onset of sister chromatid separation and metaphase-anaphase transition by degradation of specific substrates (30, 32). Another Rb-associated protein, protein phosphatase 1α catalytic subunit (13), is important for kinetochore function, chromosome segregation, and M phase progression, as demonstrated by the abnormal phenotype resulting from the mutational inactivation of its yeast homolog (2, 3, 49, 51). Lastly, mitosin (also called CENP-F), a kinetochore protein (60), also interacts specifically with Rb during M phase.

However, the mechanisms by which Rb plays a role in chromosome segregation and M phase progression remain elusive. The current approach of counting total chromosome numbers by karyotyping or detecting a specific chromosome by fluorescent in situ hybridization can only display the status of chromosome instability, which may not necessarily be a direct consequence of a certain gene defect in mammalian cells. To determine whether the loss of a gene function is responsible for improper chromosome segregation, a method for monitoring the dynamic transmission of a specific chromosome marker is required. Any attempt to select for mammalian cells carrying an integrated exogenous chromosome marker, however, bears the risk of immortalizing a primary cell line or making the genetic content of a tumor cell line even more unpredictable. Studies of chromosome segregation in mammalian cells are therefore complicated. On the other hand, methods for monitoring the dynamic transmission of chromosomes in yeast cells are feasible, and the genetic manipulation of a given gene in yeast can be accomplished without affecting the rest of the gene population and chromosome structures. Moreover, the

basic machinery for chromosome segregation is conserved between mammals and yeast (42).

In this study, a yeast assay system for investigating the role of Rb in chromosome segregation was established, based on the study of hsHec1p. hsHec1p, isolated from a screen for proteins interacting with Rb (10, 13), is a coiled-coil protein crucial for proper mitosis (10, 11, 59). Inactivation of hsHec1p leads to disruption of M phase progression (10). The homolog of hsHec1p in *Saccharomyces cerevisiae*, scHec1p (also called Ndc80 or Tid3), has a similar essential function (57, 59), and hs*HEC1* is able to rescue the lethality caused by the null mutation of sc*HEC1* (59). Yeast cells carrying a mutant allele of human or yeast *HEC1* segregate their chromosomes aberrantly (57, 59). At the nonpermissive temperature, significant mitotic delay, unequal nuclear division, and decreased viability were observed in yeast cells carrying hs*hec1-113*, a temperature-sensitive mutant allele of human *HEC1* (59). Increased frequencies of chromosome segregation errors were also detected in the hs*hec1-113* mutant at permissive temperatures. Hec1p has been found to interact physically or genetically with a number of proteins important for $G_2$/M progression and chromosome segregation, including SMC (structural maintenance of chromosomes) proteins and yeast centromere protein Ctf19p (10, 26, 59). A potential role for Hec1p in modulating chromosome segregation in part through interactions with SMC proteins has been suggested (59). There is no protein with sequence similarity to Rb in the entire *S. cerevisiae* genome. Without interference from endogenous Rb, yeast strains in which the null mutation of sc*HEC1* has been complemented by hs*HEC1* (59) therefore provide useful tools to address the consequence of the interaction between Rb and hsHec1p for chromosome segregation.

The biological significance of the interaction between Rb and hsHec1p is demonstrated here by reconstitution of these proteins in a heterologous in vivo yeast system. Expression of Rb resulted in a decrease in the rate of chromosome segregation errors in cells carrying a mutant form of hsHec1p and an increase in the survival rate of *smc1* mutant cells with defects in chromosome segregation. These results suggest that Rb plays a positive regulatory role in chromosome segregation.

## MATERIALS AND METHODS

**Strains and plasmids.** *S. cerevisiae* haploid and diploid strains carrying the hs*hec1-113* mutant have been described previously (59). A new yeast strain, 4bWHL273 (*mat*x ade2 lys2 ura3 trp1 smc1-2::LEU2), is one of the meiotic segregates of the diploid strain from the mating between 3bAS273 (a gift from D. Koshland) and YPH1015 (a gift from P. Hieter). The full-length 2.8-kb *RB* cDNA, or the cDNA for the H209 mutant *RB* derivative (Cys706 changed to Phe), was inserted in two sets of plasmids, p415GAL1 (41) and pESC::*TRP1* (Stratagene), by use of *Bam*HI and *Sal*I. The resultant plasmids were used to transform the above-mentioned strains. By a procedure described previously (21), cells were cultured in 2% raffinose overnight at 25°C before Rb expression was induced in medium containing additional 2% galactose for the indicated number of hours. The YEp195-GC15C plasmid was generated by inserting the *GAL1* promoter, hs*HEC1* cDNA (59), and *CYC1* terminator (41) into the YEplac195 vector (16) for the expression of hsHec1p. The YEp195-GEKC plasmid was generated by site-directed mutagenesis for the expression of the hshec1-EK mutant (Glu234 changed to Lys). To express myc-tagged Smc1p, the full-length *SMC1* was generated by PCR, sequenced, and fused with the c-myc tag in the pESC plasmid.

**Immunoprecipitation and immunoblotting.** The preparation of yeast cell lysates, immunoprecipitation, and immunoblotting have been described previously (59). Human hsHec1p, *S. cerevisiae* Smc1p, and human Rb were precipitated or immunoblotted with anti-hsHec1p monoclonal antibody (MAb) 9G3, mouse anti-Smc1p antiserum (59), and anti-Rb MAb 11D7, respectively.

Human bladder carcinoma T24 cells were cultured and synchronized at different stages of the cell cycle as described previously (8, 10). Cells were lysed and immunoprecipitated by procedures described previously (8, 10).

For immunoprecipitation and immunoblotting of human SMC1 (hSMC1) from T24 cells, mouse anti-hSMC1 antiserum was obtained from mice immunized with glutathione *S*-transferase (GST) protein fused with the peptide region of hSMC1 isolated from a yeast two-hybrid screen (10, 11, 59).

**Colony sectoring assays.** Colony sectoring assays were used to measure the frequencies of chromosome missegregation, as described previously (33, 59). Five single pink colonies of each diploid strain that contains a homozygous *ade2-101* ochre color mutation and a dispensable chromosome fragment carrying a copy of *SUP11* were picked and cultured to log phase in histidine-free supplemented minimal medium at 25°C for 3 days. Cells were diluted and incubated at 30°C for 4 h (one generation) in fresh medium supplied with histidine and containing 2% galactose and 2% raffinose to induce the expression of Rb or the H209 mutant. An aliquot of culture was then removed and plated on medium containing 6 mg of adenine per liter. The plates were incubated at 30°C for 6 days and at 4°C overnight before observation. The remaining cultures were used for detecting the expression of Rb or for examining the interaction between Rb and hsHec1p as described above.

**Immunoaffinity purification.** Yeast cell lysate was prepared as described previously (59). Smc1p was partially purified from this lysate with mouse anti-Smc1p polyclonal antibodies by immunoaffinity chromatography, according to modification of a procedure described previously (25, 29). Antibodies were incubated with 50 μl of protein A-Sepharose beads for 2 h at 4°C and washed twice with 1 ml of Tris-buffered saline (50 mM Tris [pH 8.0], 125 mM NaCl). A 1.5-ml portion of cell lysate (about 50 mg of total protein) was added to the antibody–protein A-Sepharose beads and incubated for another 1 h at 4°C. The mixture was then loaded on a minicolumn and washed sequentially with 4 ml of XBE2 buffer (20 mM potassium HEPES [pH 7.7], 0.1 M KCl, 10% glycerol, 2 mM $MgCl_2$, 5 mM EGTA), 0.5 ml of XBE2 with 0.4 M KCl, and 0.5 ml of XBE2. For elution, 150 μl of XBE2 containing a 4-μg/μl concentration of a GST fusion with the C-terminal region of Smc1p (59) was used. Fifty microliters of elution buffer was first allowed to flow in, and then the other 100 μl was loaded. After incubation at 4°C for 4 h, the elution buffer was allowed to flow through and collected. The elution product was incubated with 100 μl of glutathione-Sepharose that was prewashed with XBE2 for 1 h at 4°C three times to completely remove the GST fusion protein.

For multiple samples in the same experiment, equal numbers of yeast cells that contained comparable amounts of total proteins were lysed. The cell lysates were added to the antibody–protein A-Sepharose beads for immunoaffinity purification as described above. The eluted products from each sample were calibrated with comparable protein concentrations for use in gel shift assays. To minimize the effect of any quantitative variations, the amount of Smc1p in each purified product was adjusted according to the immunoblotting results.

**Gel mobility shift assay.** Approximately 2 μl of the purified product described above was incubated with the 230-bp M13 replicative-form (RF) DNA fragment in 20 μl of XBE2 buffer with 0.5 mg of bovine serum albumin per ml. The 230-bp M13 RF DNA fragment was digested from the same region of M13 genomic DNA described previously (1), although *Hin*dIII was used instead of *Eco*RI. The DNA fragment was end labeled with [$\alpha$-$^{32}$P]dCTP by the fill-in reaction with Klenow enzymes. DNA fragments labeled with 10,000 to 20,000 cpm were used as substrates. For competition, unlabeled 230-bp M13 fragment, a 220-bp pUC19 fragment digested with *Ava*II from a pUC19-derived vector (1), and a 240-bp CEN3 fragment (bp, 113925 to 114168) generated by PCR amplification from yeast genomic DNA with the primers described previously (40) were used. The DNA-protein reaction mixtures were loaded on a 5.5% acrylamide gel and run at 4°C in a buffer containing 20 mM HEPES [pH 7.5] and 0.1 mM EDTA. The results were quantified using a densitometer and ImageQuant v1.1 (Molecular Dynamics).

## RESULTS

**hsHec1p specifically interacts with Rb through the IxCxE motif.** hsHec1p was originally identified using Rb as the bait in a yeast two-hybrid screen (10, 13). In order to determine the specific region of Rb required for binding hsHec1p, a deletion set that had previously been used to delineate the binding domain for protein phosphatase 1α was employed (13). Amino acids 301 to 928 of the Rb protein and several carboxy-terminal deletion mutants, as well as the H209 point mutant with residue 706 changed from cysteine to phenylalanine, were fused with the yeast Gal4 DNA-binding domain. Full-length hsHec1p protein was fused with the Gal4 transactivation domain. The results showed that Rb uses the same T-antigen-binding domain to interact with hsHec1p, and the H209 point mutation abolished this binding (Fig. 1A). hsHec1p sequences required for binding Rb were also determined in a reciprocal manner, using a series of hsHec1p deletion mutants. These mutants showed that the central region of hsHec1p, from amino acids 128 to 251, binds to Rb (Fig. 1B). Two Rb-related

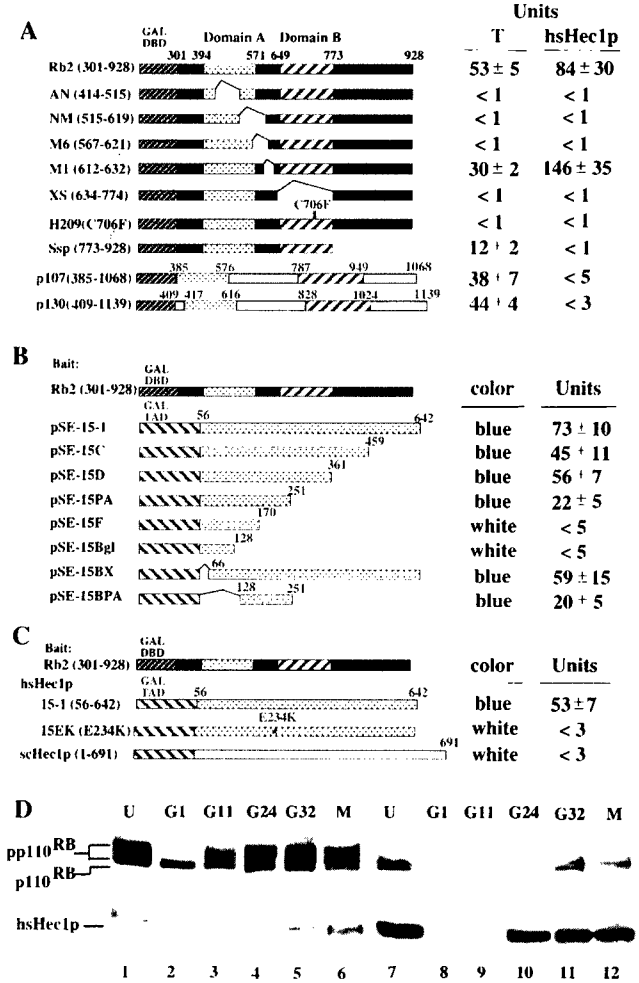


FIG. 1. Specific interaction between Rb and hsHec1p. (A) hsHec1p and T antigen bind to similar regions of the Rb protein. The Gal4 DNA-binding domain (DBD) (amino acids 1 to 147; stippled box) was fused to various Rb mutants, p107 (amino acids 385 to 1068), or p130 (amino acids 409 to 1139). The simian virus 40 T-antigen-binding domains A and B are shown as shaded and hatched boxes, respectively. hsHec1p or T antigen (13) was expressed as a Gal4 transactivation domain fusion protein and used to test for interaction with Rb fusion proteins in yeast two-hybrid assays. Transformants were grown in liquid cultures and used for *o*-nitrophenyl-β-D-galactopyranoside quantitation of β-galactosidase activity as described previously (13). (B) Various hsHec1p mutants were fused with the Gal4 transactivation domain (TAD) (hatched box). Rb (amino acids 301 to 928) was expressed as the fusion with the Gal4 DNA-binding domain used for panel A. (C) Rb was expressed as the same fusion used for panel B. Wild-type hsHec1p (15-1), an hsHec1p mutant with amino acid 234 changed from E to K (15EK), and scHec1p were fused with the Gal4 transactivation domain. (D) Cell cycle-dependent interaction between Rb and hsHec1p. T24 cells were density arrested at $G_1$ (lanes 2 and 8) and then released for reentry into the cell cycle. At different time points after release as indicated above the lanes, $5 \times 10^6$ cells were collected, lysed, and immunoprecipitated with anti-Rb MAb 11D7 (lanes 1 to 6) or with anti-hsHec1p MAb 9G3 (lanes 7 to 12). The immune complexes were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by immunoblotting with MAb 11D7 (upper panel) or with 9G3 (lower panel). G11 represents 11 h after release and corresponds to $G_1$, G24 marks 24 h after release and corresponds to S, and G32 marks 32 h after release and corresponds to $G_2$. M phase lysates (lanes 6 and 12) were obtained from cells treated with nocodazole (0.4 μg/ml). Lanes 1 and 7, unsynchronized cells.

proteins, p107 and p130 (14, 20, 36, 39), did not interact with hsHec1p (Fig. 1A).

The region of hsHec1p that binds Rb was not conserved in yeast scHec1p. Thus, it is likely that the interaction between Hec1p and Rb is not conserved in yeast. To test this notion, a yeast two-hybrid assay was performed using the above-described construct, with the Rb sequence fused with the Gal4 DNA-binding domain and a plasmid for the expression of yeast scHec1p sequence fused with the Gal4 transactivation domain. As predicted, yeast scHec1p failed to bind Rb (Fig. 1C).

An examination of the hsHec1p sequence showed that it contains an IxCxE motif, which has been implicated as the specific Rb-binding site in many proteins (reviewed in reference 5). This motif is not found in yeast scHec1p, suggesting that the inability of Rb to bind to scHec1p may be due to the lack of the IxCxE sequence. To verify this possibility, a point mutant with residue 234 changed from glutamic acid to lysine in this motif was tested in a yeast two-hybrid assay. This mutation abolished the ability of hsHec1p to bind to Rb (Fig. 1C). However, hs*hec1-EK*, with this mutation, was able to rescue the yeast sc*hec1* null mutant (data not shown) and generate the strain WHL101EK. This indicated that hsHec1p proteins, with or without an Rb-binding site, are able to perform their essential cellular function in yeast.

**Rb and hsHec1p interact at $G_2$/M phase in mammalian cells.** The interaction between Rb and hsHec1p was also examined by coimmunoprecipitation following cell cycle progression. As shown in Fig. 1D, hsHec1p binds to Rb specifically at $G_2$/M phase in human bladder carcinoma T24 cells, which were synchronized as described previously (8). Similar to most of other Rb-associated proteins, hsHec1p binds specifically to the hypophosphorylated form of Rb that reappears during M phase.

**The specific interaction between Rb and hsHec1p is reconstituted in yeast.** Wild-type Rb and the H209 mutant Rb were expressed under control of the *GAL1* promoter through *LEU2*-selectable plasmids (p415GAL1) in the same yeast strain that carries an hs*hec1-113* mutant allele (59). As a negative control, wild-type Rb was also expressed in a yeast strain carrying the hs*hec1-113EK* allele, which encodes a hshec1-113p without Rb-binding activity. The hs*hec1-113EK* cells demonstrated no apparent difference in the temperature-sensitive (ts) phenotype compared with the hs*hec1-113* cells (59).

Wild-type Rb coimmunoprecipitated with hshec1-113p but not with hshec1-113EK. The H209 mutant of Rb failed to form a complex with hshec1-113p (Fig. 2A, panel b). Consistent with a previous report (21), both hypophosphorylated and hyperphosphorylated forms of Rb were detected in these unsynchronized cells, but the H209 mutant was deficient in hyperphosphorylated forms. The abundance of hshec1-113p protein did not vary significantly when either Rb or the H209 mutant was expressed (Fig. 2A, panel c). These results suggested that the specific interaction between hsHec1p and Rb could be reconstituted in yeast cells.

To explore whether M phase-specific binding exists in yeast cells expressing Rb, the interaction was examined during cell cycle progression. Cells from the strain carrying the hs*hec1-113* allele were induced to express Rb and then synchronized in early S phase by treatment with hydroxyurea. After release from treatment, an equal aliquot of cells was taken out every 20 min (Fig. 2B; lanes 1 to 10). hshec1-113p was coimmunoprecipitated by anti-Rb MAb in cells that entered M phase, according to DNA content analysis (Fig. 2C and D), and morphology was observed under the microscope. Similarly, hshec1-113p and Rb were coimmunoprecipited in cells synchronized at metaphase with nocodazole (Fig. 2B, lanes 11 and 12) but not in cells released from nocodazole treatment for 1 h. These results indicated that the M phase-specific interaction between Rb and hsHec1p can also be reconstituted in yeast cells.

**Rb specifically enhances the fidelity of chromosome segregation.** The reconstitution of the specific interaction between Rb and hsHec1p in yeast cells provided an in vivo system for

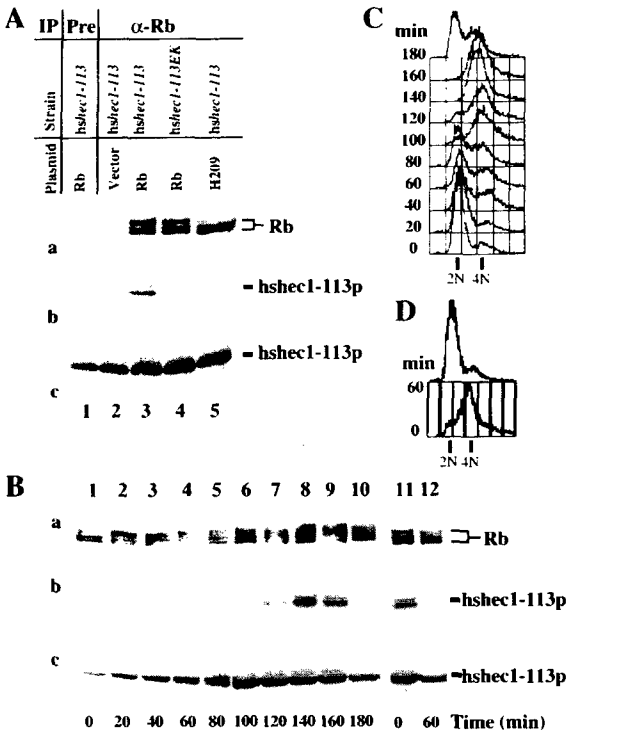


FIG. 2. Reconstitution of the interaction between Rb and hsHec1p in yeast. (A) Specific interaction between hsHec1p and Rb. Yeast cells were diluted to an optical density at 600 nm of 0.75 in fresh medium with 2% galactose and 2% raffinose and then cultured at 30°C for 4 h. Aliquots of cell lysate were immunoprecipitated (IP) with nonspecific IgG (lane 1) or with anti-Rb (α-Rb) MAb 11D7 (lanes 2 to 5) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The same blot was probed with MAb 11D7 for Rb (a) or MAb 9G3 for hshec1-113p (b). Panel c shows the endogenous level of hshec1-113p in the cells used in panels a and b. For each lane in panel c, aliquots of the same lysates used in panel a were immunoprecipitated and immunoblotted with 9G3. The yeast strains and plasmids used to express Rb are indicated for each lane. (B) The yeast cells carrying the hs*hec1-113* allele and an Rb expression vector were treated for 5 h with 0.1 M hydroxyurea or 20 μg of nocodazole per ml in medium containing 2% galactose and 2% raffinose. At different time points after release (indicated under each lane [lanes 1 to 10, release from hydroxyurea; lanes 11 and 12, release from nocodazole]), cells were collected, lysed, and immunoprecipitated with α-Rb MAb 11D7. The immunoprecipitates were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by immunoblotting with 11D7 for Rb (a) or with 9G3 for hshec1-113p (b). Aliquots of the same lysates were immunoprecipitated and immunoblotted with 9G3 (c). hshec1-113p was co-precipitated by Rb specifically at 120 to 160 min after release from hydroxyurea treatment, corresponding to $G_2$/M phase, or metaphase arrest by nocodazole (time zero, lane 11). (C and D) The DNA content of the same cells used for panel B was analyzed by fluorescence-activated cell sorting as described previously (59).

investigation of the consequence of this interaction. If hsHec1p plays a crucial role in maintaining the fidelity of chromosome segregation as described previously (59), we surmised that Rb modulates hsHec1p and enhances this activity. To test this hypothesis, we examined the rate of chromosome missegregation by using the colony sectoring assay (33) after induction of Rb expression in the hs*hec1-113* diploid strain. This strain was chosen because of its higher rate of chromosome segregation errors during mitosis (59). The total numbers of pink colonies (representing 1:1 segregation of a single dispensable chromosome fragment carried by this yeast strain), half-pink, half-red sectored colonies (representing 1:0 segregation), and half-white, half-red sectored colonies (representing 2:0 segregation) were counted. The rates of chromosome loss and nondisjunction in the first division were determined by the

TABLE 1. Rb reduces chromosome segregation errors

| Relevant genotype | Plasmid | No. of colonies[a] | Missegregation rate (%) | |
|---|---|---|---|---|
| | | | 1:0 events[b] | 2:0 events[c] |
| hs*HEC1* | | 6,096 | 0.02 | 0.01 |
| hs*hec1-113* | Vector | 6,687 | 1.47 | 0.56 |
| hs*hec1-113* | Rb | 12,641 | 0.31 | 0.10 |
| hs*hec1-113* | H209 | 4,299 | 1.54 | 0.60 |
| hs*hec1-113EK* | Vector | 5,392 | 1.48 | 0.46 |
| hs*hec1-113EK* | Rb | 6,554 | 1.51 | 0.50 |

[a] Total number of pink colonies.
[b] Number of half-red, half-pink colonies divided by total number of pink colonies.
[c] Number of half-red, half-white colonies divided by total number of pink colonies.

frequencies of half-pink, half-red colonies and half-white, half-red colonies, respectively. As shown in Table 1, Rb expression decreased the frequency of chromosome segregation errors due to chromosome loss or nondisjunction by approximately fivefold. In contrast, expression of the H209 mutant of Rb or the vector alone had no effect. As another control, we examined the influence of Rb expression on the strain carrying the hs*hec-113EK* mutant; it had no significant effect.

The observed difference in the frequencies of chromosome missegregation is not due to the variable cell growth or cell cycle status, because expression of Rb or H209 has no significant effects on these processes in yeast cells carrying either wild-type *HEC1* alleles (21) or the mutant hs*hec1-113* allele (data not shown). Therefore, the fidelity of chromosome segregation is enhanced specifically by interaction between Rb and hsHec1p.

**Rb enhances the ability of hsHec1p to suppress lethality caused by an *smc1* mutation.** hsHec1p plays an essential role in chromosome segregation in part through interacting with SMC1 protein, which, in a complex with SMC3, is involved in sister chromatid cohesion (24, 37, 54). The mutated hec1p fails to interact with Smc1p physically in the hs*hec1-113* mutant cells at the nonpermissive temperature (59). Overexpression of Hec1p suppresses the lethal phenotype of the *smc1-2* mutant strain (1-1bAS172) at 37°C (55, 59). If Rb enhances the activity of mutated hshec1p in the maintenance of proper chromosome segregation, it is likely that Rb also enhances the activity of wild-type hsHec1p in suppression of defective chromosome segregation due to the *smc1* mutation. To test this hypothesis, we employed the yeast strain 2bAS273, which also carries the *smc1-2* mutant allele and has a lethal phenotype at temperatures above 33°C (D. Koshland, personal communication). The isogenic strain 4bWHL273, carrying the same *smc1-2* allele, was generated from 2bAS273 and transformed by plasmids expressing hsHec1p and Rb under control of the *GAL1* promoter. Cells overexpressing hsHec1p grew at 34°C, a nonpermissive temperature for this *smc1* mutant strain, while cells not overexpressing hsHec1p failed to grow, whether Rb was expressed or not (Fig. 3A). Interestingly, if the temperature was raised further to 35 to 36°C, cells overexpressing hsHec1p also failed to grow. Cells overexpressing both hsHec1p and Rb, however, continued to grow, whereas cells expressing the H209 mutant hardly survived at this higher temperature. Overexpression of hshec1-EK suppressed the *smc1-2* mutant at 34°C, but coexpression of Rb and hshec1-EK did not suppress it if the temperature was raised further (Fig. 3B). These results suggested that the specific interaction between Rb and wild-type hsHec1p results in the enhancement of the fidelity of

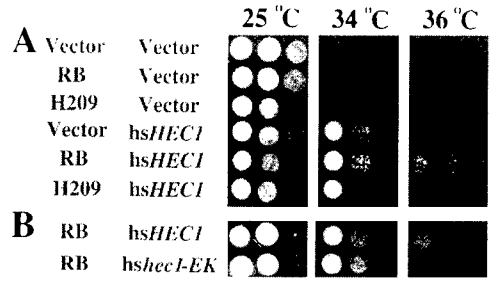


FIG. 3. Rb suppresses the ts phenotype of the *smc1-2* mutant through hsHec1p. (A) 4bWHL273 (*smc1-2*) cells were double transformed by hs*HEC1* in a *GAL1*-inducible and *URA3*-selectable vector (a YEplac195-based vector), by *RB* or the H209 *RB* mutant cDNA in a *GAL1*-inducible and *TRP1*-selectable vector (pESC::*TRP1*), or by the vectors alone. (B) 4bWHL273 cells were double transformed by hs*HEC1* or hs*hec1-EK* in the *GAL1*-inducible and *URA3*-selectable vector and by Rb in the *GAL1*-inducible and *TRP1*-selectable vector. Different dilutions of log-phase cells grown at 25°C were inoculated on three plates with 2% galactose in the same manner and incubated at 25, 34, or 36°C.

chromosome segregation, probably mediated by the interaction between Hec1p and Smc1p.

**Specific binding of Smc1p to highly structured DNA.** SMC1 protein has been suggested to associate preferentially with highly structured DNA regions of chromatin, such as AT-rich DNA, bent DNA, and scaffold-associated regions (1, 22, 24, 29), and to mediate intermolecular cross-linking in sister chromatid cohesion (24). An in vitro binding assay for investigation of SMC1 DNA-binding activity has been established by using a 230-bp M13 RF DNA fragment (bp 6001 to 6231), which has a very high potential to form secondary structures, e.g., stem-loops, and therefore mimics highly structured DNA regions (1). The carboxyl-terminal region of SMC1 protein had been shown to mediate this specific DNA-binding activity (1).

To partially purify the Smc1p protein complex from yeast cell lysates, anti-Smc1p polyclonal antibodies (59) and a single-step immunoaffinity approach (25, 29) were employed. The affinity-bound proteins were eluted by the use of a highly concentrated GST fusion protein that had been used as the antigen to raise the antibodies (59). Excessive GST fusion protein was subsequently removed with glutathione-Sepharose. The affinity-purified fraction (APF) was incubated with the 230-bp M13 RF DNA fragment. Specific DNA-binding activity for the APF was detected by gel mobility shift assays (Fig. 4A, lanes 1 to 3). The abundance of the specific DNA-protein complex increased when more APF was added. Meanwhile, the mobility of the DNA-protein complex decreased and formed a more slowly migrating band (Fig. 4A, lane 3, bar). This stoichiometric effect is consistent with previous observations for the DNA-binding activity of another SMC-containing complex, the 13S condensin in *Xenopus* (29). This DNA-protein complex is not likely to be contaminated by the eluting antigen, which, encompassing the C-terminal DNA-binding region of Smc1p (1), formed a faster-migrating complex with the same DNA substrate (data not shown).

In order to determine the specificity of this DNA-binding activity, we also tested the APF from the *smc1-2* mutant cells cultured at 37°C, with smc1p inactivated (55). Our observation suggested that this mutated protein is unstable and barely detectable in these mutant cells cultured for 6 h at 37°C (Fig. 4B). No Smc1p-containing complex was obtained from these cells using the same purification procedure (Fig. 4C), and therefore, no DNA-binding activity was detected (Fig. 4A, lanes 4 to 6).

To determine whether this Smc1p-associated activity is spe-

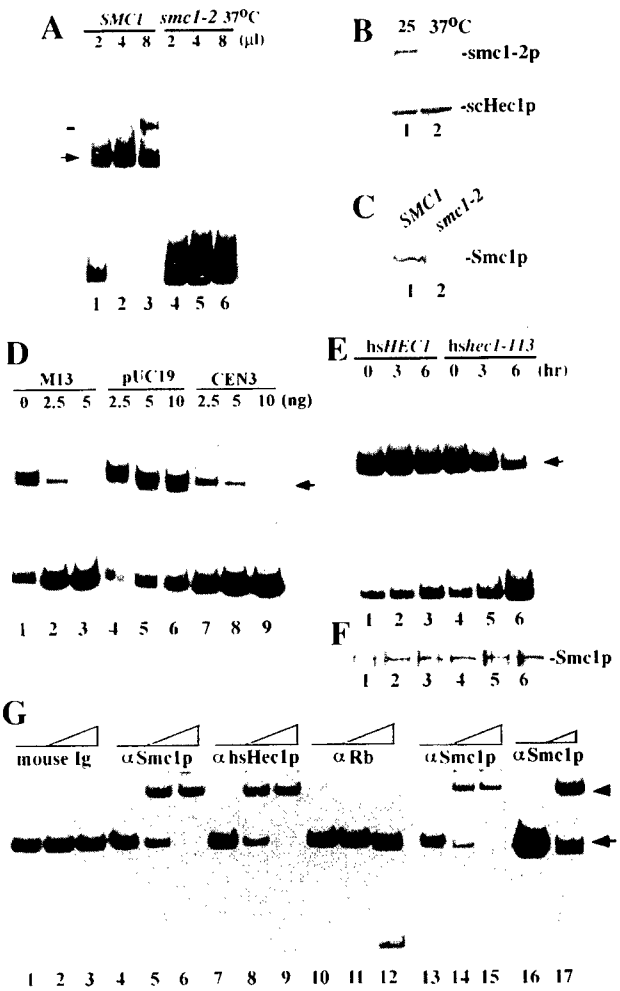


FIG. 4. DNA-binding activity of Smc1p and Hec1p complexes. (A) DNA-binding activity of Smc1p purified from equal numbers of wild-type cells (lanes 1 to 3) and *smc1-2* ts mutant cells (lanes 4 to 6) with a 230-bp M13 RF DNA fragment. The amount of concentrated protein in each lane is shown, and the position of the DNA-protein complex is indicated (arrow). Note that a slower-mobility complex (bar) appeared when more protein was added. (B) Immunoblotting by mouse anti-Smc1p polyclonal antibodies (upper panel) or by anti-scHec1p polyclonal antibodies (lower panel) of lysates from *smc1-2* ts mutant cells cultured at 25°C (lane 1) or 37°C (lane 2) for 6 h. (C) Immunoblotting of Smc1p purified from wild-type (lane 1) or *smc1-2* mutant (lane 2) cells cultured at 37°C for 6 h. (D) Competition of DNA-binding activity by unlabeled DNA fragments. The amount of competitor DNA added in each reaction is indicated above each lane. (E) DNA-binding activity of Smc1p purified from wild-type hs*HEC1* cells (lanes 1 to 3) or from the hs*hec1-113* mutant cells (lanes 4 to 6) with the 230-bp M13 fragment. Cells were cultured at 25°C until log-phase growth and then shifted to 37°C for 0, 3, and 6 h before harvest. (F) Comparable amounts of Smc1p in each of the APFs were measured by immunoblotting with anti-Smc1p antibodies and were used for panel E. (G) Antibody supershift assay. Anti-Smc1p (αSmc1p) antibodies and anti-hsHec1p MAb 9G3 supershifted the DNA-protein complex formed by APF from hshec1-113 cells expressing Rb (lanes 1 to 12), but mouse IgG or anti-Rb MAb 11D7 did not. Anti-Smc1p also supershifted the DNA-binding complex formed by APF from hshec1-113 cells not expressing Rb (lanes 13 to 15) and by APF from the wild-type hsHEC1 cells (lanes 16 and 17). Lanes 1, 4, 7, 10, 13, and 16, no antibodies; lanes 2 and 3, 0.5 and 1 μg of mouse IgG, respectively, lanes 5 and 6, 0.5 and 1 μg anti-Smc1p antibody, respectively; lanes 8 and 9, 0.5 and 1 μg of 9G3, respectively; lanes 11 and 12, 0.5 and 1 μg of 11D7, respectively; lanes 14 and 15, 0.5 and 1 μg of anti-Smc1p antibody, respectively; lane 17, 0.5 μg of anti-Smc1p antibody. The original shift is indicated by an arrow, and the antibody supershift is indicated by an arrowhead.

cific to the highly structured DNA, the DNA-binding activity was competed by unlabeled DNA fragments containing the scaffold-associated region of *S. cerevisiae* CEN3. This centromere region was suggested to be a preferential binding site of SMC proteins and was able to compete with the M13 fragment in the in vitro DNA-binding assay of recombinant SMC1 (1). As shown in Fig. 4D, the Smc1p-associated DNA-binding activity that we detected in the yeast cells can also be competed by unlabeled CEN3 DNA and M13 DNA fragment but not by the region on pUC19 DNA with the least potential to form the secondary structures (1).

We also used a similar procedure to partially purify myc-tagged Smc1p from yeast cells overexpressing myc-Smc1p by use of anti-c-myc MAb and elution with the corresponding peptide. myc-Smc1p has the same DNA-binding activity (data not shown).

**Hec1p modulates specific DNA-binding activity of Smc1p.** To test whether a deficiency in Hec1p activity affects the function of Smc1p, we examined the activity of Smc1p in the hs*hec1-113* mutant yeast cells and compared it with that in cells expressing wild-type hsHec1p. Cells expressing wild-type hsHec1p or mutant hshec1-113p were cultured at the permissive temperature (25°C) and then shifted to 37°C for different periods of time. Equal numbers of cells were harvested and lysed. The resultant cell lysates from different samples contained comparable amounts of total proteins and were subjected to affinity purification of Smc1p. The DNA-binding activity of Smc1p in the wild-type cells did not change significantly after the cells were shifted to 37°C. In the hs*hec1-113* mutant cells, however, this Smc1p activity dramatically decreased, and only less than 20% remained after 6 h at 37°C (Fig. 4E). The amount of Smc1p expressed in the hshec1-113 cells was comparable to that in the wild-type cells (Fig. 4F), suggesting that the functional defect resulted specifically because of the mutated hec1p. The mobilities of the DNA-binding complexes from the wild-type cells and the mutant hs*hec1-113* cells were very similar; only if gel electrophoresis was prolonged more than usual could they be distinguished (data not shown). It is therefore likely that Hec1p is present in the DNA-binding complex. As shown in Fig. 4G, anti-Hec1p antibodies and anti-Smc1p antibodies, but not anti-Rb antibodies or mouse immunoglobulin G (IgG), were able to supershift the DNA-binding complex. These results suggest that Hec1p is present in the complex with Smc1p to mediate the DNA-binding activity. Similar results were observed with APF from yeast cells expressing the wild-type Hec1p or cells not expressing Rb (Fig. 4G, lanes 13 to 17).

**Rb, through hsHec1p, enhances the DNA-binding activity of Smc1p.** If Rb enhances the fidelity of chromosome segregation, which may be mediated by the DNA-binding activity of Smc1p through hsHec1p, this Smc1p activity should increase in the cells expressing Rb. To test this hypothesis, we examined the DNA-binding activity of Smc1p in the same Rb-reconstituted yeast strains that had been tested for frequencies of chromosome missegregation. As in the colony sectoring assay, the cells were cultured at 30°C for 8 h while either Rb or the H209 mutant was induced. In the hs*hec1-113* cells carrying only the empty vector, the DNA-binding activity of Smc1p decreased dramatically, to 30 to 40% of the wild-type level (Fig. 5A and C). These results are consistent with the abnormally high frequencies of chromosome missegregation in the same cells at the permissive temperature (Table 1). In cells expressing wild-type Rb, however, Smc1p DNA-binding activity was restored nearly to normal levels. Expression of the H209 mutant had no such effect, nor did wild-type Rb expression alone affect cells carrying the hs*hec1-113EK* allele, which encodes a protein that cannot bind to Rb.

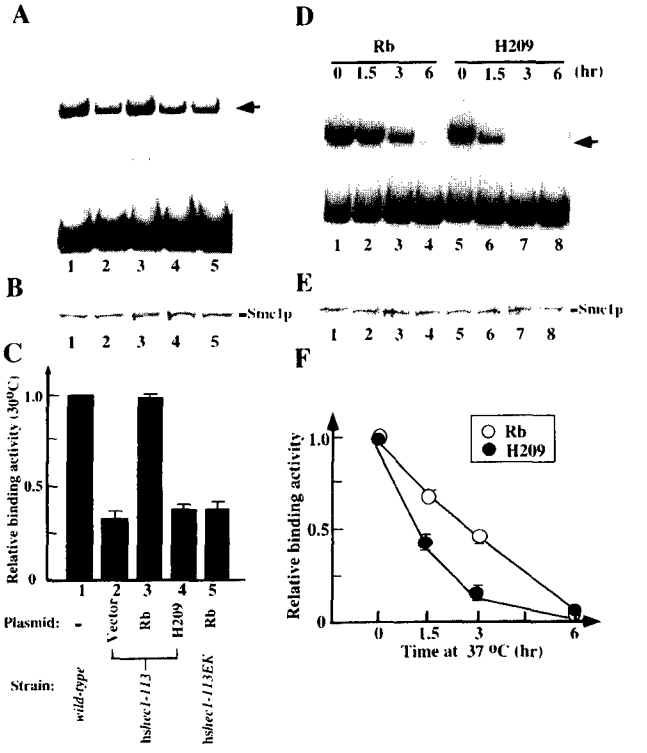


FIG. 5. Rb enhances the DNA-binding activity of Smc1p through hsHec1p. (A) DNA-binding ability of Smc1p purified from equal numbers of cells expressing various forms of Rb and hsHec1p. Expression of Rb and the H209 mutant was induced by addition of 2% galactose to the medium, and cells were cultured at 30°C in this medium for 8 h before harvest. (B) Immunoblot showing that comparable amounts of Smc1p were detected in each of the affinity-purified products used for panel A. (C) Histogram showing relative binding activity of Smc1p in each lane of panel A. (D) DNA-binding ability of Smc1p purified from *hshec1-113* cells expressing Rb (lanes 1 to 4) or the H209 mutant (lanes 5 to 8). Cells were cultured at 25°C in medium containing 2% galactose for 8 h to induce the expression of wild-type Rb or the H209 mutant and then shifted to 37°C for 0, 1.5, 3, or 6 h before harvest. (E) Immunoblot showing comparable amounts of Smc1p in each of the APFs used for panel D. (F) Effect of Rb on the DNA-binding activity of Smc1p in hs*hec1-113* cells. The relative DNA-binding activity of Smc1p indicates the ratio between the quantified density result of each lane in panel D and that of lane 1 for Rb or lane 5 for H209. Bars represent standard errors from three separate experiments.

To further corroborate this finding, the dynamic effect of Rb on the activity of hsHec1p was examined. hs*hec1-113* cells were cultured at 25°C in medium containing 2% galactose to induce the expression of Rb or the H209 mutant and then shifted to 37°C for different periods of time. As in the previous experiment (Fig. 4E), the DNA-binding activity of Smc1p began to decrease after cells were shifted to 37°C (Fig. 5D and F). This decrease of Smc1p activity, however, was significantly retarded during the first 3 h at 37°C in the cells expressing Rb compared with the cells expressing H209 mutant (Fig. 5F). By 6 h, Smc1p activity in both strains was very low. This result suggested that Rb can restore much of the activity impaired by mutation of hshec1p but cannot by itself complement the complete loss of hshec1p. Interestingly, Rb was not found in the Smc1p DNA-binding complex, since anti-Rb antibodies were not able to supershift the complex formed by the APF from hs*hec1-113* cells expressing wild-type Rb (Fig. 4G). Rb thus appears to function like a chaperone, consistent with a previous proposal (6).

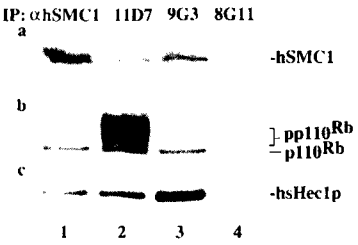


FIG. 6. Rb, hsHec1p, and hSMC1 protein form a complex in human cells. Asynchronous fast-growing human T24 cells ($6 \times 10^6$) were lysed and immunoprecipitated (IP) by mouse anti-hSMC1 (αhSMC1) antiserum (lane 1), anti-Rb MAb 11D7 (lane 2), anti-hsHec1p MAb 9G3 (lane 3), and anti-GST MAb 8G11 (lane 4). The immunocomplexes were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by immunoblotting with anti-hSMC1 antiserum to detect human SMC1 (a), with 11D7 to detect Rb (b), and with 9G3 to detect hsHec1p (c).

The above results suggest that a potential role of Rb in the modulation of SMC1 through hsHec1p exists and that both Hec1p and SMC1 proteins are functionally conserved from yeast to humans (24, 54, 59). It is therefore likely that Rb, Hec1p, and SMC1 may form a single complex in mammalian cells. To test this notion, hsHec1p and human SMC1 protein were coimmunoprecipitated with each other in human T24 cells (Fig. 6), consistent with our previous observation showing that the Hec1p-SMC1 interaction is conserved (59). Interestingly, the hypophosphorylated form of Rb was also coimmunoprecipitated. As a control, anti-GST MAb 8G11 did not coimmunoprecipitate any of these proteins (Fig. 6). These results suggest that Rb is present in a complex with Hec1p and SMC1 and support a potential role for Rb in modulating the activity of SMC1.

## DISCUSSION

In this study, we have employed a yeast system to address the function of Rb in chromosome segregation. Expression of Rb reduced chromosome segregation errors in cells carrying a mutant form of hsHec1p and enhanced the survival rate of *smc1* mutant cells with defects in chromosome segregation. Complexes of Hec1p and Smc1p play essential roles in chromosome segregation. Rb appears to chaperone Hec1p and indirectly to enhance the DNA-binding activity of Smc1p. These results reveal a novel biological activity of Rb intimately linked to its role in carcinogenesis and cancer progression.

The lack of an Rb homolog in yeast allowed us to address Rb function using yeast machinery as a powerful assay tool, without interference from endogenous Rb. Mechanisms similar to those governing Rb phosphorylation in mammalian cells have been demonstrated in yeast (21). However, no significant differences in cell morphology, growth rate, cell cycle progression, or mating pheromone response were observed in yeast cells expressing human wild-type or mutant Rb. These results suggest that Rb does not exert a function when yeast lacks specific cellular mediators of the antiproliferation and differentiation functions of Rb during $G_1$ phase. Alternatively, potential mediators of such functions in yeast are unable to interact with Rb; such is the case with yeast Hec1p, which has no Rb-binding motif. In either case, the lack of both Rb and mediators of Rb function in yeast made it possible to exploit the yeast cell as an assay system for chromosome segregation and to reconstitute the interaction between hsHec1p and Rb in this system. This assay system ensures that the observed phenomena are direct and specific consequences of Rb expression and are specifically mediated by hsHec1p.

Expression of Rb decreased the frequency of chromosome segregation errors fivefold but was insufficient alone to rescue the yeast cells completely from aberrant mitosis. The fivefold enhancement is likely reminiscent of the physiological effect from a high-level regulator on the basic machinery for chromosome segregation. This improvement in the fidelity of chromosome segregation, however, would be quite significant in higher organisms, considering the millions of cells undergoing mitosis or meiosis daily. In the case of a lack of functional Rb, chromosome segregation errors in mammalian cells are expected to occur at a frequency similar to that for the wild-type yeast. Apparently, a higher fidelity of chromosome segregation is required for higher organisms to avoid errors in the more complicated chromosome segregation.

The biochemical mechanisms by which Rb modulates the activity of Hec1p and by which Hec1p modulates the activity of Smc1p remain to be elucidated. Our studies of DNA-binding activity of Smc1p from cells with different genetic backgrounds have provided some important clues leading to the understanding of these biochemical mechanisms. The DNA-binding activity of SMC1 is suggested to serve as a biochemical basis for its function in the chromatin assembly essential for sister chromatid cohesion and chromosome segregation (24). The modulation of this activity will undoubtedly affect the biological function of SMC1 in chromosome segregation, although other functions of Smc1p may also be influenced. It has been suggested that SMC1 forms complexes with various proteins, most of which, however, have not been revealed (24, 54). Our results indicate that Hec1p is present in the DNA-binding complex of Smc1p and also suggest that Hec1p is important for the biochemical activity of this complex. Consistently, the interaction between Hec1p and Smc1p is critical for proper chromosome segregation (59). Although purified recombinant protein containing the C-terminal region of SMC1 was shown to have the DNA-binding activity (1), it is likely that SMC1 requires other cofactors, such as Hec1p, to enhance its activity for a more stable binding of structured DNA. Rb appears to enhance the DNA-binding activity of Smc1p through Hec1p. This positive regulatory effect of Rb has also been observed in a number of transcription factors, such as MyoD (47), the glucocorticoid receptor (53), C/EBPβ (6), NF-IL6 (7), and c-jun (45). Our results showing that Rb is not a component of the DNA-binding complex formed by Smc1p and Hec1p suggest that Rb may serve as a chaperone for Hec1p, presumably by stabilizing its active conformation. Taken together, the results presented here suggest a potential role of Rb in regulation of SMC1 through hsHec1p.

The functional analysis of Rb in the heterologous yeast system is further supported by the in vivo interaction between Rb, Hec1p, and SMC1 in mammalian cells. The presence of Rb in a complex with Hec1p and Smc1p suggests the relevance of the novel Rb function revealed by the yeast study to mammalian cells where Rb exists. The complex formed between Rb and SMC1 indicates a biological role of Rb in the SMC1 activity, although Rb is not present in the DNA-binding complex of SMC1. Rb thus appears to modulate the activity of SMC1 before SMC1 binds to chromatin DNA. Unlike the activities of Hec1p and SMC1, this M phase activity of Rb does not appear to be required by either yeast or mammalian cells for their basic machinery of chromosome segregation or for cell survival. Nevertheless, such an activity of Rb in regulating SMC1 could be important for higher fidelity of chromosome segregation and higher integrity of mitotic chromosome structures in mammalian cells.

Whether loss of Rb function leads to a decrease in the fidelity of chromosome segregation in mammalian cells re-

mains to be explored. Nonetheless, such an activity for Rb may explain in part the abnormal process of mitosis observed in Rb-deficient fibroblasts and the chromosome abnormalities observed in Rb-deficient tumor cells (12, 28). It may also provide some clues for explaining observations that the majority of human retinoblastomas losing the wild-type allele and reduplicating the mutant allele early in the course of carcingenesis result from nondisjunction and misproportioning of sister chromatids (4, 19). Interestingly, yeast or human cells lacking functional Hec1p complete mitosis with unseparated or unequally separated chromosomes and enter a new cell cycle, leading to hyperploidy and aneuploidy (10, 57, 59). This is similar to the phenomenon observed in Rb-deficient fibroblasts treated with nocodazole (12, 28). Since neither loss of Rb function nor loss of Hec1p function appears to affect the mitotic checkpoint control (28, 59), microtubule-destabilizing agents probably challenge the chromosome segregation process in Rb-deficient cells and thereby induce a high frequency of aberrant mitosis. These results indirectly support the role of Rb in chromosome segregation.

Taken together, the results of this study, using a heterologous yeast system, provide a useful assay and more direct evidence for revealing the mechanistic process of a novel function of Rb in chromosome segregation. The study thereby contributes to explaining a new critical role of Rb in human carcinogenesis.

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The first two authors contributed equally to this report.

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